Biochemical Pharmacology, Vol. 35, No. 23, pp. 4139–4152, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

BIOCHEMICAL CHANGES ASSOCIATED WITH THE POTENTIATION OF ACETAMINOPHEN HEPATOTOXICITY BY BRIEF ANESTHESIA WITH DIETHYL ETHER*

ESTHER C. A. TO† and PETER G. WELLS‡
Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada M5S 1A1

(*Received* 30 *July* 1985; *accepted* 22 *April* 1986)

Abstract—Acetaminophen hepatotoxicity in male CD-1 mice was enhanced markedly by brief anesthesia with diethyl ether (ether), and particularly so if acetaminophen was given several hours after ether. The present study was conducted to examine the possible biochemical mechanisms behind this delayed toxicologic synergism. *In vitro* biochemical studies indicated that ether anesthesia produced a delayed reduction in the activities of glucuronyl transferase and glutathione (GSH) *S*-transferase, and in the hepatic content of GSH. The hepatic content but not activity of the cytochromes P-450 was initially reduced by ether but recovered by the time of maximal toxicologic enhancement. *In vivo* studies showed that ether produced a small decrease in the plasma concentrations of glucuronide and sulfate conjugates of acetaminophen, with a concomitant, minor increase in the half-life of acetaminophen, and a major increase in the bioactivation of acetaminophen, as determined by an early, 2-fold increase in the plasma GSH and cysteine conjugates of acetaminophen, and a 3-fold increase in the covalent binding of acetaminophen to hepatocellular protein. Decreases produced by ether in the *in vivo* production of acetaminophen glucuronide correlated with increasing plasma concentrations of unmetabolised acetaminophen, decreasing hepatic GSH content and increasing covalent binding of acetaminophen to hepatocellular protein when these measurements were performed in the same animals. The biochemical mechanisms underlying the potentiation of acetaminophen hepatoxicity as measured by plasma glutamic pyruvic transaminase concentrations appeared to be due to delayed, complex effects of ether upon multiple enzymatic pathways of acetaminophen elimination and detoxification.

Diethyl ether (ether) is used widely as an anesthetic in animal research, and it remains a convenient human anesthetic in some third world countries. Although there have been reports as early as 1958 [1] that ether can inhibit drug-metabolising enzymes in liver homogenates, it has been only recently that the effects of ether on metabolism have received more attention. Umeda and Inaba [2] found that ether inhibits diphenylhydantoin elimination *in vivo*, and Johannessen *et al.* [3] showed that brief ether anesthesia significantly reduces the *in vivo* elimination of acetaminophen in rats. Hempel *et al.* [4] showed *in vitro* that ether inhibits the oxidative metabolism of *p*-nitroanisole. Ether was also shown *in vitro* to cause a marked depletion of uridine diphosphoglucuronic acid (UDPGA§), the essential cofactor for glucuronidation [5, 6]. More recently, ether given for 1 hr has been shown to cause immediate decreases in the *in vivo* and *in vitro* glucuronidation of acetaminophen in rats [7], and rat hepatocytes incubated in the presence of ether have reduced glucuronidation of acetaminophen [8].

Acetaminophen (paracetamol, *N*-acetyl-*p*-aminophenol, APAP, Tylenol) is a widely used analgesic/antipyretic drug. Acetaminophen can cause centrilobular hepatic necrosis in humans [9, 10] and in animals [11, 12]. As shown in Fig. 1, saturable, enzymatically catalysed conjugations of acetaminophen with glucuronic acid and sulfate prevent the formation of the toxic reactive metabolite and account, respectively, for about 60 and 30% of acetaminophen metabolism, depending upon the species [12, 13]. A small fraction (5–10%) of acetaminophen is bioactivated by hepatic cytochromes P-450 to the potentially toxic reactive intermediate. Under normal conditions, this reactive intermediate is evanescent, being immediately detoxified by enzymatic conjugation with hepatic reduced glutathione (GSH) and subsequently excreted as cysteine and *N*-acetylcysteine conjugates [14]. However, if the amount of acetaminophen bioactivated is increased, or if detoxification of the reactive intermediate is reduced, then the available reactive intermediate arylates hepatic macromolecules, which is thought to initiate processes leading to hepatocellular necrosis [12, 15].

Since glucuronidation constitutes the major nontoxic elimination pathway for acetaminophen (Fig.

* A preliminary report of this work was presented at the Third International Symposium on Biological Reactive Intermediates (Abstract No. 64), University of Maryland, College Park, MA, U.S.A., June 1985. Supported by grants from the Atkinson Foundation, the Banting Research Foundation, the Hospital for Sick Children Foundation, the Connaught Fund, and the Medical Research Council of Canada.

† Recipient of an Ontario Graduate Scholarship.

‡ To whom correspondence should be addressed.

§ Abbreviations: UDPGA, uridine diphosphoglucuronic acid; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloronitrobenzene; GSH, reduced glutathione; APAP, acetaminophen, paracetamol, *N*-acetyl-*p*-aminophenol; and PAPS, adenosine 3′-phosphate-5′-phosphosulfate.

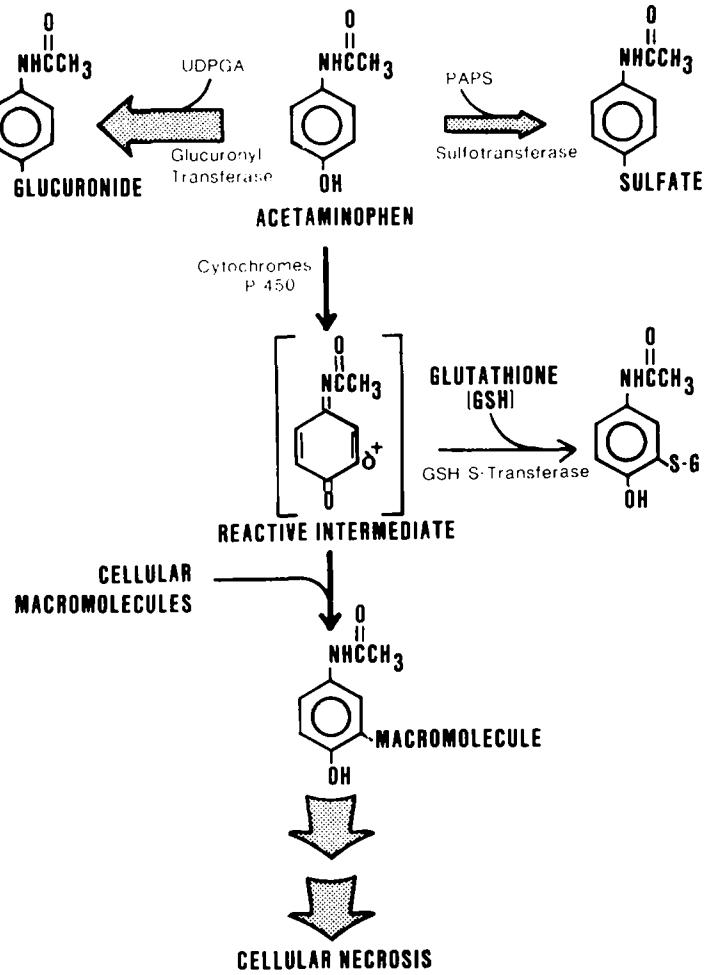

Fig. 1. Postulated relation of acetaminophen (APAP) biotransformation to hepatotoxicity. Over 60% of APAP is eliminated via enzymatic conjugation with glucuronic acid, producing a nontoxic, water soluble metabolite. A small amount (5–10%) is bioactivated by the hepatic cytochromes P-450 to a reactive intermediary metabolite which, if not detoxified by conjugation with glutathione, can bind covalently to essential hepatocellular macromolecules, thereby initiating a process leading to cellular necrosis (see text).

1), and since ether inhibits the glucuronidation of a number of substrates, previous murine studies in this laboratory examined whether ether could enhance the hepatotoxicity of acetaminophen, ostensibly by inhibiting glucuronidation and forcing more acetaminophen through the toxifying pathway catalysed by the cytochromes P-450 [16, 17]. However, ether also can inhibit the activity of the toxifying cytochromes P-450, as cited above. Therefore acetaminophen was given to separate groups of NIH general strain male mice at different times after ether administration to determine whether P-450 bioactivation might recover before glucuronidation. For this hypothesis, enhancement of the hepatotoxicity of acetaminophen by ether would occur only if acetaminophen was given at that time following ether administration when bioactivation had recovered while glucuronidation remained suppressed (Fig. 2, upper panel). The results of those studies supported the above hypothesis, in that acetaminophen hepatotoxicity was enhanced markedly by brief anesthesia with ether, and particularly so if acetaminophen was given several hours after ether [17].

The present study was conducted to confirm this delayed toxicologic synergism and to examine the possible biochemical mechanisms.

METHODS AND MATERIALS

Animals. Male CD-1 mice (Charles River Canada Inc., St. Constant, Quebec, Canada) weighing 25–30 g were housed in plastic cages containing ground corn cob bedding (Northeastern Products Corp., Warrensburg, NY). A 12-hr light cycle was maintained automatically, and animals were provided *ad lib.* with food (Purina Rodent Chow, Woodlyn Laboratories Ltd. Guelph, Ontario) and tap water. All animals were maintained for 1 week before receiving any treatment.

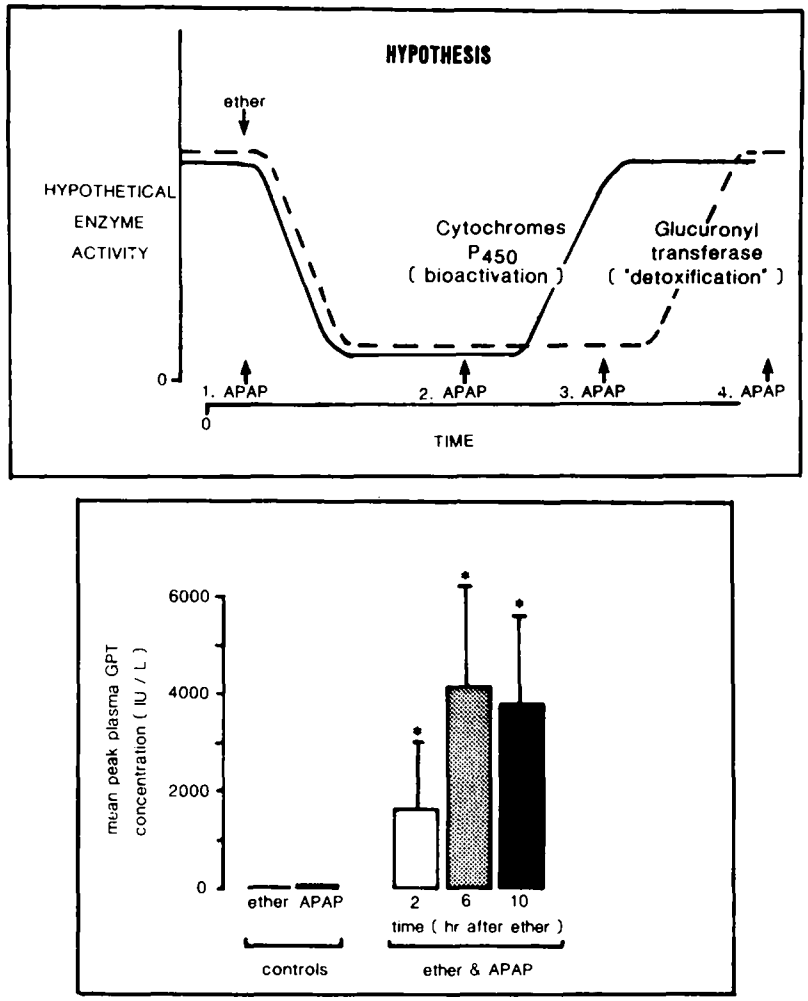

Fig. 2. Upper panel: Biochemical hypothesis for the potentiation of acetaminophen (APAP) hepatotoxicity by general anesthetics. General anesthesia inhibits the enzymatic pathways for both the bioactivation (P-450 toxification) and "detoxification" (elimination via the alternative, nontoxifying glucuronyl transferase pathway) of APAP. If bioactivation recovers first, then administration of APAP at this time (case 3) would cause enhanced hepatotoxicity. APAP given at other times after anesthesia, either when both bioactivation and detoxification were unaffected (case 1), inhibited (case 2) or recovered (case 4), would cause the same hepatotoxicity as APAP given alone. Lower panel: Temporal effect of ether pretreatment on acetaminophen (APAP) hepatotoxicity. APAP, 300 mg/kg i.p., was administered to separate groups of male CD-1 mice at different times following 5 min of anesthesia with ether. Plasma GPT concentrations were measured repetitively in each mouse, and each bar represents the mean ± S.D. of the peak GPT concentration from each mouse. Asterisks indicate significant differences from the control group given APAP alone ($P < 0.05$).

Toxicologic potentiation. Preliminary experiments were conducted to confirm the potentiation of acetaminophen hepatotoxicity by diethyl ether (ether) in CD-1 male mice. Ether (BDH Laboratories, Toronto, Ontario, Canada) was administered over 5 min to groups of six mice housed in an inhalation chamber, with anesthetic efficacy monitored by abolition of the righting reflex. Following recovery from anesthesia, animals were transferred to their original cages. Acetaminophen (*N*-acetyl-*p*-aminophenol, Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in normal saline and adjusted to pH 9.5 with NaOH, the drug concentration being calculated to deliver the appropriate dose in a vehicular volume of 0.01 ml/g of body weight. Acetaminophen, 300 mg/kg, was injected intraperitoneally (i.p.) into different groups of animals at 2, 6 or 10 hr after pretreatment with ether anesthesia. All groups received acetaminophen at the same time of day. Control animals were injected with an equal volume of the vehicular solution adjusted to the corresponding pH.

Hepatocellular damage was assessed by measuring the amount of glutamic pyruvic transaminase (GPT) released into the blood by injured hepatocytes. Plasma GPT was quantified according to the colourimetric method of Reitman and Frankel [18] using a standardised assay kit (Sigma Chemical Co.). Using

heparinised microcapillary tubes (Microcaps, Drummond Scientific Co., Bloomall, PA, U.S.A.), sequential plasma samples were obtained from each mouse via the tail vein at 6, 12, 24 and 36 hr after acetaminophen injection. Repetitive microsampling from the same mouse has been shown to permit accurate temporal identification and quantification of the maximal plasma GPT concentration, which varies substantially in time of occurrence among different mice treated identically [19]. Accurate assessment of the peak value for plasma GPT concentrations can be essential for a valid indirect assessment of hepatocellular damage.

Pilot study: Temporal biochemical effects of ether. Prior to the fully controlled study, a pilot experiment was conducted in a small number of animals to establish the temporal nature of biochemical effects caused by ether anesthesia. Groups of four mice were pretreated with ether and killed by cervical dislocation at various time intervals afterwards. Control groups were studied at 0, 12 and 24 hr after treated groups received ether. The liver was flushed via the hepatic portal vein with isotonic KCl, excised, and homogenised in isotonic KCl on ice using a probe homogeniser (Ultra-Turrax, Janke & Kunkel, Terochem Laboratories, Toronto, Canada). An aliquot was used to quantify hepatic GSH content according to the method of Sedlak and Lindsay [20] as previously described [21]. The liver homogenate was then centrifuged at 9,000 *g* for 20 min at 4°. The 9,000 *g* supernatant fraction was centrifuged at 100,000 *g* for 60 min at 4° to separate the microsomal and cytosolic fractions, which were immediately frozen in liquid nitrogen and stored at −80° until used to measure enzymatic activities as described below.

Full study: Combined treatment with ether and acetaminophen. Based in part upon the temporal results from the above pilot study using ether alone, a series of *in vitro* and *in vivo* studies were conducted using larger groups of eight mice to evaluate the combined effects of ether and acetaminophen. Animals were pretreated with ether at the same time of day as before to minimise the potential effects of diurnal cycles. Since the *in vivo* toxicological potentiation study showed a maximal effect when acetaminophen was given 6 hr after ether anesthesia (Fig. 2, lower panel), this timing of administration was employed for all subsequent studies.

For the *in vitro* biochemical studies, animals were killed and studied at 2 hr and 8 hr after ether anesthesia. The 2-hr time interval was chosen to contrast this time of minimal toxicologic enhancement with those changes observed with the 8-hr interval, when maximal potentiation occurred. In addition, the 8-hr interval (2 hr after acetaminophen) permitted a correlative evaluation of biochemical changes with concomitant *in vivo* studies of acetaminophen metabolism and covalent binding. In perspective, the 2-hr sampling time occurred 4 hr prior to acetaminophen administration, while the 8-hr sampling time occurred 2 hr after acetaminophen administration (Fig. 3). Thus, the 2-hr group was evaluated only for controls and ether-treated animals, while the 8-hr group was evaluated for controls, animals receiving ether alone, acetaminophen alone, and ether in combination with acetaminophen (Fig. 3).

Previous studies employing multiple blood sampling from the tail vein of each mouse indicated that acetaminophen and its metabolites achieved peak plasma concentrations within 0.25 to 1.0 hr after i.p. injection of acetaminophen [22]. Accordingly, plasma samples were obtained at 0.5, 1.0 and 2.0 hr following i.p. injections of unlabeled acetaminophen combined with radiolabeled acetaminophen for covalent binding studies. Tritiated acetaminophen, randomly labeled with a specific activity of 1.8 Ci/mmole (New England Nuclear, Lachine, Quebec, Canada) was given in a dose of 2 μCi/g body weight, or about 50 μCi/mouse. Following the 2.0-hr plasma sample, animals were killed by cervical dislocation.

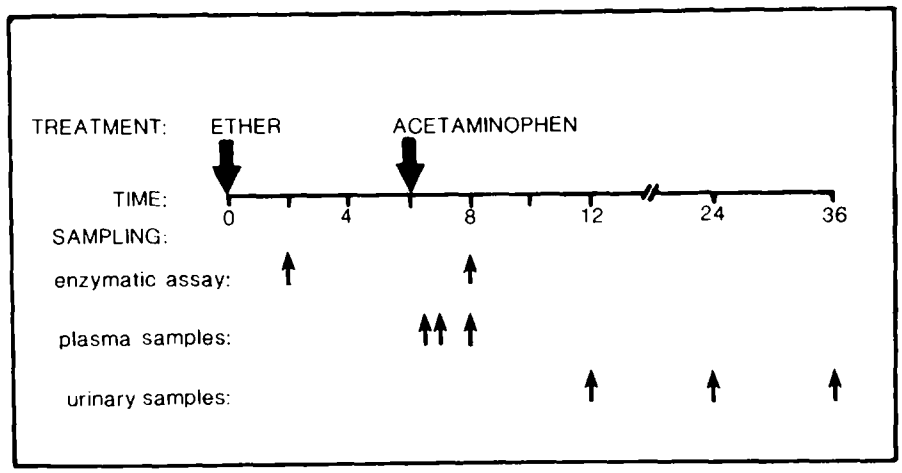

Fig. 3. Timing of treatments and sampling in the full study (see Fig. 5). Acetaminophen (APAP), 300 mg/kg i.p., was administered 6 hr following 5 min of general anesthesia using diethyl ether (ether). Mice were killed at 2 and 8 hr following ether anesthesia. Plasma samples were obtained at 0.5, 1.0 and 2.0 hr following APAP administration. Urinary samples were collected at 6, 12, 24 and 36 hr following APAP administration

and hepatic tissue was analysed for GSH content and covalently bound radiolabeled acetaminophen [21]. Microsomal and cytosolic fractions were prepared and frozen as described above for subsequent biochemical analyses.

In a subsequent experiment, groups of eight mice received the ether and acetaminophen combination treatment but were killed 36 hr after acetaminophen injection. In this instance, only plasma acetaminophen metabolite concentrations, covalently bound radiolabeled acetaminophen, plasma GPT and hepatic GSH content were measured. No enzymatic assays were performed at this time.

Analytical methods. Covalent binding of radiolabeled acetaminophen to hepatocellular protein was determined in hepatic homogenates using exhaustive washing with hot methanol as previously described [21]. Hepatic protein concentration was measured [23] using a standardised assay kit (Bio-Rad Laboratories Canada Ltd., Mississauga, Ontario) with bovine serum albumin (Sigma Chemical Co.) as the protein standard. A 0.8-ml aliquot of liver homogenate was precipitated with a volume of 5% trichloroacetic acid (TCA). The volume of TCA added was proportional to the amount of liver tissue contained in the 0.8-ml aliquot (i.e. volume of TCA = six times the liver weight). The mixture was vortexed and centrifuged at 1000 *g* for 15 min at 4°. The supernatant fraction was removed and assayed for hepatic GSH content. The pellet was washed with 2.0 ml of methanol, vortexed for 1 min, and shaken in a water bath at 56° for 10 min. The sample was then centrifuged again at 1000 *g* for 10 min and resulting supernatant fraction was discarded. The washing procedure was repeated for six to eight times until the supernatant fraction contained only a background level of radioactivity. The pellet was then dissolved with 3.0 ml of 2 N NaOH and shaken in a 37° water bath overnight. After digestion, 0.5 ml was placed in a scintillation vial, diluted with 10 ml of scintillation fluid (Ready-Solv HP/b, Beckman Laboratories, Toronto, Ontario) and neutralised with 0.5 ml of 2 N HCl. Radioactivity was measured in a liquid scintillation spectrometer (model LS 7500, Beckman Instruments, Inc., Toronto, Ontario). Results were expressed as picogram equivalents of radiolabeled acetaminophen bound per mg of protein. To obtain total binding (radiolabeled plus unlabeled acetaminophen), the radiolabeled binding data should be multiplied by 2565.

Glucuronyl transferase activity was assayed by high performance liquid chromatography (HPLC) using both 1-naphthol [24] and acetaminophen (To and Wells, submitted) as substrates. For measurement of glucuronyl transferase activity, 1-naphthol (Sigma Chemical Co.) to a final concentration of 5.0 mM was dissolved in a 50 mM Tris buffer, pH 7.4, with 10 mM magnesium chloride and 3.0% (v/v) dimethyl sulfoxide (DMSO). After addition of uridine diphosphoglucuronic acid (UDPGA) (Sigma Chemical Co.) to a final concentration of 4.0 mM, the mixture was incubated at 37° for 5 min, and microsomal protein was added at a concentration of 1.0 mg/ml. The suspension was incubated in a shaking water bath at 37° for 10 min. The reaction was stopped by the addition of ice-cold methanol containing 0.04 mg/ml of 2-naphthol (Sigma Chemical Co.) as the internal standard. The suspension was centrifuged at 1000 *g* for 20 min at 4° and the supernatant fraction was saved. The pellet was rewashed with methanol and centrifuged, and this second supernatant fraction was added to the first. The supernatant was dried under nitrogen, redissolved in methanol and injected into the HPLC. All samples were analysed using a 15 cm × 4.6 mm i.d. reverse phase C-18 column with a particle size of 5 μm (Beckman Instruments, Toronto, Ontario). For analysis of the 1-naphthol glucuronide, the optimal solvent system consisted of 0.1 M acetic acid and methanol (55:45, v/v) with a flow rate of 1.5 ml/min. Absorbance was monitored at 240 nm using a spectrophotometric detector (model LC 85, Perkin-Elmer Canada Ltd.).

Glucuronyl transferase activity also was assayed using acetaminophen as the substrate. Acetaminophen was dissolved to a final concentration of 5.0 mM in a 50 mM Tris buffer, pH 7.4, with 10 mM magnesium chloride and 0.05% (v/v) Triton X-100. After the addition of UDPGA to a final concentration of 4.0 mM, the mixture was incubated at 37° for 5 min. Microsomal protein, 1 mg/ml, was added and the suspension was incubated at 37° for 10 min. The reaction was stopped by the addition of ice-cold methanol containing 0.04 mg/ml of *p*-hydroxybenzoic acid (Eastman Kodak Co., Rochester, NY) as the internal standard. The suspension was centrifuged at 1000 *g* for 20 min at 4° and the supernatant fraction was saved. The pellet was rewashed with methanol and centrifuged, and this second supernatant was added to the first. The supernatant was dried under nitrogen, redissolved in methanol, and injected in the HPLC. The optimal solvent system consisted of 0.1 M acetic acid and methanol (92:8, v/v) with a flow rate of 1.5 ml/min. Absorbance was measured at 248 nm.

Sulfotransferase activity was assayed using 2-naphthol as the substrate [24]. The reaction mixture contained 0.125 mM 2-naphthol (Sigma Chemical Co.), 6.0 mM 2-mercaptoethanol and 0.2 mM adenosine 3'-phosphate-5'-phosphosulfate (PAPS) (Sigma Chemical Co.) in 5.0% (v/v) acetone in 0.25 M sodium phosphate, pH 6.5. This mixture was preincubated at 37° for 5 min before the reaction was initiated by the addition of cytosolic protein from the 100,000 *g* fraction to give a final concentration of 2.0 mg/ml. This suspension was incubated in a shaking water bath at 37° for 10 min. The reaction was stopped by the addition of ice-cold methanol containing 0.04 mg/ml of acetaminophen as the internal standard. The suspension was centrifuged at 1000 *g* for 20 min and the supernatant fraction was saved. The pellet was rewashed with methanol, centrifuged, and this second supernatant added to the first. The supernatant was dried under nitrogen and redissolved in water. The unconjugated 2-naphthol was extracted out with chloroform to prevent it from interfering with subsequent assays due to its long retention time (30 min). The aqueous layer was injected into the HPLC. For analysis of the 2-naphthol sulfate, the optimal solvent system consisted of 0.1 M acetic acid and acetonitrile (85:15, v/v). Absorbance was monitored at 235 nm.

The hepatic content of cytochromes P-450 in microsomal preparations was determined according to the method of Omura and Sato [25]. The activity of the cytochromes P-450 was quantified according to a modified method of Imai *et al.* [26], which determines the rate of hydroxylation of aniline by the cytochromes P-450. GSH *S*-transferase activity was measured using both 3,4-dichloronitrobenzene (DCNB) (Sigma Chemical Co.) according to a modified method of Booth *et al.* [27], and 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma Chemical Co.) according to a modified method of Habig *et al.* [28]. Hepatic GSH was quantified as the soluble sulfhydryl content according to the method of Sedlak and Lindsay [20].

Plasma sample collection and preparation. Blood samples were obtained in order to measure the concentration of acetaminophen and its metabolites in the plasma at various time intervals after an i.p. injection [22]. Plasma samples were obtained as described above for plasma GPT measurements. For analysis of acetaminophen and metabolites, a 150-μl aliquot of methanol containing 0.01 mg/ml acetanilide as the internal standard was added to 75 μl of plasma to precipitate the proteins. The mixture was vortexed and then centrifuged for 15 min at 1000 *g*. The supernatant fraction was removed and the pellet was washed with 100 μl of methanol. The supernatant fractions were pooled and evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 75 μl of methanol and centrifuged again if cloudy.

Acetaminophen and metabolites in biological samples were separated by HPLC (model Series 4, Perkin-Elmer Canada Ltd., Toronto, Ontario) using a 15 cm × 4.6 mm i.d. reverse phase C-18 column with a particle size of 5 μm (Beckman Instruments), preceded by a reverse phase C-18 guard column with the same particle size (Supelco Canada Ltd., Oakville, Ontario) [22]. The solvent system consisted of a linear gradient which was started 3.0 min after injection, increasing from 5% methanol and 95% 0.1 M acetic acid to 15% methanol and 85% acetic acid, over a period of 4.0 min. The final solvent conditions were then kept constant until the end of the run. HPLC peak metabolite concentrations were detected spectrophotometrically at 248 nm and quantified by the chromatographic peak area-under-the-curve.

Urinary sample collection and preparation. Acetaminophen and its metabolites in the mouse are excreted predominantly by the kidneys [22]; therefore, urine was collected over 24 hr to measure the amount of acetaminophen metabolites produced *in vivo*. Metabolic cages with non-wetting, polymethylpentene collecting surfaces (Nalgene, Sybron Corp., Rexdale, Ontario) were used to house the mice over a 24-hr period for separate collection of urine and feces. Urine collected at the end of 6, 12, and 24 hr was removed, and the cage and collection receptacles were rinsed with methanol at the end of each collection time. The methanol rinses were added to the urine sample. To an aliquot of urine, 4 vol. of methanol containing 0.1 mg/ml of acetanilide as the internal standard were added to precipitate the proteins. The sample was centrifuged for 20 min at 1000 *g*. An aliquot of the supernatant fraction was removed and blown down to dryness under a stream of nitrogen. The sample was redissolved in sufficient methanol to yield the original concentration before methanol precipitation. This solution was centrifuged at 1000 *g* for 15 min followed by filtration through a 5 μm teflon filter (type FH, Millipore, Mississauga, Ontario). A 10-μl aliquot of the filtered sample was injected into the HPLC and analysed as described above.

Statistical analysis. The plasma area-under-the-curve (AUC) for acetaminophen and metabolites was calculated by the trapezoidal rule [29], and the clearance$_{ip}$ of acetaminophen was calculated as the dose/AUC.

Statistical comparisons of differences and correlations between groups were determined using a standard, computerised statistical program (SPSS Inc., Chicago, IL, U.S.A.) modified for microcomputers (SPSS-PC). Multiple comparisons among groups were determined by analysis of variance followed by a range test, whereas paired data were analysed by Student's *t*-test. A probability of $P < 0.05$ was chosen as the minimal level of significance.

RESULTS

Toxicology. Ether pretreatment produced a significant increase in the peak plasma GPT concentration induced by acetaminophen (Fig. 2, lower panel). A maximal, 20-fold toxicologic enhancement was observed if acetaminophen was administered 6 hr after ether. This enhancement was evident whether the timing of either ether or acetaminophen administration was held constant. Ether alone did not produce an elevation in plasma GPT concentrations at any time (Figs. 2 and 4).

Biochemical and pharmacological interrelationships. The biochemical effects of ether over time as determined in the pilot study occurred primarily between 6 and 10 hr with regard to decreases in enzymatic activities and GSH (Fig. 4). Results from control groups sampled at 0, 12 and 24 hr were not significantly different; therefore, these data were pooled.

Based upon the above pilot results (Fig. 4) and the toxicological data (Fig. 2), the full study with combination treatment groups and respective controls was sampled at 8 hr to study the maximal toxicologic enhancement, and at 2 hr to provide comparative data at a time of minimal enhancement (Fig. 5). In the full study, brief ether pretreatment was followed 8 hr later by significant decreases in the activities of glucuronyl transferase using acetaminophen as the substrate (reduced 20%), GSH *S*-transferase activity using DCNB (reduced 24%) and hepatic GSH content (reduced 15%), while a concomitant, significant 3-fold increase was observed in the covalent binding of acetaminophen (Fig. 5). The significant 3-fold increase in covalent binding after ether remained evident in animals studied 36 hr after acetaminophen administration.

The glucuronidation of 1-naphthol, the sulfation of 2-naphthol, the content and activity of the cytochromes P-450, and the conjugation of CDNB were

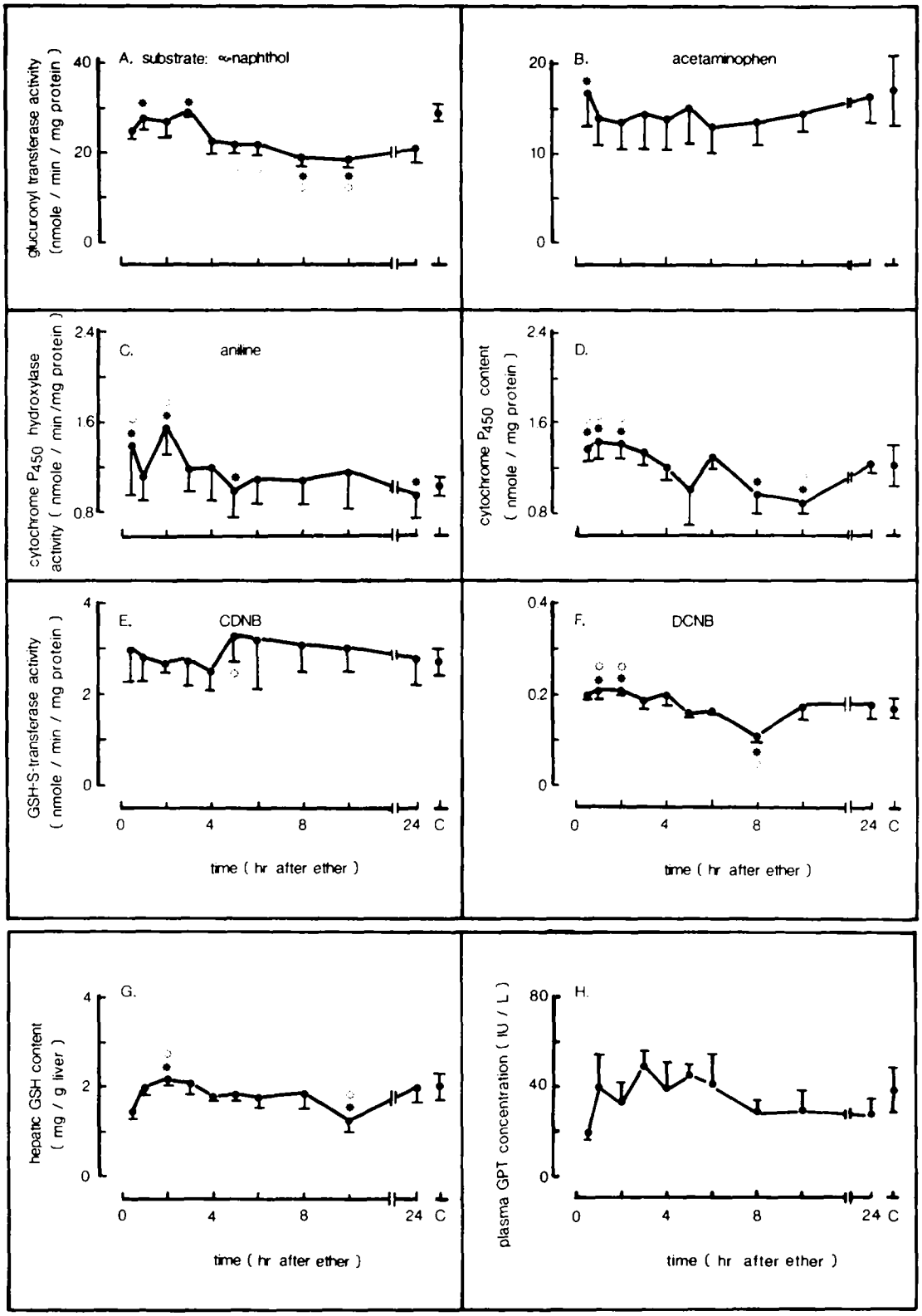

Fig. 4. Pilot study: temporal biochemical effects of 5-min general anesthesia with ether. Data points represent the mean ± S.D. for four animals. The open asterisks indicate significant differences from controls, and the solid asterisks indicate significant differences from the overall mean of the treated group ($P < 0.05$).

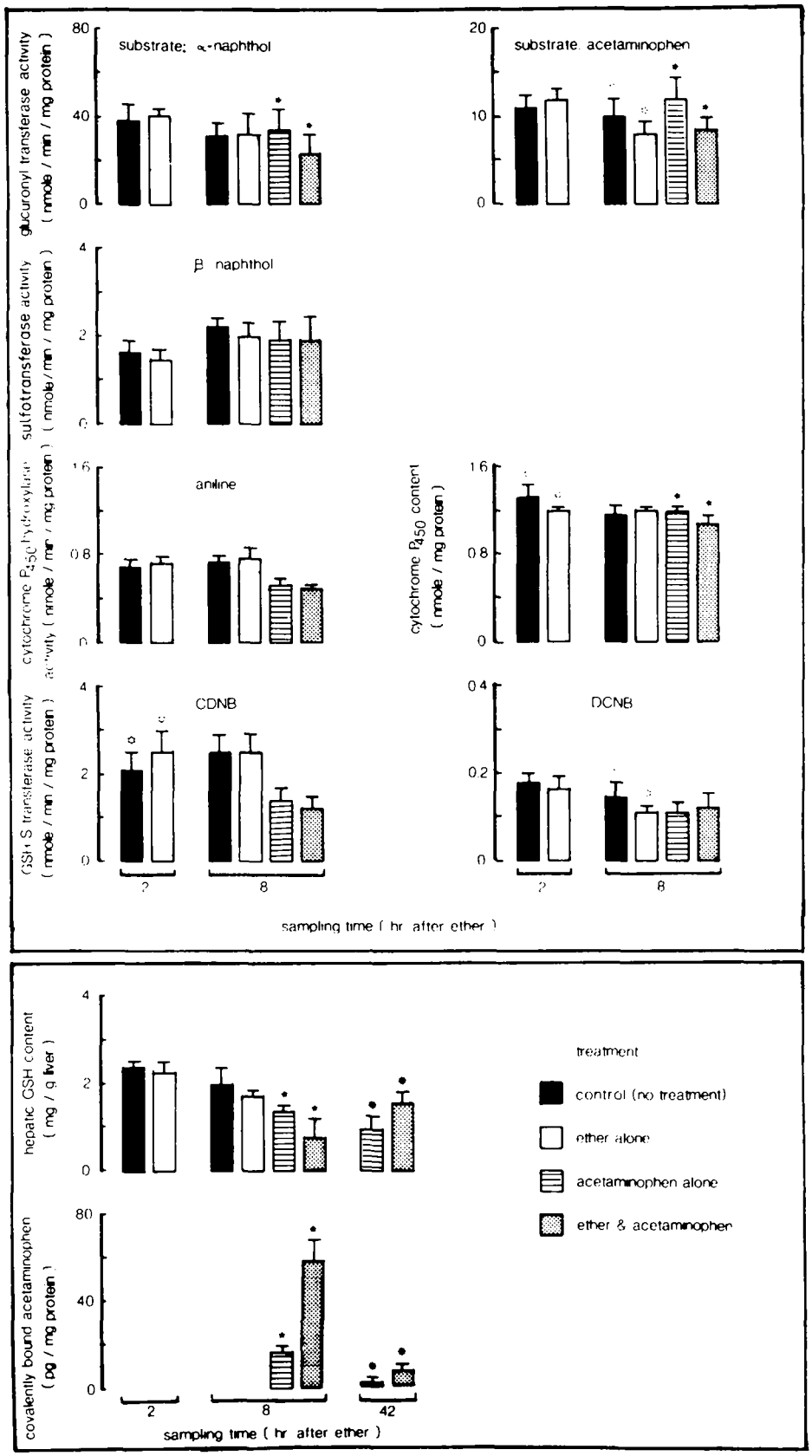

Fig. 5. Full study: biochemical effects of ether and acetaminophen (APAP) alone and in combination. Drugs were administered as described in Fig. 3. Enzymatic activities, cytochrome P-450 content, hepatic GSH content, and covalently bound acetaminophen were assessed at 2 hr after ether anesthesia, and 8 hr after ether anesthesia (i.e. 2 hr after APAP administration). GSH and APAP covalent binding also were measured at 42 hr after ether (i.e. 36 hr after APAP). Bars represent the mean + S.D. for eight animals. Pairs of identical asterisks indicate a significant difference between adjacent bars ($P < 0.05$).

unaffected at 8 hr by treatment with ether alone (Fig. 5). At 2 hr after ether alone, there was a small but significant 16% decrease in P-450 content and a 24% increase in the GSH *S*-transferase activity using CDNB; other activities were unchanged. Animals treated with acetaminophen alone had, 2 hr later (8 hr after ether would have been given), a 29% decrease in P-450 activity ($P < 0.05$), a 42% decrease in GSH-transferase activity using CDNB ($P < 0.05$), and a 32% decrease in hepatic GSH content ($P < 0.05$). Animals treated with ether plus acetaminophen had a further, significant 31% decrease in glucuronyl transferase activity using acetaminophen as the substrate, and a 36% decrease using 1-naphthol, compared with animals receiving acetaminophen alone. The group receiving ether plus acetaminophen also had a significant 43% decrease in hepatic GSH content compared with animals receiving acetaminophen alone (or a 61% decrease compared with controls), as well as a small (13%) but significant decrease in hepatic P-450 content.

Ether pretreatment in the full study produced a small decrease in the mean plasma concentrations of the sulfate and glucuronide conjugates of acetaminophen, with a concomitant, small increase in the concentrations of acetaminophen and significant, up to 2-fold increases in the concentrations of GSH-derived metabolites (GSH and cysteine conjugates) within the first hour following acetaminophen administration (Fig. 6). The plasma half-life of acetaminophen was increased 29% by ether pretreatment, from 0.31 ± 0.10 to 0.40 ± 0.14 hr (mean ± S.D.) ($P = 0.31$); the plasma clearance was decreased 10%, from 70 ± 22 to 63 ± 12 ml/min ($P = 0.24$).

In pooled urinary samples, ether pretreatment decreased the cumulative 6-hr recovery of the sulfate conjugate by 22%, while increasing the recovery of unmetabolised acetaminophen by 100% and the cysteine conjugate by 13% (Fig. 7). The 6-hr urinary recovery of the glucuronide and GSH conjugates of acetaminophen appeared to be unchanged by ether pretreatment. In the 6- to 12-hr urinary sample, ether pretreatment appeared to reduce the recovery of sulfate, glucuronide and cysteine conjugates, although the amount of drug and metabolites recovered in this sampling period represented only 1.7% of the total urinary chemical recovery. Less than 3% of acetaminophen and metabolites was recovered in the feces.

Since the biochemical and pharmacological measurements were carried out simultaneously in the same animals, their interrelationship was examined (Fig. 8). For plasma area-under-the-curve data among individual mice, the decreasing glucuronide production correlated significantly with increasing parent acetaminophen concentrations, which correlated with decreasing hepatic GSH content, which correlated with increasing covalent binding of acetaminophen at 2 hr.

DISCUSSION

Brief general anesthesia using ether significantly potentiated the hepatotoxicity of acetaminophen administered hours later, as evidenced by the 20-fold

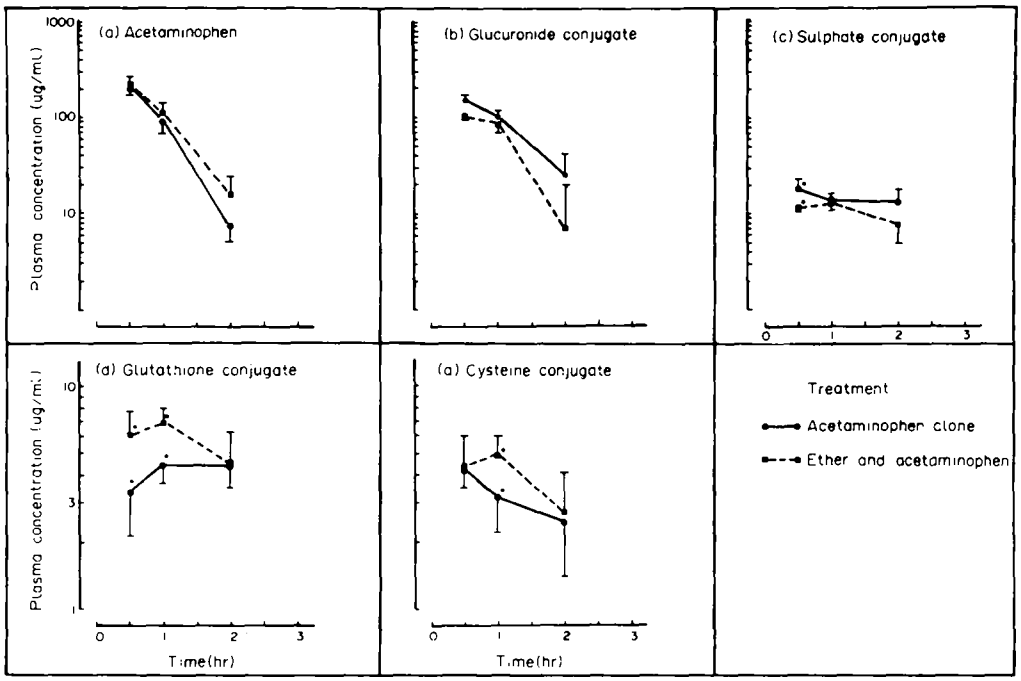

Fig. 6. Disposition of acetaminophen (APAP) and metabolites in plasma. Ether and APAP were administered as described in Fig. 3. Concentration of APAP and metabolites was quantified by high performance liquid chromatography at 0.5, 1.0 and 2.0 hr following APAP administration. Data points represent the mean ± S.D. for eight animals. Asterisks indicate significant differences between the two groups ($P < 0.05$).

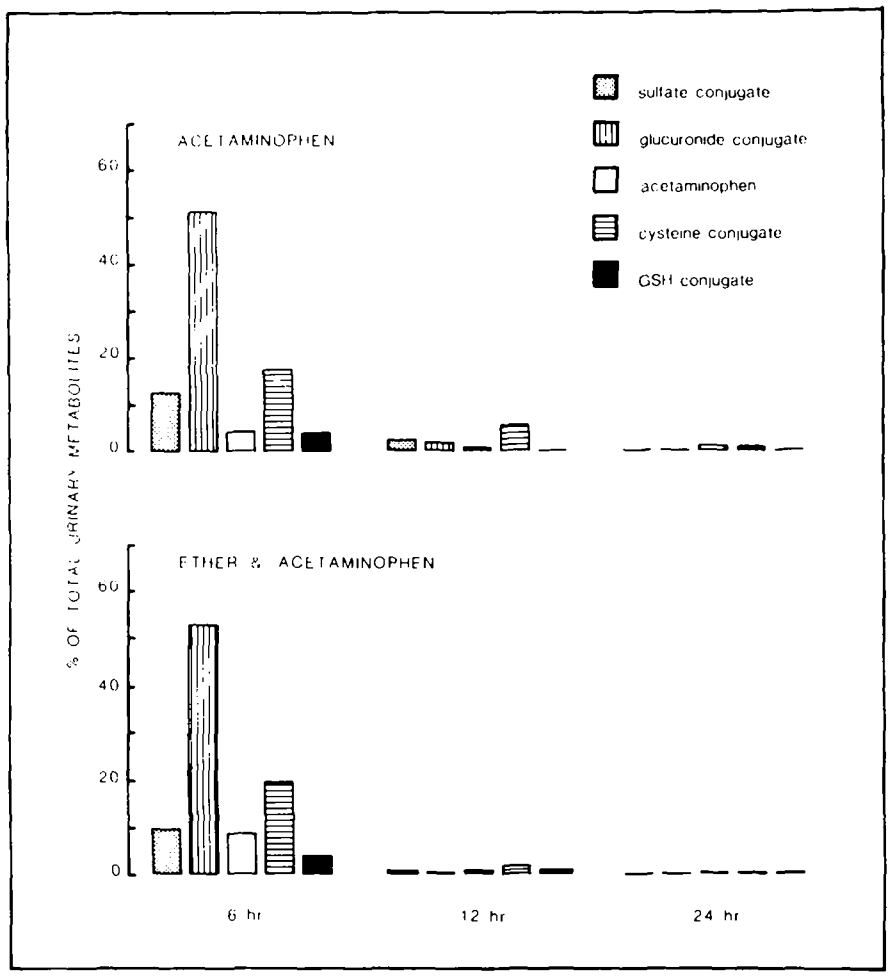

Fig. 7. Urinary profile of acetaminophen (APAP) and metabolites. Ether and APAP were administered as described in Fig. 3. Urine was collected from two groups of mice from 0 to 6 hr, 6 to 12 hr, and 12 to 24 hr following APAP administration. Bars represent the pooled urinary data from three animals housed together in a metabolic cage.

enhancement in peak plasma GPT concentrations. These results with acetaminophen always given at the same time of day concur with those from previous studies demonstrating a similar potentiation of acetaminophen hepatotoxicity by ether or halothane, where the anesthetic always was given at the same time of day [19]. In the present study, in order to elucidate the mechanism of this potentiation, biochemical changes measured *in vitro* were studied concurrently with pathologic and metabolic changes measured in the same animals *in vivo*. This study was designed to permit a study of the correlation of data obtained from *in vitro* assays of enzymatic activity, *in vivo* analyses of plasma and urinary concentrations of acetaminophen and metabolites, and *in vivo* assessment of hepatic damage. Whenever possible, each mouse was assessed for enzymatic activities, hepatic GSH content, plasma and urinary concentrations of acetaminophen and metabolites, plasma GPT concentration, and covalent binding of the reactive intermediate of acetaminophen to hepatocellular proteins. Therefore, any dependence of the severity in hepatic damage upon quantitative biochemical changes could be studied without the confounding influence of large interanimal variability.

The potentiation of acetaminophen hepatotoxicity by ether pretreatment required a delay between treatments, with maximal potentiation occurring when acetaminophen was administered 6 hr after ether anesthesia. Under normal circumstances, a nontoxic dose of acetaminophen produces little hepatic damage because bioactivation is balanced by: (1) competing pathways of elimination, namely conjugation with sulfate and glucuronic acid; and (2) a major detoxifying pathway involving enzymatic conjugation of the reactive intermediate with hepatic GSH. The ability of ether to potentiate acetaminophen hepatotoxicity could result from an effect on acetaminophen metabolism via changes in enzymatic activities, or from an effect on hepatocyte vulnerability due to depletion of hepatic GSH content. Ether anesthesia has been reported to alter the metabolism of acetaminophen [3, 7, 8, 16] and

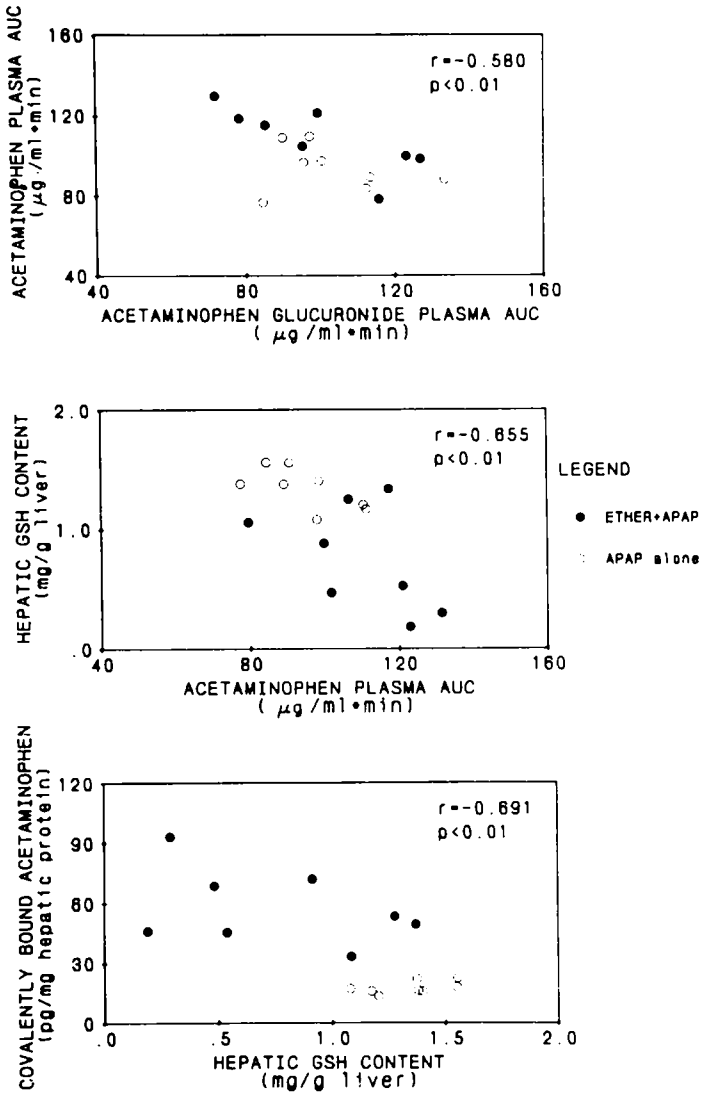

Fig. 8. Interrelationship of plasma area-under-the-curve (AUC) data over 2 hr for the glucuronide conjugate of acetaminophen (APAP), AUC data for unmetabolised APAP, the hepatic content of reduced glutathione (GSH) and covalent binding of APAP to hepatocellular protein at 2 hr. All parameters were measured in the same animals at the same time. Data for individual animals were derived from the studies presented in Figs. 5 and 6.

other drugs such as antipyrine [30], iopanoic acid [31] and diphenylhydantoin [2] by modifying activities of different metabolising enzymes.

This study was designed to test the hypothesis that ether would inhibit both bioactivating and detoxifying enzymes, with an earlier recovery of bioactivating activity accounting for the delayed toxicologic enhancement (Fig. 2, upper panel). We were particularly interested in the toxicological relevance of early reports of the reduction by ether of UDPGA [5, 6], which since has been associated with a decrease in acetaminophen glucuronidation [7, 8]. Glucuronidation not only is the major (> 50%) elimination pathway for acetaminophen (Fig. 7), but also diverts acetaminophen away from the quantitatively minor but toxicologically critical P-450 bioactivating pathway (Fig. 1). Since sulfation is a minor, saturable pathway, a relatively small decrease in the major glucuronidating pathway for acetaminophen could result in a relatively substantial increase in the percentage of hepatotoxic reactive intermediate produced. This concept is somewhat analogous to the *in vivo* anticoagulant activity of warfarin, where a quantitatively minor decrease of as little as 2% in the binding of warfarin to plasma proteins results in a substantial increase in anticoagulant activity. This apparently disproportionate response arises because activity is related only to the unbound fraction of warfarin in plasma, which is only about 2%. Thus, a small, 2% decrease in protein binding doubles the concentration of the quantitatively minor but pharmacodynamically critical free drug concentration.

The significant 20% decrease in *in vitro* glucuronyl

transferase activity in the group studied 8 hr after receiving ether alone (Fig. 5), together with the small *in vivo* reductions by ether in the mean plasma concentrations of acetaminophen glucuronide and sulfate conjugates (Fig. 6), were consistent with the small, albeit nonsignificant increase in the plasma concentration of acetaminophen (Fig. 6), the decrease in acetaminophen clearance, and the increased urinary recovery of unmetabolised acetaminophen after ether pretreatment (Fig. 7). Glucuronidation and sulfation were the major pathways of acetaminophen elimination, together accounting for over 60% of acetaminophen elimination (Fig. 7). The 2-fold increase in *in vivo* plasma concentrations of GSH-derived metabolites (Fig. 6), together with the 3-fold increase in the amount of acetaminophen covalently bound to hepatocellular protein (Fig. 5), support the hypothesis that minor reductions in the major elimination pathways of glucuronidation and to a lesser extent sulfation can result in a major percentage increase in the production of the acetaminophen reactive intermediate. The plasma observations were consistent with the urinary data from 0 to 6 hr (Fig. 7), during which time the percentage recovery of sulfate conjugate was decreased, while recovery of unchanged acetaminophen and its cysteine conjugate was increased. It is interesting to note the relative insensitivity of cumulative, pooled urinary samples in discriminating potentially important but short-lived changes. Up to a 2-fold increase in the plasma concentrations of the GSH conjugate of acetaminophen was produced by ether pretreatment (Fig. 6), reflecting a major increase in acetaminophen bioactivation. However, these changes observed in plasma occurred entirely within 1 hr, and were completely obscured even in the first 6-hr cumulative urinary sample (Fig. 7).

The above trends derived from mean data were supported by the correlations observed among the above parameters, all of which were studied in each mouse. The correlations of decreasing plasma acetaminophen glucuronide production with increasing unmetabolised acetaminophen, decreasing hepatic GSH content, and increasing acetaminophen covalent binding 2 hr following acetaminophen administration (Fig. 8) were compatible with a causal interrelationship. Furthermore, in other *in vivo* studies, the individual peak plasma GPT concentrations, which accurately reflect the maximal hepatotoxicity in each mouse, correlated with the amount of acetaminophen covalently bound to hepatocellular protein in the same animals 36 hr after acetaminophen administration ($r = 0.83$, $N = 20$, $P < 0.05$) [19].

The reductions 8 hr after ether in the *in vitro* activity of GSH *S*-transferase using DCNB, and in the hepatic content of GSH (Fig. 5), suggest that additional mechanisms could be contributing to the potentiation of acetaminophen hepatotoxicity by ether. The possibilities would include additive depletion of intracellular GSH pools leading to derangement in calcium homeostasis [32] or reduced protection of protein thiols [33], and/or an impairment in the detoxification of the acetaminophen reactive intermediate by enzymatic conjugation with GSH [14, 34, 35]. However, one cannot be certain that DCNB is reflective of the substrate activity for the reactive intermediate of acetaminophen, since the GSH *S*-transferase enzyme system is composed of a multitude of isoenzymes [28, 36]. The decrease in GSH *S*-transferase activity for CDNB at 8 hr following treatment with acetaminophen either alone or in combination with ether is interesting, since a similar decrease was not observed for DCNB, suggesting differential effects of acetaminophen covalent binding or early events in hepatocellular necrosis upon isoenzymatic activities. Interestingly, substantial covalent binding of acetaminophen to GSH *S*-transferase has been observed without affecting enzymatic activity as measured by the substrate CDNB [37], although isoenzymatic differences were not evaluated. The increased transferase activity observed for CDNB at 2 hr could have contributed to the lack of potentiation of acetaminophen hepatotoxicity by ether at that time.

Brief ether anesthesia has been shown to cause acute inhibition of glucuronidation via immediate depletion of the essential cofactor, uridine diphosphoglucuronic acid (UDPGA) [5, 6, 38]; however, this cofactor was repleted within 1 hr after ether anesthesia. Thus, it is unlikely that the potentiation by ether of acetaminophen hepatotoxicity, which was maximal when acetaminophen was given 6 hr after ether, was due to this mechanism. Nevertheless, there still is the question of whether inhibition of glucuronidation would significantly alter hepatotoxicity. Moldeus *et al.* [39] found that inhibition of sulfation or glucuronidation using restricted extracellular sulfate or galactosamine in isolated hepatocytes did not alter the production of GSH-derived metabolites reflecting bioactivation of acetaminophen to the reactive intermediate. This would suggest that inhibition of the major elimination pathways should not alter the rate of cytochrome P-450-catalysed bioactivation of acetaminophen, and therefore should not affect hepatotoxicity, which was not assessed by Moldeus. However, depending upon underlying mechanisms, the inhibitors and *in vitro* conditions employed by Moldeus may not be extrapolatable generally to all transferase inhibitors, particularly with regard to *in vivo* effects and their toxicological implications. This would appear to be supported by the work of Smith and Jollow [40], who demonstrated an *in vivo* enhancement of acetaminophen-induced hepatic centrilobular necrosis by galactosamine in hamsters. In the case of the *in vivo* potentiation of acetaminophen-induced hepatotoxicity by ether, which has complex biochemical effects, the ultimate toxicological consequences appear to depend upon the altered balance of bioactivation and detoxification, together with the temporal relation of treatment times. It is interesting that ether inhibited the activity of both soluble and membrane-bound enzymes. Our results are qualitatively consistent with a recent report that ether can inhibit the glucuronidation and sulfation of acetaminophen, as well as the conjugation of its reactive intermediate with GSH [7], although that study involved nontoxic doses of acetaminophen in rats pretreated with a more prolonged, 1 hr exposure to ether.

While the initial reduction in the hepatic content of the cytochromes P-450 at 2 hr after ether, followed by recovery to control levels by 8 hr (Fig. 5), would

fit with our hypothesis (Fig. 2, upper panel), this was not accompanied by a congruent change in enzymatic activity, at least as measured by aniline hydroxylation. Conversely, there was no evidence at 8 hr of enzymatic induction of cytochromes P-450 with a 5-min exposure to ether, as has been reported for prolonged exposure over 7 to 28 hr [41, 42]. In light of the early decrease in P-450 content, it is possible that the P-450 isoenzyme responsible for acetaminophen bioactivation was similarly decreased at 2 hr, although this was not indicated by the aniline hydroxylation assay. However, the isoenzymatic multiplicity of the cytochromes P-450 system [43, 44] must be considered. Aniline hydroxylase predominantly reflects activity of the P-450 rather than the P-448 isoenzymatic group [45], and since acetaminophen was not used as the substrate, the possible involvement of a different P-450 isoenzyme cannot be excluded. Steele *et al.* [46] examining the metabolic activation of acetaminophen by purified forms of cytochrome P-448 and cytochrome P-450 found that the bioactivation of acetaminophen in their *in vitro* system primarily was due to cytochrome P-448. These results are difficult to rationalise with the reported *in vivo* potentiation of acetaminophen hepatotoxicity by the P-450 isoenzyme inducer phenobarbital in animals [12] and humans [47]; nevertheless, any effect of ether on the isoenzyme P-448 may not be detected either by the aniline hydroxylation assay or by the determination of overall content of cytochromes P-450. The significant decrease in cytochrome P-450 content observed at 8 hr after ether anesthesia in the group receiving both ether and acetaminophen may have been due to enhanced destruction of hepatocytes by acetaminophen, since similar decreases in cytochrome P-450 activity and content have been observed after hepatotoxic doses of acetaminophen [48, 49]. Therefore, this decrease likely was secondary to early events in acetaminophen-induced hepatic necrosis, rather than to a biochemical potentiation by ether.

In summary, our working hypothesis (Fig. 2, upper panel) for designing this study involved early inhibition by ether of P-450-dependent bioactivation and glucuronyl transferase-dependent "detoxification", with an earlier recovery of bioactivation. While other mechanisms cannot be excluded, the combined *in vivo* and *in vitro* results indicate that the increased susceptibility to acetaminophen hepatotoxicity may have been due to a combination of delayed decreases induced by ether in the activities of glucuronyl transferase, sulfotransferase and GSH *S*-transferase, along with a depletion of hepatic GSH. The small decrease in hepatic content of cytochromes P-450 at 2 hr when toxicologic enhancement was minimal, together with repletion at 8 hr when enhancement was maximal and the above "detoxification" pathways were inhibited, is compatible with our hypothesis (Fig. 2, upper panel). However, the lack of an accompanying change in the activity of P-450 suggests either that a different P-450 isoenzyme is involved, or that P-450 activity was not toxicologically limiting. The toxicological imbalance in the bioactivation and detoxification of acetaminophen observed after ether pretreatment was evidenced by significant increases both in the plasma concentrations of GSH and cysteine conjugates, and in the covalent binding of acetaminophen to hepatocellular protein.

REFERENCES

1. R. Baekland and N. M. Green, *Anesthesiology* **19**, 724 (1958).
2. T. Umeda and T. Inaba, *Can. J. Physiol. Pharmac.* **56**, 241 (1978).
3. W. Johannessen, G. Gadeholt and J. Aarbakke, *J. Pharm. Pharmac.* **33**, 365 (1981).
4. V. Hempel, C. von Kuegelgen and H. Remmes, *Anaesthesist* **24**, 400 (1975).
5. G. Eriksson and D. Strath, *Fedn. Eur. Biochem. Soc. Lett.* **124**, 39 (1981).
6. J. B. Watkins and C. D. Klaassen, *J. pharmac. Meth.* **7**, 145 (1982).
7. J. W. Watkins, C. Siegers and C. D. Klaassen, *Proc. Soc. exp. Biol. Med.* **177**, 168 (1984).
8. H. Aune, P. A. Hals, B. I. Hansen and J. Aarbakke, *Pharmacology* **28**, 67 (1984).
9. A. T. Proudfoot and N. Wright, *Br. med. J.* **3**, 557 (1970).
10. J. D. Barker, D. J. DeCarle and S. Anuras, *Ann. intern. Med.* **87**, 299 (1977).
11. E. M. Boyd and G. M. Bereczky, *Br. J. Pharmac. Chemother.* **26**, 606 (1966).
12. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 185 (1973).
13. J. E. Mrochek, S. Katz, W. H. Christie and S. R. Dinsmore, *Clin. Chem.* **20**, 1086 (1974).
14. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 211 (1973).
15. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 195 (1973).
16. P. G. Wells and P. Ramji, *Proc. Can. Fedn. biol. Soc.* **26**, 228 (1983).
17. P. G. Wells, P. Ramji and M. S. W. Ku, *Fund. appl. Toxic.* **6**, 299 (1986).
18. S. Reitman and S. Frankel, *Am. J. clin. Path.* **28**, 56 (1957).
19. P. G. Wells and E. C. A. To, *Fund. appl. Toxic.* **7**, 17 (1986).
20. J. Sedlak and R. H. Lindsay, *Analyt. Biochem.* **25**, 192 (1968).
21. P. G. Wells, R. C. Boerth, J. A. Oates and R. D. Harbison, *Toxic. appl. Pharmac.* **54**, 197 (1980).
22. E. C. A. To and P. G. Wells, *J. analyt. Toxic.* **9**, 217 (1985).
23. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
24. E. C. A. To and P. G. Wells, *J. Chromat.* **301**, 282 (1984).
25. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
26. Y. Imai, A. Ito and N. Sato, *J. Biochem. Tokyo* **60**, 417 (1966).
27. J. Booth, E. Boyland and P. Sims, *Biochem. J.* **79**, 516 (1961).
28. W. H. Habig, M. J. Pabst and W. B. Jakoby, *J. biol. Chem.* **249**, 7130 (1974).
29. M. Gibaldi and D. Perrier, *Pharmacokinetics*, p. 293. Marcel Dekker, New York (1975).
30. H. Aune, H. Olsen and J. Moreland, *Br. J. Anaesth.* **53**, 621 (1981).
31. W. J. Cooke and L. M. Cooke, *J. Pharmac. exp. Ther.* **225**, 85 (1983).
32. S. Orrenius, S. A. Jewell, G. Bellomo, H. Thor, D. P. Jones and M. T. Smith, in *Functions of Glutathione: Biochemical Physiological Toxicological and Clinical Aspects* (Eds. A. Larsson, S. Orrenius, A. Holmgren

and B. Mannervik), p. 261. Raven Press, New York (1983).
33. E. Albano, M. Rundgren, P. J. Harvison, S. D. Nelson and P. Moldeus, *Molec. Pharmac.* **28**, 306 (1985).
34. D. E. Rollins and A. R. Buckpitt, *Toxic. appl. Pharmac.* **47**, 331 (1979).
35. A. R. Buckpitt, D. E. Rollins and J. R. Mitchell, *Biochem. Pharmac.* **29**, 2941 (1979).
36. M. J. Pabst, W. H. Habig and W. B. Jakoby, *J. biol. Chem.* **249**, 7140 (1974).
37. A. Wendel and P. Cikryt, *Res. Commun. Chem. Pathol. Pharmac.* **33**, 463 (1981).
38. Z. Gregus, J. B. Watkins, T. N. Thompson and C. D. Klaassen, *J. Pharmac. exp. Ther.* **225**, 256 (1983).
39. P. Moldeus, B. Anderson and V. Gergely, *Drug Metab. Dispos.* **7**, 416 (1979).
40. C. Smith and D. Jollow, *Pharmacologist* **18**, 156 (1976).
41. H. W. Linde and M. L. Berman, *Anesthes. Analg.* **50**, 657 (1971).
42. B. R. Brown and A. M. Sagalyn, *Anesthesiology* **40**, 142 (1978).
43. P. E. Gooding, J. Chayen, B. Sawyer and T. F. Slater, *Chem. Biol. Interact.* **20**, 299 (1978).
44. S. Ahmad, *Drug Metab. Rev.* **10**, 1 (1979).
45. M. R. Juchau, in *The Biochemical Basis of Chemical Teratogenesis* (Ed. M. R. Juchau), p. 63. Elsevier/North Holland, New York (1981).
46. C. M. Steele, H. A. Masson, J. M. Battershill, G. G. Gibson and C. Ioannides, *Res. Comm. Chem. Pathol. Pharmac.* **40**, 109 (1983).
47. N. Wright and L. F. Prescott, *Scott. med. J.* **18**, 56 (1973).
48. S. S. Thorgeirsson, H. A. Sasame, J. R. Mitchell, D. J. Jollow and W. Z. Potter, *Pharmacology* **14**, 205 (1976).
49. R. A. Wilson and F. E. Hart, *Gastroenterology* **73**, 691 (1971).