AWARD LECTURE

Acetaminophen Hepatotoxicity: The First 35 Years*

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ABSTRACT

The acetaminophen nomogram including its uses and limitations is discussed as well as the development of the N-acetylcysteine protocol. While it has taken many years to elucidate the genetic variability and true multiplicity of the cytochrome P450 "mixed function oxidase system" many publications early on looked at the enzyme system as a single entity. Numerous articles indicated that barbiturates, anticonvulsants, and others could induce "P450" and add to the toxicity of acetaminophen. It rapidly became apparent that just because "P450" was induced when measured as a whole, not all other substrates would have changed metabolic activity. The role of diet and ethanol induction and inhibition on CYP2E1, the enzyme of greatest interest for acetaminophen is multifaceted. The lack of enhancement of acetaminophen toxicity by phenytoin and in fact, the potential for reduction of toxicity with that agent is a good example of the evolution of our knowledge. Further complicating our understanding is the introduction of misleading terms such as "therapeutic misadventure" and other expressions of molecular intent. A critical understanding of the literature makes it clear that therapeutic doses of acetaminophen either alone or in the presence of inducers do not produce toxicity. While the community of clinical toxicologists is small, it needs to be more aggressive in making sure that physicians from other specialties and non-clinical toxicology colleagues understand the significance and implications of this science.

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PROLOGUE

Before getting to the scientific portion of this presentation it is appropriate to provide a few thoughts on Matthew J. Ellenhorn, M.D., whose memory this talk honors.

He changed the very nature of the scientific presentations at this annual meeting because of his presence. When we rehearsed the talks and poster discussions at the Rocky Mountain Poison and Drug Center in the weeks before the meeting, his potential comments were the gold standard. As we convened the fellows and faculty to practice, it was my job following each presentation to proclaim loudly, "Ellenhorn!" and then ask the most difficult possible series of questions. Anticipation of his insight changed the degree of our conclusions and made sure that we stayed closer to our data. He did not disappoint us but appeared at the microphone following many platform presentations and usually began his questions with, "What is the evidence…?" Clinical Toxicology is better because of him.

ACETAMINOPHEN NOMOGRAM

The history of the development of the nomogram and its uses and limitations as well as the fact that it was not drawn originally as a 4-hour half-life line is important to its continued use (1).

It was my privilege to spend the latter part of 1973 at the Royal Infirmary of Edinburgh Poisons Unit (Ward 3), which was headed by Henry Matthew, M.D. Each day he conducted rounds on all patients in both the male and female portions of Ward 3 (2). Every patient was his ultimate responsibility and physicians at faculty, postgraduate, and student levels of training participated in those rounds. Dr. Matthew would address questions to various attendees and make observations usually concluding with a treatment or a diagnostic plan. The two poisons of greatest interest and greatest severity at that time were paracetamol (acetaminophen) and the herbicide paraquat.

I had been asked to write an article for Pediatrics about acetaminophen (APAP), which was being used in children increasingly. The editor of Pediatrics asked me to include a clear way of determining toxicity for the journal's readers. While laboratory methods for APAP were not routinely available in most hospitals in the United States, the incidence of toxicity in the United Kingdom had led to use of accurate analytical techniques in that country. There was a great deal of interest in understanding why patients were showing toxicity in the United Kingdom but not in the United States. Prior to my traveling to Edinburgh, Irving Sunshine, Ph.D. had asked me to bring back a sample of paracetamol to see if there was a difference in formulation from the versions sold in the United States. As expected Dr. Sunshine's analysis found no difference between the UK and US preparations.

While preparing the article, we decided to include a nomogram as an answer to the request by the editor. We utilized all of the 30 cases in the previous publication from the Poisons Unit, which we referenced in our article (3). We then added 34 additional unpublished cases for which the poisons unit had records. We plotted the initial plasma levels of all the cases vs. time since ingestion by history. We then drew a discriminant line best separating those who demonstrated subsequently hepatotoxicity [defined as a serum glutamate oxaloacetate transferase (SGOT) (aspartate transferase—AST) of greater than 1000 IU] at any time during their hospital course and those who did not develop that AST elevation. All 64 cases utilized in preparation of this nomogram were untreated in terms of antidotes including *N*-acetylcysteine (NAC), methionine, or cysteamine but were afforded normal care.

The publication in 1971 had stated that levels greater than 300 μg/mL at 4 hour and 50 μg/mL at 12 hour were associated with toxicity although it also suggested there was a range at the upper level. The authors of that article also suggested that a half-life greater than 4 hour would likely produce toxicity and a half-life greater than 12 hour would produce hepatic coma likely. The additional 34 untreated cases suggested that we should lower the 4-hour level to 200 μg/mL above which toxicity was likely. Thus, inclusion of that data resulted in our drawing a nomogram line between the plotted cases starting at 4 hour and 200 μg/mL and intersecting 50 μg/mL at 12 hour (Fig. 1). The slope of the line was different than that which would have been suggested by the data from the first 30 published cases utilizing 300 and 50 μg/mL, which would have been a 3-hour half-life. The fact that our published line turned out to have the slope of a 4-hour half-life was not based on APAP kinetics. Since it was drawn to discriminate between two groups of patients, the half-life had nothing to do with its construction. However, plotting repeated patient levels on the nomogram could indicate readily if the half-life is shorter or longer than 4 hour. An increase in half-life reflects a change in hepatic metabolism. The nomogram intended to provide clinicians a method to predict whether patients would develop hepatic toxicity following an *initial* plasma level. Given the importance of absorption, interpretation of exposure risk via use of the
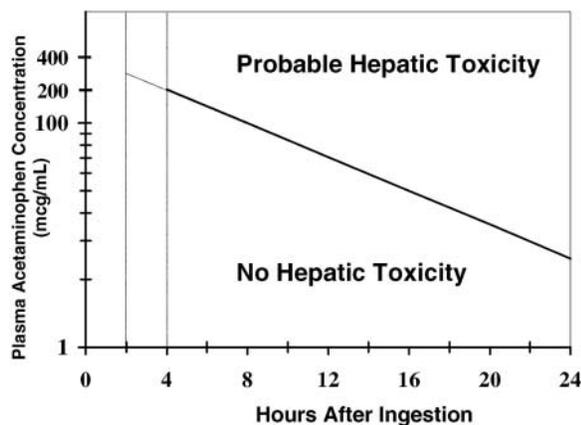
Figure 1. Acetaminophen nomogram (1).

nomogram was restricted to levels obtained 4 or more hours after a single ingestion of APAP. The nomogram was only intended to guide an early clinical decision irrespective of the patient's half-life following a single acute overdose and was not intended to provide information in chronic ingestions.

A dotted line was added from 2 to 4 hour to permit some interpretation of earlier levels although it was unclear whether such levels would be valid. Absorption of APAP following an oral ingestion of tablets in a therapeutic dose ranges from 70 to 90% of the amount consumed and peak plasma values occur between 20 and 90 minutes (4). Interestingly the percent absorbed was higher with the higher doses consumed and lower with the minimum doses consumed orally. There was concern regarding co-ingested drugs such as propoxyphene, which delayed gastric emptying. It seemed safer to obtain a plasma level at 4 hour or later and increase our confidence that a measurement at that time would result in no higher peaks.

In that same article, we reported 156 additional cases in the United States although many of them did not have plasma levels. The intent of that report was simply to suggest that this toxicity was not unique to the United Kingdom and to encourage clinicians in the United States to begin looking for them.

THE *N*-ACETYLCYSTEINE PROTOCOL

Given the pioneering work of Mitchell et al., in working out the mechanism of APAP hepatotoxicity it appeared that treatment of these patients was a real possibility (5). The best prospect seemed to be with those agents containing a sulfhydryl group. Elliott Piperno and

colleagues at McNeil Consumer Products Company laboratories developed an animal model and proved that Mucomyst® (NAC) was an effective agent to protect against APAP hepatotoxicity (6).

The decision was made to develop a multicenter open protocol in the United States to determine as rapidly as possible if NAC was safe and effective as a treatment for APAP overdose and those details have been published (7). Although the Food and Drug Administration (FDA) rejected our request for a double blind study, we were given approval to utilize NAC orally in an open study. Due to several presentations at meetings and published letters to the editor following the use of cysteamine it was considered unethical to withhold treatment (8–11).

The FDA reviewers required an alteration of the original nomogram as part of the NAC protocol. They wanted some additional safety built in and required that a new "safety" line be added 25% below the original line. Thus, the second study design nomogram was developed and utilized for the entire protocol (Fig. 2). A higher line 100% above the original nomogram line was also incorporated in an attempt to define higher risk but did not affect the treatment decision. The protocol required that plasma levels be obtained at 4 or more hours after ingestion and that treatment with NAC was begun prior to 24 hour after ingestion. In some cases, treatment was begun prior to obtaining a plasma level and discontinued if it was below the lower treatment line. This occurred since we believed that it was safer to treat and stop than await plasma levels, which in some locations took more than 24 hour. Additionally some patients had a plasma level in time but were begun on NAC after 24 hour and
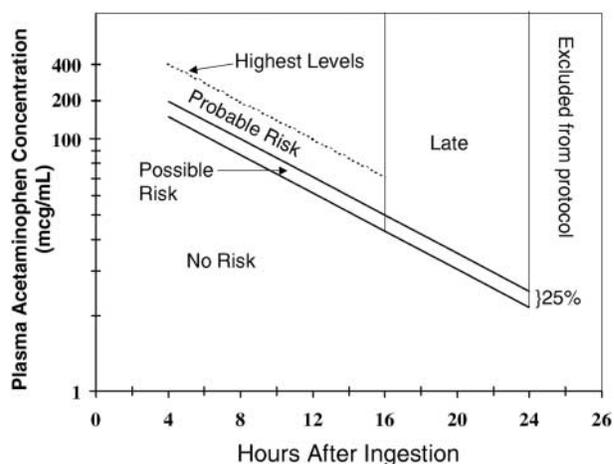

Figure 2. Study design nomogram (7).

still received the full protocol dose. Those patients who were treated "unnecessarily" provided additional safety data for the use of NAC and a few did develop hepatotoxicity.

Acetaminophen levels were not available at all institutions and many times those assays were colorimetric and either easily interfered with or inaccurate by as much as 700% (12). The multicenter study required that plasma samples for APAP had to be shipped to our laboratory regardless of whether the analysis was performed locally or not (7). We also performed and reported stability studies confirming the ability to transport samples across the United States. Development of a new technique permitted analysis on very small samples (13). By performing all these analyses in one laboratory, we were confident that consistent and comparable results would be obtained for the protocol.

The 1988 NEJM publication based on 11,195 exposures, with 2023 meeting inclusion criteria reported many outcome parameters (14). That publication utilized a risk analysis nomogram, which had the original nomogram line, the 25% safety line and a 50% higher line at 4 hour of 300 μg/mL (Fig. 3). All 3 lines were extended to 24 hour. This nomogram, a modification of the study design nomogram in Fig. 2, was utilized to divide patients into the risk analysis groups. Multiple cuts of the data, in addition to the nomogram, were done to look at various outcomes from the protocol and compare them to previously published studies. Hepatotoxicity was minimal regardless of the initial APAP concentration when NAC was begun within 8 hour of ingestion. There was an increase in the frequency of hepatotoxicity when the treatment delay reached 8–16 hour. The reduction of the severity of this increase between 16 and 24 hour indicates that there is a diminution but not complete loss of effectiveness after 16 hour. While it is clear that treatment prior to 8 hour is superior to treatment after 8 hour, work published since this study indicates that NAC may offer some benefit even beyond the 24-hour protocol cutoff (15,16).

As part of the evaluation of the 11,195 reported exposures, we noted that 517 had APAP levels below the study nomogram line or otherwise did not meet protocol criteria (14). Those cases were mentioned only briefly in that report. Some of those patients were administered a full course of NAC even though they had nontoxic APAP levels or entered late. All cases with an SGOT of 1000 IU or greater were plotted on the study design nomogram (Fig. 2), including the nonprotocol patients, and a "treatment outcome" nomogram resulted. This previously unpublished outcome nomogram shows the
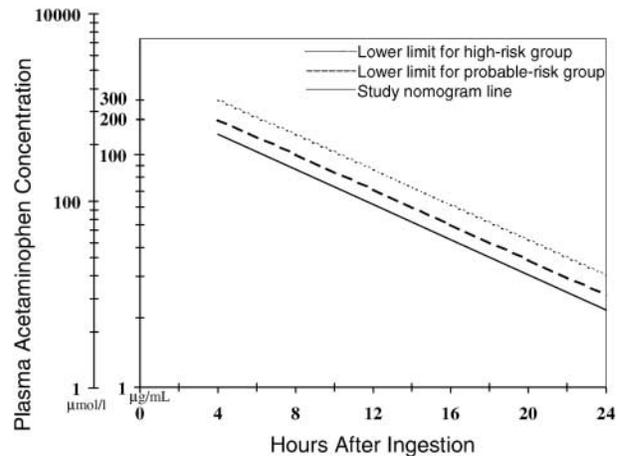

Figure 3. Risk analysis nomogram (14).

percent of toxic cases related to the original plasma level and historical time of ingestion (Fig. 4). The original nomogram indicated that there should be no toxicity below the nomogram line. Given the inaccuracy of patient histories and the dependence on time it is not surprising that some cases whose initial plasma level was below the line showed toxicity. A small difference in historical time of ingestion can change the location on the nomogram and the likelihood of toxicity significantly in either direction. This outcome nomogram only indicates the toxicity of those patients who have been treated with a full course of NAC.

The dosing of NAC for the protocol was based upon our observations of the half-life of APAP in 75 preprotocol patients from our center (some of which were treated and
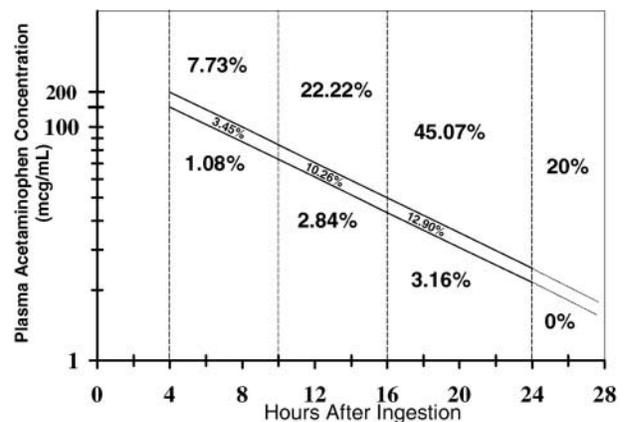

Figure 4. Outcome nomogram—additional data from the National Multicenter Study.

some of which were not) as well as the understanding of APAP metabolism (5,17–20). There was a substantial range of half-life with some as high as 60 hour in previously reported untreated patients who were hepatotoxic (3). Utilizing the disappearance of APAP after five half-lives with a 4-hour protocol provided a 20-hour treatment regimen. Given the fact that we had seen a number of patients with longer half-lives the FDA reviewers were not comfortable with that length of NAC administration. Eventually it was decided that a 3-day protocol, which allowed disappearance of APAP with a half-life modestly longer than 12 hour would be implemented.

At that time we had substantial difficulties measuring the mercapturic acid, cysteine, and other conjugates in the urine of large numbers of humans and were unable to relate the disappearance of the toxic metabolite to time in a statistically valid number of patients. It would have been desirable to measure conjugates and determine a more accurate protocol related to length of treatment and initial APAP level. It had been our initial intent to provide guidance for the length of time of NAC administration related to the height of the APAP level but we were unable to do so. There did not appear to be a direct correlation between dose and enzyme level although higher doses, as documented with plasma levels, generally produced higher levels of hepatic enzymes as shown on the nomogram. We had wanted to state that if the level was, for example, 300 μg/mL, treatment should last X hour and if it was 400 μg/mL it should last Y hour and that was not possible. Our observations indicated that toxicity occurred above certain levels but the height above those levels did not relate necessarily to a further refinement of severity. We therefore concluded that we were seeing a threshold effect and were unable to define it. It was our opinion that it was important to maintain a NAC level as long as the liver was capable of producing the toxic metabolite. There were a few of the initial patients on the protocol who terminated NAC early (and were therefore excluded from the protocol) and showed a second increase in enzymes.

It was established that the hepatic damage would occur upon 70% depletion of glutathione (17). Given an average 1.5-L liver with 6 mmols of glutathione, then 4.2 mmols would have to be depleted to reach the threshold of toxicity. Since approximately 4% of a dose of APAP is converted to *N*-acetyl-*p*-benzoquinone imine (NAPQI), we could calculate how much APAP would be expected to produce toxicity. Since there is 151.2 mg/mmol of APAP then (4.2)(151.2)/0.04 equals 15.9 g which would have to be absorbed in a 70 kg human to arrive at *threshold* toxicity (21). Further, 4% of 15.9 g

results in 0.635 g of NAPQI produced assuming the same molecular weight.

Based on the estimated absorption and turnover rate of glutathione it was determined that approximately 6 mg/kg/h of NAC would be required. Given the desire by the FDA to have a safety factor of 3 and given the problems with continuous administration of oral medication it was eventually decided that 70 mg/kg every 4 hour would be utilized. The concept of the 140 mg/kg loading dose was added since it was felt that getting a high dose into the liver earlier and maintaining it was likely to be more successful.

Mucomyst, brand name for NAC, was widely available in institutions in the United States for other purposes. Although it was a sterile solution, it was only approved for oral use. Though some centers gave NAC intravenously after passing it through a Millipore filter, it was not approved for that purpose. Parenteral NAC was available in the United Kingdom.

The 72-hour oral NAC protocol is probably unnecessary in many cases where the drug has a shorter half-life and disappears before the full dosage is completed. There is, however, no convincing evidence that a shorter treatment is effective. Stopping treatment earlier without an adequate study may mean that some patients will have a relatively asymptomatic and a possibly, but not assuredly, modest rise in enzymes. The only way to be certain that NAC can be stopped earlier is to perform a prospective trial in which patients have enzymes measured well past the point at which NAC is terminated. One retrospective study looked at 75 patients and found three of 33 (9%) who developed an increase in aminotransferase levels within 24 hour of the early cessation of NAC (22). It is unknown whether any of the remaining patients had an increase in enzymes. The authors proposed future studies to evaluate the early termination of NAC therapy.

METABOLISM

The metabolism of APAP is well described and there is little question that the toxic metabolite is the electrophile NAPQI. Although several other metabolites were considered, including epoxide formation, semi-quinone radical, and 3-hydroxy APAP, evidence has discounted those possibilities (23–25).

Since the first articles regarding APAP hepatotoxicity were published in 1966 more than 30,000 additional articles with keywords related to this phenomenon have appeared in the medical literature (26–28). Our under-

standing of APAP metabolism and toxicity as well as its inducers and inhibitors has grown dramatically during these past 35 years. Unfortunately, many early observations, which later turned out to be incorrect have been perpetuated. In order to address some of those misperceptions a clear understanding of our current knowledge regarding the enzymes responsible for the metabolism of APAP NAPQI is imperative.

Several isoenzymes including CYP1A2, CYP2E1, and CYP3A4 are capable of producing NAPQI in vitro under various circumstances in both animals and humans (29). Recently it was questioned as to which of these isoenzymes is responsible for NAPQI production in humans following ingestion of APAP (30). It is, however, now clear that CYP2E1 is the primary enzyme overwhelmingly responsible for this metabolite in humans (31).

The CYP2E1 enzyme located primarily intracellularly in the endoplasmic reticulum contains 493 amino acids and has a molecular weight of 56,820. There is only one gene for the CYP2E subfamily in humans and it is located on chromosome 10 spanning 11,413 base pairs (32,33). While there are at least three genotypes it is currently unclear as to the significance of this polymorphism (34,35). It is known to metabolize over 75 different compounds. It is unique among these CYP enzyme families in its ability to produce reactive oxygen radicals through reduction of dioxygen and it is the only one strongly induced by ethanol which itself is oxidized. This oxidation of ethanol through this enzyme is one way of generating aldehyde, which in turn is capable of producing membranous lipid peroxidation (25). These free radicals can stimulate immune cells to produce cytokines and Ito cells to produce collagen. Macrophages, which can also accumulate centrilobularly following APAP hepatotoxicity, are capable of enhancing toxicity by release of hydrogen peroxide, superoxide anion, and hydroxyl radicals. Further, antibodies to tumor necrosis factor (TNf-α) a cytokine, reduce liver injury induced by APAP as does pre-treatment with dextran sulfate (36). Interestingly interferon inhibits expression of the enzyme while interleukin seems to play a variable role in expression of the enzyme.

Interleukin 18 (IL-18) is detected following APAP injection in the mouse peaking at 8 hour without complete reversal from IL-18 antibodies (37). The authors conclude that the apoptosis is Fas independent. This same group showed a decrease in Interleukin-6 (IL-6) in association with APAP hepatotoxicity in humans. Acute phase proteins such as C-reactive protein (CRP) are IL-6 dependent. The authors suggest that measuring IL-6 or CRP may serve as a prognostic factor in APAP overdoses (38).

The CYP2E1 has variable concentrations in humans ranging from 48.8 to 368 (relative immunoblot density) IOD/nmol total P450 (29). In the rat, the levels have a range of 20-fold and in humans, an even greater range (39–41). These ranges may be due to induction by different exposures to various xenobiotics and the effects of diet or a combination of both. The significance of these ranges is unclear in that specific enzymes have been shown to limit increases following induction. For example, CYP2E1 has a 2-fold maximum increase when measured in alcoholics utilizing a chlorzoxazone probe (42). The effect is not consistent since 78% showed an increase over controls but 22% of alcoholics with similar ethanol consumption histories have no increase in CYP2E1 over controls (43).

CYP2E1 is located in most tissues with the largest concentration in the liver. Differential immuno-histochemical staining studies have demonstrated that CYP2E1 is located primarily in the hepatocytes surrounding the hepatic venules, which are also referred to as perivenular, zone 3 or the centrilobular region (44). Only insignificant amounts are found in other regions of the hepatic lobule outside of approximately five cells around the central venules. Alcoholics have an increased concentration in the centrilobular region. Work in ethanol-induced rats indicates a concentration as high as 0.1 mmol in zone 3 (45).

To think about hepatotoxicity, centrilobular necrosis, and inducers it is instructive to examine carbon tetrachloride (CCl_4), which was very well studied. Once absorbed CCl_4, under low oxygen tension is reduced by CYP2E1 to the trichloromethyl radical $CCl_3^{\bullet}$ (25). When the oxygen tension is increased the trichloromethyl radical is oxidized to $CCl_3OO^{\bullet}$ (trichloromethylperoxy radical). Both of these radicals are direct alkylating agents and can induce lipid peroxidation. The most interesting aspect of this is the predominantly centrilobular hepatotoxicity. This is primarily due to the oxygen gradient, which is high at the portal triad and gradually is reduced to near zero in zone 3. At higher oxygen concentrations, the $CCl_3OO^{\bullet}$ radical is favored and this is capable of reacting with thiols. Since the thiol glutathione is readily available, it reacts and detoxifies the free radical resulting in little cellular damage in zone 1 near the portal triad. In the zone 3 anaerobic area $CCl_3^{\bullet}$ is the primary radical formed and it does not readily react with thiols. Both covalent binding and lipid peroxidation occur in zone 3 resulting in a picture of centrilobular hepatotoxicity (46,47). Additionally, since there is little

CYP2E1 found in zone 1, less of the carbon tetrachloride is metabolized to a radical in that area.

The effect of induction and inhibition on carbon tetrachloride is very clear in animals. The LD_{50} is increased 7-fold when rats are pretreated with phenobarbital or acetone and reduced 4-fold when treated with a low protein diet (48). When rats are treated with both a low protein diet and phenobarbital the LD_{50} is returned toward control levels. In contrast to humans, phenobarbital induction of CYP2E1 carbon tetrachloride metabolism is well demonstrated in animals. CYP2A5 in the mouse and CYP2B4 in the rabbit are inducible by phenobarbital and are capable of metabolizing carbon tetrachloride among other enzymes. It is therefore possible that isoenzymes other than CYP2E1 may be related to the mechanism since phenobarbital does not induce CYP2E1 in humans.

Like other enzymes CYP2E1 induction and inhibition can be seen at transcriptional, pretranslational, translational, and posttranslational points. The role of transcriptional expression is probably minimal for this particular CYP enzyme in terms of APAP. It appears that the most important induction mechanism is posttranslational protein stabilization by xenobiotics although all four mechanisms may play a role in determination of the total expression of the enzyme. CYP2E1 turnover has a biphasic pattern with half-lives of 7 and 37 hour. Acetone, binding as a ligand substrate, dramatically extends the fast component degradation so that the half-life appears to have a single 37-hour component (49) Similarly ethanol, 4-methylpyrazole, pyrazole, and isoniazid produce the same effect all without any transcriptional change in mRNA for CYP2E1 (50–52). The ligand attachment of substrate to the enzyme prevents phosphorylation and instead auto-phagocytosis followed by slower lysosomal degradation occurs. If glucagon or epinephrine is utilized the degradation of the enzyme is faster. This is due to activation of kinase, which leads to phosphorylation of the enzyme (53).

Following use of inducers the enzyme is increased in the same perivenous site indicating a local increase in the enzyme rather than development in other cells. However, when growth hormone is inhibited by hypophysectomy, recruitment of cells in zone 2 occurs, and CYP2E1 can be produced indicating transcriptional activity on the apoprotein. Additionally, growth hormone repression of CYP2E1 may be related to the levels of the enzyme seen in the young animal. At birth there is a substantially higher level of enzyme than later in life, which may correlate with increasing levels of growth hormone (54).

Acetone, ethanol, pyrazole, isoniazid, and a few others serve as both an inducer and substrate for CYP2E1. Isoniazid initially inhibits and then enhances formation of NAPQI (55). This is what would be expected of ligand stabilization, which inhibits the degradation of the enzyme. Acetaminophen, chlorzoxazone, carbon tetrachloride, and some others are substrates for the enzyme but do not induce it. As such, chlorzoxazone is used as a marker for CYP2E1 metabolism (56). Imidazole compounds (e.g., ketoconazole) are examples of agents that induce the enzyme but do not serve as a substrate (57). This is an important distinction when considering long-term use of APAP. Self-induction does not occur, and APAP does not have cumulative kinetics. Thus, changes in long-term use of this drug can only be affected by other mechanisms.

Acetone and other ketones are important examples in our understanding of the activity, induction, and inhibition of the CYP2E1 enzyme. Several nutritional processes that generate ketones may produce effects on the enzyme through this indirect process. Since induction of the enzyme is seen in diabetic and obese patients who have increased ketone levels, it was suggested that acetone serves as a mediator. However, the levels of acetone achieved in these circumstances may not be sufficient to simply substrate stabilize the enzyme but may additionally produce some transcriptional enhancement.

Unsaturated fats clearly enhance the induction of CYP2E1 when coupled with ethanol. Early work from Lieber's laboratory examining ethanol replacement of carbohydrates in the diet of rats showed induction of the enzyme (originally referred to as "microsomal ethanol oxidizing system") (58,59). Further work has indicated that a high fat to carbohydrate diet enhances the effect of ethanol while ethanol in a more balanced diet has substantially less effect. It is well known that alcoholics with high consumption of saturated fat have a lower incidence of liver damage than alcoholics with a high consumption of unsaturated fats (60). Administration of linolenic acid, which is the major unsaturated component of corn oil, is twice as likely to produce liver damage than those with diets in which highly saturated tallow was substituted.

Imidazoles inhibit CYP2E1 and prevent the conversion of acetone to glucose as part of this enzymes role in gluconeogenesis. This effect would be seen in starvation or high fat diets where ketones abound. Insulin-dependent diabetes induces CYP2E1, which can be reversed by insulin (61). In fasting, starvation and diabetes the indirect mediator capacity of acetone on

CYP2E1 is likely. This CYP2E1 increase in diabetics may also be related to the decrease in growth hormone in those patients. Thus several mechanisms appear to play a role in levels of the enzyme.

The important question as to which enzymes produce NAPQI following an APAP overdose in humans has been resolved recently. Several studies have looked at the production of NAPQI from CYP2E1, CYP1A2, and CYP3A4. In one in vitro study utilizing human liver samples the conclusion was that CYP1A2 and CYP2E1 were responsible for all of the production of NAPQI and that CYP2C9 and CYP2C19 contributed only negligible amounts (62). Additional work utilizing in vitro human microsomes showed that NAPQI could be produced through CYP3A4. The activity of this isoenzyme on APAP was proven utilizing the CYP3A4 specific inhibitor troleandomycin (TAO). Further, a metabolite of disulfiram (diethyldithiocarbamate or DDC) a specific inhibitor of CYP2E1 was utilized to further isolate the phenomenon. They concluded that APAP might be an inhibitor of CYP3A4 and that the enzyme played a minor role in the production of NAPQI in humans (29). They also suggest that high concentrations of APAP may inhibit CYP3A4 substrates in the gut during first-pass metabolism in this organ.

However, in vivo work from this group in human volunteers produced a more clinically relevant outcome (31). In order to isolate effects they chose to utilize rifampin as an inducer of CYP3A4 to determine if this enzyme contributes to NAPQI formation. Rifampin also induces CYP2C9, CYP2C19, and CYP1A2 all of which have been shown to have minimal NAPQI formation from APAP. Additionally, they utilized disulfiram as an inhibitor of CYP2E1. Disulfiram had a substantial effect on NAPQI production reducing the conjugates through that pathway by 69%. It was shown that disulfiram does not inhibit CYP2A6, CYP3A4, and some other isoenzymes in rats (63) and humans (64). Rifampin induction produced an insignificant change in NAPQI formation indicating that CYP3A4 plays no real role in production of the toxic metabolite from APAP. Although disulfiram may be useful in prevention of APAP hepatotoxicity, its inhibition of aldehyde dehydrogenase mitigates against its use if ethanol is present. Certainly alcoholics already on disulfiram at the time of APAP consumption will exhibit hepatoprotection (31). Work from this same laboratory demonstrated by pre-treating volunteers with omeprazole (a potent inducer of CYP1A2) that CYP1A2 was not involved by demonstrating lack of NAPQI enhancement. Caffeine clearance by demethylation

was also shown in these same patients to confirm the induction of CYP1A2 (65).

By genetic selection of mice where CYP2E1 was "knocked out," it was shown that lack of this enzyme diminishes but does not eliminate hepatotoxicity completely (66). Other work on CYP1A2 and CYP2E1 double-null mice showed that while knockout of each of these enzymes increased survival rates, those with both enzymes "knocked out" had almost 100% survival (67). This would indicate that CYP1A2 plays some role in production of NAPQI from APAP. However, just as other animal and in vitro work is inconsistent with human in vivo work this model may have no relevance to human APAP hepatotoxicity.

This is a brief presentation of CYP2E1 metabolism describing effects that may be due to direct action on the enzyme or through indirect mechanisms. The selection of information presented in this section will be utilized to explain current knowledge of aspects of induction addressed in the remainder of this paper. The differences between in vivo and in vitro work as well as animal and human studies must be considered when evaluating the literature. Each of these must be taken into account when considering induction, inhibition and mechanism of action for clinical observations and treatment.

INDUCTION AND INHIBITION

Our publication in 1975 made reference to phenobarbital inducing the P450 mixed function oxidase system (1). This had been reported in animal work and it was a serious concern that the barbiturates, then still widely utilized as soporifics would enhance the toxicity of a dose of APAP (17). The in vitro preparation of those enzymes located on the endoplasmic reticulum involves grinding of liver samples and centrifugation. Selection of the correct layer from the ultracentrifuge samples results in fragments of the endoplasmic reticulum referred to as microsomes. Prior to our understanding of isoenzymes the microsomal preparations were treated as a whole in the laboratory. This masked or confused many effects until further methods were developed to separate isoenzymes. Early literature did not take into account the different isoenzymes of P450 or what we now know are interspecies differences in metabolism.

Phenobarbital produces a pleiotropic response in the liver inducing CYP2B, CYP2C, UDP-Glucuronosyl-transferase, aldehyde dehydrogenase, and glutathione *S*-transferase among others. This is consistent with the early phenobarbital work, which showed a nonspecific

increase in metabolic activity of P450 and other hepatic metabolism. Biotransformation reactions are divided generally into Phase I functionalization reactions mostly located on the endoplasmic reticulum such as the CYP enzymes and Phase II biosynthetic reactions, which are generally cytosolic such as UDP-glucuronosyltransferase or glutathione *S*-transferase. Phenobarbital is capable of inducing many enzymes in both categories, which caused confusion in some early work.

We now know that CYP2E1 is the primary toxic metabolic enzyme for APAP and that it does not metabolize or become induced by any of the barbiturates. Additionally, there had been suggestions in the literature that phenytoin, other antiepileptics, antihistamines, diethylstilbestrol, ethacrynic acid, ethanol, promethazine, and other agents could all enhance APAP toxicity (3,68). Others suggested that environmental contaminants such as DDT, lindane, and certain food additives could all induce the activity of drug metabolizing enzymes in hepatic microsomes (69). The authors further expressed concern that such induction would affect a number of other drugs by shortening half-life and changing plasma levels.

PHENYTOIN DOES NOT INDUCE CYP2E1 BUT MAY BE HEPATO-PROTECTIVE IN ACETAMINOPHEN OVERDOSE

It has taken many years to begin to work out which drugs and chemicals induce, inhibit or are metabolized by which isoenzymes. The relationship between APAP and phenytoin is a good example of how concepts changed as knowledge and techniques improved.

A study looking at administration of APAP in epileptic patients vs. controls was reported in 1979 (69). Only 1 of the 6 patients was taking a single antiepileptic drug. The authors found no real differences in urine recovery of conjugated and free APAP between control and epileptic patients. Following intravenous administration of APAP they concluded that there was no significant difference between the control and epileptic group despite statistically significant differences in area under the curve and clearance. They concluded that there was a significant difference in plasma APAP concentration in the epileptic group between the oral and intravenous administration and concluded that it was related to lower bioavailability from first-pass metabolism. Further, they concluded that enzyme induction had occurred but did not speculate as to which enzyme(s)

that was and did not separate the known different metabolic processes.

In a report published in 1981 a single oral dose of APAP was given to patients with enzyme induction from taking anticonvulsants or rifampin (70). Thirteen of the patients were consuming phenytoin alone. They found no difference in the urine recovery of mercapturic acid and cysteine conjugates between the patients and controls. They found a significant increase in glucuronide conjugation with reduction in both sulfate and unchanged drug. They also found that the half-life of APAP was reduced in the patients, which was confirmed by a halving of the antipyrine half-life (an in vivo human measurement). The authors state that the lack of increase in mercapturic acid and cysteine conjugates (from NAPQI) was unexpected. While they did not eliminate the possibility of induction of oxidative metabolism by phenytoin they concluded that it was not likely to be of clinical significance although they were unsure in overdose patients. They concluded, correctly, that, "... patients with enzyme induction due to treatment with anticonvulsants and rifampin do not seem to be at greater risk of paracetamol (acetaminophen) hepatotoxicity ..." This work was one of the first times that consideration of differences in enzymes might be responsible and the authors concluded correctly that there had been an enhancement of glucuronide but not mercapturic acid pathways. The isoenzymes of P450 were not yet adequately understood.

In 1984, a study reported an increase of 60% in mercapturic acid conjugates in patients who were epileptic and concluded that these patients were at greater risk from APAP (71). Unfortunately, they were mixed phenytoin and carbamazepine patients, and the effect of each drug was not separated. There were other reports of mixed inducers, which have little value and simply add confusion (72). A larger report of overdoses of APAP in patients with a mixed series of antiepileptics concluded that they enhanced APAP hepatotoxicity (73). It is impossible to draw that conclusion from such a series of cases. Additionally some of the patients in the latter study consumed ethanol and all patients consumed an APAP dose in excess of therapeutic with one patient as high as 75 g. More recently, data from that same group changed the view of their earlier article concluding that "taking anticonvulsant medication or consuming excessive alcohol was associated with overall mortality rates of 33 and 37%, respectively, which were not significantly different from that of nonhigh-risk patients" (74).

A series of cases with phenytoin alone compared to a control group showed a lower recovery of mercapturic

acid, cysteine, and sulfate conjugates but a higher recovery of glucuronide metabolites. The conclusion was that there was not an increased risk from therapeutic APAP in phenytoin-treated patients (75).

Is there a phenytoin–APAP interaction? Phenytoin is metabolized primarily to p-HPPH (5-(4-hydroxyphenyl)-5-phenyhydantoin) and is then conjugated by glucuronide and excreted. The metabolism to p-HPPH is facilitated by CYP2C9 and CYP2C19. Phenytoin is a potent inducer of glucuronosyltransferase, which facilitates the metabolism of APAP to its nontoxic glucuronide metabolite as well as phenytoin to its glucuronide. This agrees with the earlier work in patients discussed above in which an increase in glucuronide and a decrease in other metabolites from APAP was shown. Production of NAPQI from APAP is not moderated by CYP2C9 or CYP2C19. As noted above, CYP3A4 does not play a role in APAP metabolism while it is central to the metabolism of phenytoin. CYP2E1 plays no role in phenytoin metabolism.

The conclusion is that phenytoin does not enhance APAP hepatotoxicity and in fact, by increasing glucuronidation may be hepato-protective. It took over 20 years to work out all of the mechanisms involved and answer the questions raised by early investigators. It is now clear that a phenytoin–APAP interaction does not increase APAP hepatotoxicity.

ETHANOL DOES NOT ENHANCE ACETAMINOPHEN HEPATOTOXICITY IN THERAPEUTIC DOSES

Ethanol has become the most interesting and important inducer and inhibitor of CYP2E1 of all those that have been studied. It was suggested as an enhancer of hepatotoxicity following APAP *overdose* early in the APAP clinical literature (68). In the first comprehensive report of the NAC protocol for APAP overdose we found a higher bilirubin in those with ethanol abuse compared to those without ethanol history as the only distinguishing feature (7). Additionally we found lesser hepatic abnormalities in those patients with acute ethanol consumption. In a series of severe APAP overdoses, the survival rates between drinkers and nondrinkers was not significantly different (74). However, in a small subset of this same study looking at those who had been treated psychiatrically for alcoholism, there was a higher mortality, which was also associated with a higher overdose of APAP ranging from 20 to 90 g and a median of 54 g.

A great deal of experimental work was done on the effect of ethanol on CYP2E1 as noted below. Eighty-six percent of alcoholics have antibodies to the alpha-hydroxyethyl CYP2E1 adduct (76).

With the indication that CYP2E1 is the only enzyme significantly related to NAPQI production from APAP and that it is induced by ethanol, an understanding of the effects on humans must be examined rigorously. Additionally, it must be recalled that ethanol itself can cause substantial hepatotoxicity without the presence of any other drugs.

Ethanol both induces and acts as a substrate for CP2E1 resulting in simultaneous inhibition and induction. There are two methods for the induction of CYP2E1 by ethanol, which appear to be concentration dependent. Below 250 mg/dL of ethanol the activity appears to be primarily enzyme stabilization by ligand formation, which reduces degradation of the enzyme. At concentrations greater than 250 mg/dL of ethanol it appears that there is de novo synthesis of CYP2E1 through mRNA stabilization and perhaps other mechanisms although apparently not transcriptional changes as noted above (40,44,77–79). Most importantly there appears to be a maximum induction of a 2-fold increase in NAPQI production following ethanol induction experimentally and in an adaptation of a computer model (42,55).

In a work that has not yet been published, it appears that a chronic alcoholic might decrease the synthesis rate of a transporter that takes glutathione from the cytosol into the mitochondria. Since the mitochondria are a target for NAPQI it is possible that depletion of glutathione in the mitochondria may help explain the toxicity in the alcoholic patient with acetaminophen overdose (80).

Although there has been a great deal written about therapeutic doses of APAP causing toxicity in alcoholics as a result of ethanol induction, a careful review of the literature does not support this view. We have reviewed more than 2000 reports in the literature dealing with the therapeutic use of APAP in the alcoholic (81). Data was separated into Class I (controlled randomized and blinded clinical trials), Class II (prospective nonrandomized or nonblinded clinical trials, cohort or well-designed case–control studies, dramatic results in uncontrolled studies and volunteer studies), and Class III (retrospective case series, case studies). Class I and II data demonstrated little if any toxicity while Class III data suggested that, "… a morbid fate awaits some alcoholic patients who ingest a therapeutic dose of paracetamol." Why such a difference?

The class III data relied exclusively on the patient history. In many instances, review articles published some new cases and added previously published cases at times misrepresenting the original data and many times duplicating it.

One example of this is found in an article published in 1986 entitled "Acetaminophen Hepatotoxicity in Alcoholics: A Therapeutic Misadventure." (82). The authors state, "There seems little doubt that none of the 25 patients deliberately took an overdose of APAP or deliberately distorted the information regarding the amount that they consumed." The authors have therefore tried to establish *intent* as part of the diagnosis. Pharmacologically, the intent of the patient has no bearing on the metabolism or toxicity of a drug and this should not be included in the medical literature as it is confusing and irrelevant. Further, they cannot know the intent of a patient by reading a case report. Perhaps a psychiatrist could delve into the matter by talking directly to each patient but these authors did not do that.

Table 1 in this study shows the details for 19 patients in addition to 6 new patients. Fifteen of those patients consumed more than 4 g in 24 hour with a range 4.5–16.5 g—clearly supratherapeutic.

One of the cases included in this article is that of Himmelstein et al. (83). The table in the 1986 paper lists the patient as having consumed 4 g of APAP. Looking at the history in the original article however, it states "The patient had been an alcoholic for 6 years, drinking up to 2 L of whiskey per day. He was well until 4 days before admission when he had several teeth extracted. Twelve APAP 325 mg with codeine tablets were prescribed for pain." It is important to note that on day 3 his APAP level was 63 μg/mL, which, if he were 70 kg, would have given him 4.5 g of APAP as a body burden at that time. Since 3 days had elapsed since admission and 7 days had elapsed since his dental procedure he clearly must have taken an overdose of APAP to still have a residual level of that height. Discrepancies are found when reviewing the originals of some of the other references.

An examination of the six new patients reported in this study shows:

- Patient 1 reported in this study reported a therapeutic dose of APAP but had none detected by the laboratory.
- Patient 2 reported in this study ingested 12.5 g of APAP together with a six-pack of beer *each day*. It is hard to understand that this patient would not have thought it an overdose to take 25 tablets per

day of extra strength or 39 tablets per day of regular strength APAP.
- Patient 3 reported in this study reported 3.8 g per day but had none detected by the laboratory.
- Patient 4 reported in this study ingested 4–6 g per day of APAP on a chronic basis.
- Patient 5 reported in this study stated that she took, "not excessive" doses of APAP.
- Patient 6 reported in this study took a therapeutic dose but had none detected by the laboratory.

In a large case registry study the concepts of therapeutic dose and therapeutic intent are once again confused (84). Review of the raw data from this study shows that many of the cases were anecdotal in nature and they would clearly not pass scrutiny when examined individually. Some were merely letters to the authors recalling various cases with inadequate data to be evaluated.

A 1997 publication reports 71 cases of which 21 were hospitalized for excessive APAP consumption without suicidal "intent" and are considered "accidental" (85). Only five were admitted to the hospital because of a specific history of excess APAP ingestion; the majority had abnormal aminotransferase levels or prothrombin times that mandated their admission. These were contrasted with 50 APAP overdose patients who were suicide attempts. The authors conclude that since there was higher morbidity and mortality in the "accidental overdose" group and that this group also had a higher incidence of alcohol abuse that alcohol plays a role in the hepatotoxicity.

In a letter published later, Walker comments "Untenable case definitions entrapped Schiodt and associates in their study of APAP toxicity in an urban county hospital. They recognized their predicament but nonetheless trumpeted a

Table 1

P450 Levels in Hepatic Disease

Type	% of Control P450
Acute viral hepatitis	
Mild	100[a]
Moderate	118[a]
Healing	92[b]
Fatty liver	102[b]
Cirrhosis	34[b]

[a] Ref. (96).
[b] Ref. (97).

conclusion that they knew was questionable. Patients with accidental overdoses became eligible for analysis because of morbidity at presentation. This is why they had higher rates of morbidity and mortality than those who attempted suicide." Thus this gives a skewed bias of the data (86). In fact, looking at a previous study, severe chronic alcoholics had a substantially greater ingestion of APAP but when properly compared to other cases showed no difference in mortality indicating that ethanol was not a determining factor (74).

A re-examination of our earlier data demonstrated that acute APAP overdose with chronic ethanol was associated with increased hepatotoxicity only in at-risk cases (87). Toxicity, as defined by AST and alanyl transferase (ALT) was worse in patients categorized in the high-risk (above the 300 μg/mL line) APAP overdose. Toxicity was no different in low-risk (below the 200 μg/mL line) overdoses whether they were alcoholics or not. This again confirms observations from a previous report by a different group (74).

Concerns regarding fasting and malnutrition have been raised in a number of articles and were addressed in a retrospective case review (88). All the patients who took APAP for therapeutic reasons and developed severe hepatotoxicity took more than 4 g/d. Seven of the 10 patients who developed hepatotoxicity after consuming between 4 and 10 g/d chronically took APAP but only one was a recent ethanol user. All of the patients who developed hepatotoxicity after consuming more than 10 g/d were ethanol abusers.

The terms fasting and malnutrition have been misused in numerous articles. Reduction of food intake for a few days has not been shown to decrease glutathione in humans. As noted below under nutritional aspects, true malnutrition is capable of reducing P450 enzymes as well as glutathione. In a prospective trial, restricting patients to 500 or 1000 calories/day for 5–13 days a dose of 2 g APAP orally produced no evidence of any change in the elimination or the metabolic pattern of APAP disposition (89). The authors, however, suggested that alcoholic and food restricted patients should be limited to 2 g/d of APAP quoting an abstract, which preceded the final published article (discussed in the next paragraph) as well as a study composed of anecdotal case reports (84,90). The stated limitation of 2 g APAP each day for alcoholics has no basis from any scientific evidence in the literature.

In a prospective trial utilizing volunteers the question was posed as to whether or not those who consume ethanol had an increase in NAPQI formation during the at-risk time shortly after ethanol is eliminated from the body (91). Acetaminophen was administered after ethanol had been metabolized. This would be the most susceptible time for APAP administration. They found an increase of 22% in mean NAPQI formation and concluded that there was an incremental increase in the risk of APAP hepatotoxicity. A 22% increase in the amount going through the CYP2E1 pathway would take NAPQI production from 4 to 4.88%. Assuming 6 mmol of glutathione in the liver and the need to deplete 4.2 mmol as calculated above, the dose of APAP required would decrease from 15.9 to 13 g of APAP for the threshold of toxicity to be reached. The calculation is 0.635 g NAPQI divided by 4.88% equals 13 g of APAP. Even considering the maximal increase of 2-fold in the amount of NAPQI produced following ethanol induction there is still a considerable safety margin. To reach *threshold* toxicity 7.9 g of APAP at a *single time* would be required. This observation is consistent with those clinical trials examining maximal therapeutic doses of APAP in alcoholics.

In a prospective, double-blind, randomized placebo-controlled trial of 201 alcoholics given maximum therapeutic doses of APAP (1 g 4 times/d) for 2 days. There was no statistical difference in AST or ALT between those alcoholics treated with APAP and those treated with a placebo (92,93). The authors concluded that their study did not support a reduction in the dose of APAP in alcoholics.

The entire issue of ethanol–APAP interaction has been reviewed recently (30). Prescott states, "Finally, and most importantly, *there has never been a single documented instance of any degree of acute liver damage produced by therapeutic doses of paracetamol given as a challenge in any chronic alcoholic under properly controlled conditions*. If paracetamol is as dangerous in the chronic alcoholic as claimed by so many investigators, why has no such example been published?" In reviewing the many articles produced Prescott also states, "However convincing the numerous reports of liver damage following paracetamol overdose in chronic alcoholics may be, they are purely anecdotal and the inescapable fact remains that exactly the same severe and fatal liver damage occurs after overdosage in patients who are not chronic alcoholics."

In conclusion, patients who have ethanol in their hepatic tissue at the same time as they consume APAP will have a hepato-protective effect by the inhibition of CYP2E1 from ethanol (94). Ethanol consumed on a chronic basis may maximally induce CYP2E1 2-fold. Some depletion of glutathione may occur in those alcoholics with chronic high-ethanol consumption and a chronically poor diet.

Chronic heavy alcoholics are probably at greater risk for toxicity following an *overdose* of APAP. There is no evidence that therapeutic doses of APAP cause toxicity in an ethanol induced patient.

NUTRITIONAL ASPECTS

The significance of nutritional factors on APAP metabolism cannot be looked at as a simple process. While there are changes in glutathione levels in starvation, that isolated fact must be examined within the knowledge that CYP2E1 is also undoubtedly reduced as shown in earlier work. In malnourished monkeys the levels of P450 were reduced substantially although the enzyme kinetics of the remaining P450 was normal (95). Cytochrome P450 reductase activity and ethylmorphine-stimulated reductase were both half the levels of controls. Reduction in P450 levels reduces substrate for NAPQI formation concomitantly, which then reduces the utilization and requirement for glutathione.

P450 levels from liver biopsies in patients with hepatic disease are shown in Table 1 (96,97). Glutathione levels in human liver biopsies from patients with hepatic disease are shown in Table 2 (98,99). Glutathione is increased in all forms of hepatitis except for steatosis while P450 levels are at normal levels in all except cirrhosis. A patient with cirrhosis having 108–186% of normal glutathione levels while at the same time having 34% of P450 levels would produce substantially less NAPQI while having more glutathione to detoxify it. This may help explain some of the seemingly disparate findings in patients with anorexia nervosa although the data in this disease state are not adequate (100,101). The expectation was that these patients would have reduced

Table 2

Glutathione Levels in Hepatic Disease

Hepatitis	% of Control
Toxic	231[a]
Viral	176[a]
Chronic	216[a]
Chronic active	142[a]
Cirrhosis	186[b]
	108[b]
Fatty liver	153[b]
Steatosis	78[b]

[a] Ref. (98).
[b] Ref. (99).

quantities of glutathione and would therefore be at greater risk for toxicity following an APAP overdose. The fact that the reverse is true most likely indicates that the concentration of the enzyme CYP2E1 is also reduced. Lack of direct correlation between rat, mouse, and human data in this area complicates our understanding. Substantially more work remains to be done to determine dietary effects and this should now be possible in vivo given the probe techniques available.

CLINICAL TOXICOLOGY

There was a lack of understanding of the pharmacokinetics in many cases of APAP toxicity. We described the normal time course and phases of toxicity in 1975 and provided a diagram in 1981 (7). Appreciation of this time course in relationship to the ingestion and laboratory findings is helpful in sorting out various issues in evaluation of a case. The following are common misconceptions that can be corrected by considering APAP kinetics and metabolism:

1. In the preclinical phase (an asymptomatic patient before we have any abnormal laboratory results or APAP levels) we must make diagnostic and therapeutic decisions based entirely on history. This history-based decision-making is a fundamental poison center activity. The usual guideline used in the triage of a patient exposed to APAP is to refer for evaluation anyone exposed to 150 mg/kg or greater.

2. Once the first plasma APAP level (C_p) is determined, it should be correlated with the ingestion history. Since APAP has a volume of distribution of about 1 L/kg, the plasma level in mg/L can be "read" as the mg/kg body burden at that time, e.g., a C_p of 200 μg/mL corresponds to a 200-mg/kg-body burden. Thus for a 70 kg patient this represents a 14,000 mg (14 g body burden). For comparison, a therapeutic dose (1 g/70 kg) produces a 2 hour C_p of about 10 μg/mL. Since C_p typically falls by half every 2 hour, it is important to relate the time of ingestion in considering the history.

3. If two levels can be obtained (three or more are even better) a half-life can be estimated. Acetaminophen half-life following a therapeutic dose (and most overdoses) is approximately 2 hour. Even following a large overdose, patients do not have an instantaneous increase in half-life—as much as 12 hour or more. Thus if a

patient comes in with a prolonged half-life and the history of 4–8 hour since overdose, the time of ingestion must have been much earlier or they have another reason for the increased half-life. In most patients following a large overdose, the half-life at the time of ingestion is normal and increases over several days due to hepatic toxicity.

4. Aspartate transferase levels take time to rise and peak levels are not expected until 72 or 96 hour after overdose. A patient who arrives with a peak level and a history of having taken an overdose 4 hour before either consumed the toxic dose considerably earlier or has some other cause for the AST elevation.

5. Chronic excessive consumption of APAP in supratherapeutic doses is harder to quantify. The plasma level will still reflect the body burden and the half-life will be a superior method of determining potential of toxicity.

Consideration of these factors as well as the remainder of the clinical picture will provide a more accurate understanding of the toxicity being treated.

CONCLUSION

Specific enzymes must be considered when evaluating a patient for the possibility of induction or inhibition. Case reports provide an interesting indication as to where further work may be considered. An understanding of inter-species differences as well as in vitro vs. in vivo data is important when evaluating the literature. Confusing terms such as "therapeutic misadventure" and those indicating molecular intent should be ignored and the data evaluated pharmacologically and toxicologically.

There is no evidence that therapeutic doses of APAP can cause hepatotoxicity in either induced or noninduced patients.

ACKNOWLEDGMENTS

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