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A STUDY OF ACETAMINOPHEN ANALOGUES' TOXICITY

by

Gamal Soliman, M.B., B.Ch.

Thesis presented to the School of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology.

UNIVERSITY OF OTTAWA

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ABBREVIATIONS

A4AP	Acetaminophen, N-Acetyl-4-Aminophenol
A3AP	N-Acetyl-3-Aminophenol
A2AP	N-Acetyl-2-Aminophenol
GSH	Glutathione, reduced form
NAC	N-acetylcysteine
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
SEM	Standard error of the mean
PGE_2	Prostaglandin E_2

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Abstract

Toxicity of acetaminophen analogues on human lymphocytes:

Acetaminophen (A4AP) is toxic to the liver in either intentional or accidental overdose. The cytotoxicity of two structural analogues of acetaminophen has been examined by using a human lymphocyte/mouse hepatic microsomal culture technique. The human cells were challenged with different drug concentrations for 16 hours. The 2 analogues studied were N-Acetyl-3-Aminophenol (A3AP) and N-Acetyl-2-Aminophenol (A2AP). Cytotoxicity was determined by trypan blue dye exclusion. A2AP was much more toxic than A4AP, while A3AP lacked any toxic effect. A4AP is oxidized by hepatic cell cytochrome P-450 mixed function oxidase to a toxic metabolite, postulated to be N-acetyl-4-iminoquinone. The susceptibility to oxidation was compared for A4AP, A3AP and A2AP using an electrochemical cell. The nanoampere current generated by the two electron oxidation process was amplified and recorded on a chart recorder. Consistent with the structural constraints upon quinone formation, A4AP and A2AP were readily oxidized by the electrochemical cell while A3AP was resistant to oxidation. The two observations on A3AP: 1) lack of cytotoxicity and 2) lack of oxidation in vitro, lead us to conclude that A3AP has much less potential for hepatic injury than A4AP or A2AP.

Introduction

1) History: Acetanilide is the parent member of the class of non-salicylate antipyretics/analgesics to which acetaminophen belongs. Acetanilide was first synthesized in 1852 by acetylation of aniline. It was introduced into medicine in 1886 by Cahn and Hepp. Cahn and Hepp were investigating the action of naphthalene on intestinal parasites. When their supplies ran out they ordered more from the pharmacy in the town but, in error, acetanilide was substituted for naphthalene. The physicians were surprised to find that their treatment with this new material did not behave like the old but at the same time noted that it was powerfully antipyretic. Investigations showed the mistake made by the pharmacy and within a short time acetanilide was introduced as an antipyretic under the trade name of Antifebrin but it was excessively toxic (Spooner and Harvey, 1976). Acetaminophen (Paracetamol, N-Acetyl-4-Aminophenol, A4AP) was first used in medicine by Von Mering in 1893. In 1949 it was recognized to be the major metabolite of both acetanilide and phenacetin by Brodie and Axelrod. Acetaminophen has been available in the United States without a prescription since 1955 and several studies have reported its administration, even to newborn infants, without untoward effects (Cornely and Ritter, 1956; Windorfer, 1972).

2) Pharmacological Effects: Acetaminophen has analgesic and antipyretic effects that do not differ significantly from those of aspirin, but has only weak anti-inflammatory effects. Acetaminophen is well-tolerated and lacks many of the side effects of aspirin. It is generally assumed that the antipyretic activity of acetaminophen resides in the aminobenzene structure.

3) Pharmacokinetics and Metabolism: Acetaminophen is rapidly and almost completely absorbed from the gastrointestinal tract. It reaches its peak concentration in plasma in 30 to 60 minutes and has a half-life of 1.9 to 2.2 hours in adults while in full term infants it is 3.5 hours (Levy et al, 1975). A biphasic decline in plasma acetaminophen concentration has been observed after oral doses of 500 mg or 1000 mg or after an intravenous dose of 1000 mg. This suggested a two-compartment open model described by the equation: $C_t = 13.8e^{-2.55t} + 13.0e^{-0.28t}$. The total plasma clearance was 352 $\pm$ 40 ml/minute calculated from the intravenous dose divided by the area under the curve (AUC). Oral bioavailability increases from 63% after 500 mg to 89% and 87% after 1000 mg and 2000 mg respectively (Rawlins et al., 1977). No binding to plasma protein occurs after normal therapeutic doses (plasma levels up to 60 mcg/ml). At very high plasma concentrations (280 mcg/ml), similar to those observed in man after ingestion of toxic amounts of the drug , the drug is 15 to 21% bound to plasma protein. No binding to red cells occurs after therapeutic or toxic doses of

acetaminophen (Gazzard et al., 1973). The drug is metabolized in the liver (see section 4, below) and the metabolites excreted in the urine. The volume of distribution and half-life is the same for adults and children (Peterson and Rumack, 1978).

4) Hepatic Metabolism: Acetaminophen is conjugated in the liver to glucuronic acid (60%) and sulfate (35%) (Potter et al, 1974). Glucuronidation is less in children than in adults (Alam et al, 1977). Acetaminophen also undergoes oxidative metabolism, postulated to be N-hydroxylation followed by dehydration, to form a toxic intermediate metabolite (N-Acetyl-p-benzoquinoneimine). (Figure 1). Glutathione detoxifies this reactive metabolite and the subsequent urinary metabolite is an acetaminophen mecapturate (Potter et al, 1974). It has been possible to separate by HPLC five different acetaminophen metabolites from in vitro experiments using rat hepatocytes. The five metabolites were conjugates of sulphate, glucuronide, cysteine, glutathione (GSH), N-Acetyl cysteine. The first three metabolites were obtained from incubation of acetaminophen with hepatocytes isolated from phenobarbital treated rats and the last two conjugates from incubation of acetaminophen with microsomes in the presence of GSH or N-Acetyl cysteine respectively (Moldeus et al, 1978).

5) Mechanism of Action: The thermoregulatory center located in the anterior and posterior hypothalamus regulates internal

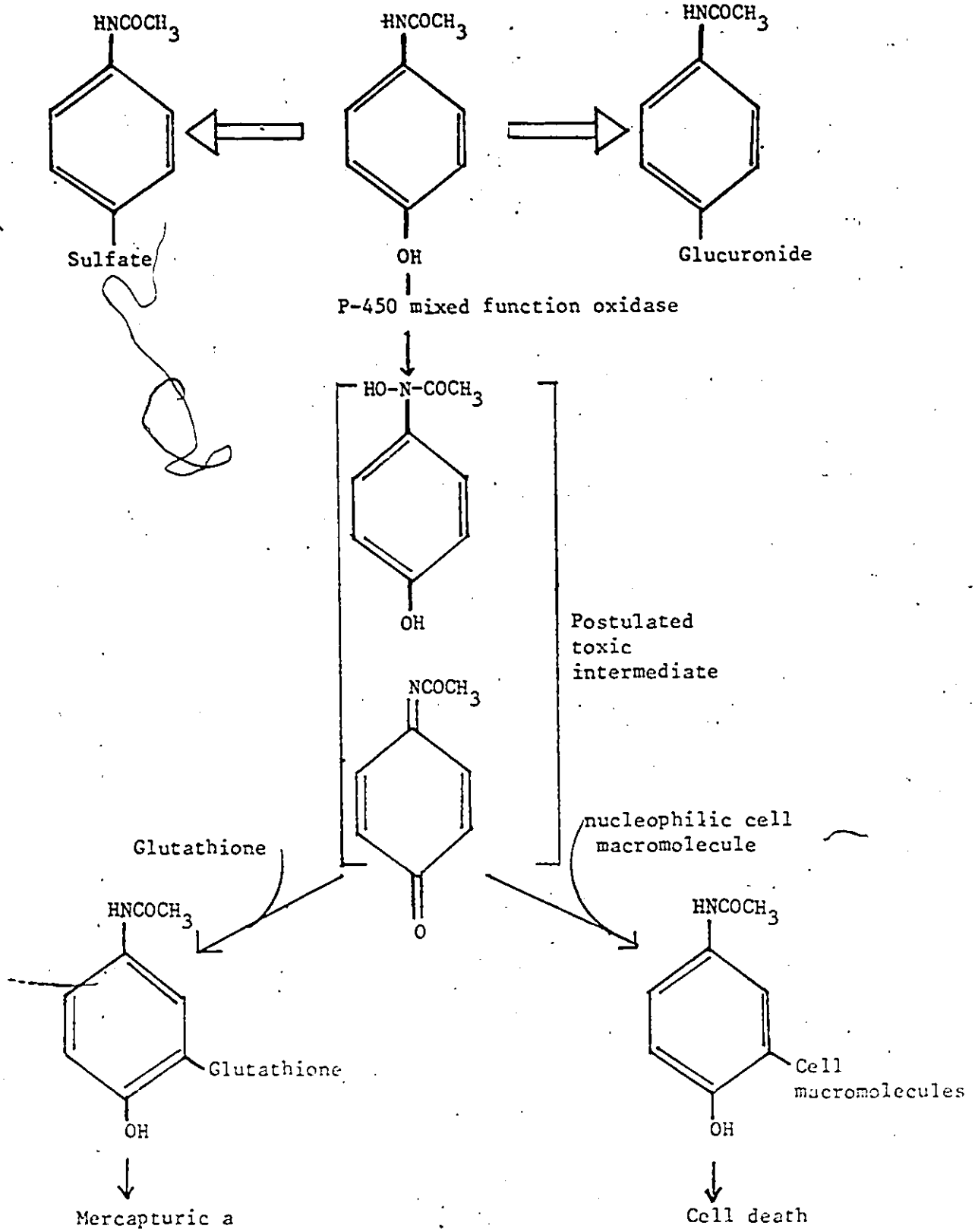


Fig. 1. Proposed metabolism of acetaminophen

temperature at 37°C primarily by its ability to balance heat production against peripheral heat loss. During fever the thermostat setting in the hypothalamic center shifts upwards. This results in signals from the posterior hypothalamus to increase heat production and decrease peripheral heat loss. Endogenous pyrogen, a small molecular weight protein, was first described by Beeson in 1948. Following injection of exogenous pyrogen (e.g. lipopolysaccharide of gram negative bacteria) phagocytes produce the endogenous pyrogen and release it into the circulation. It is the endogenous pyrogen rather than the many exogenous pyrogens that is responsible for the upward resetting of the hypothalamic thermostat and fever. Synthesis of prostaglandin, particularly of the E series, may be an important result of the action of the endogenous pyrogen on the hypothalamus. Aspirin and other antipyretics have no effect on the synthesis and release of endogenous pyrogen from phagocytic leukocytes. The potency of an antipyretic in reducing fever is proportionately related to its ability to inhibit the synthesis of prostaglandins, and thus the primary action of antipyretics is the inhibition of prostaglandin synthesis in the hypothalamus (Milton, 1980).

Further evidence for endogenous pyrogen was reported by Herman and Vane in 1976. They found increased production of prostaglandin by jejunum isolated from rabbits which had received endotoxin intravenously, whereas no such stimulation of prostaglandin synthesis is seen after adding endotoxin to

bath fluids. A possible explanation for this difference of activity *in vitro* and *in vivo* could be that endotoxin interacts with macrophages and releases an intermediate metabolite (endogenous pyrogen) which in turn can switch on prostaglandin synthesis (Herman and Vane, 1976). For some time a puzzling feature of the antipyretic action of acetaminophen compared with the action of well-known anti-inflammatory agents was the inability to demonstrate an inhibition of prostaglandin synthesis by acetaminophen. This puzzle was resolved by the demonstration that prostaglandin synthetase of different organs show different sensitivities to acetaminophen and that, in particular, the synthetase derived from brain tissue differs from the synthetase of other tissues in being inhibited by acetaminophen (Flower and Vane, 1972). This demonstration, taken together with the report that the rise in prostaglandin concentration in the CSF during fever can be inhibited by acetaminophen during reduction of the fever (Feldberg et al, 1972), indicated that acetaminophen exerts its antipyretic action by the same general mechanism as the anti-inflammatory agents. Flower and Vane in 1972 showed inhibition of prostaglandin synthetase in both rabbit and dog brain and a similar inhibition has also been shown in gerbil brain (ID_{50} = 20 µg/ml) (Willis et al, 1972). Very recently it has been demonstrated that very high concentrations (1.5 mM) of acetaminophen inhibit prostaglandin synthesis by sheep seminal vesicles, whereas low concentrations can stimulate the synthesis of prostaglandin (Dalpe-Scott et al, 1984).

6) Toxicity of Acetaminophen: Skin rash or allergic reactions occur occasionally and are sometimes accompanied by drug fever and mucosal lesions.

A. Renal Toxicity: It has been suggested that acetaminophen may be involved in two types of nephrotoxicity. In patients who have taken massive overdoses, in addition to the hepatic lesion, many instances of acute tubular necrosis have been reported (Proudfoot and Wright, 1970). With chronic abuse the initial lesion is in the medulla (Mudge et al, 1978). In Fischer 344 (F344) rats, acetaminophen (A4AP) produces renal necrosis restricted to the straight segment of the proximal tubule. On the other hand, Sprague-Dawley (SD) rats are extremely resistant to the nephrotoxic effects (Newton et al, 1983, a and b). P-aminophenol administration in both strains of rats has caused renal lesions indistinguishable from A4AP induced renal lesions in F344 rats. The p-aminophenol-induced renal lesions in F344 rats, however, were generally more severe than those in SD rats. Covalent binding of p-aminophenol to renal microsomes in vitro was much greater in F344 rats than in SD rats at substrate concentrations less than 5mM. This suggests that A4AP-induced nephrotoxicity may be related to strain differences in the activation of the nephrotoxic metabolite p-aminophenol (Newton et al, 1983, a and b). Also, it has been shown that male SD rats are more susceptible to A4AP toxicity than female SD rats due to a significant difference in glutathione utilization (Raheja et al, 1983). The

covalent binding of tritiated acetaminophen to tissue protein has been demonstrated in liver and renal cortex and papillae in CD1 mice and Sprague-Dawley rats. This binding was greater in liver than in kidneys, but the duration of this binding was longer for renal papilla than for renal cortex or for liver (Mudge et al., 1978). It has also been reported that in rabbit kidneys (in vitro) the binding of acetaminophen is greater in cortex than medulla. In vivo studies have shown that greater binding of acetaminophen occurs in renal inner medulla than renal cortex or liver. However, cytochrome P450 could not be detected in rabbit renal inner medulla. Hence, another metabolic pathway for the activation of acetaminophen was presumed to be operative in renal inner medulla. This alternate pathway was suggested to be co-oxidation of acetaminophen mediated by prostaglandin endoperoxide synthetase, requiring arachidonic acid and O_2. This alternate pathway could be a significant contributing factor for the genesis of papillary necrosis (Mohandas et al., 1981). Pre-treatment of CD1 mice and SD rats with 3-methyl-cholanthrene increased the covalent binding of tritiated A4AP in the liver but not in the kidney. Phenobarbitone had a paradoxical effect, increasing binding in the liver and decreasing it in the kidney. Further evidence for an alternate pathway comes from experiments showing that SKF 525-A, an inhibitor of Cytochrome P450, had no significant toxic effect except on renal papillae (Mudge et al, 1978). Thus, the pathway leading to renal toxicity is unlikely to be P450 dependent.

Acetaminophen inhibits prostaglandin production by the renal inner medulla. This inhibition of prostaglandin synthesis is reversible, dose related and does not require glutathione (Zenser et al, 1978). Acetaminophen could inhibit prostaglandin production either by reducing the release of arachidonic acid from tissue lipid or by a direct effect on prostaglandin cyclooxygenase. Acetaminophen inhibits the conversion of ^{14}C arachidonic acid to ^{14}C PGE_2 in the renal inner medulla of male Sprague-Dawley rats. In the presence of acetaminophen there was a decrease in ^{14}C PGE_2 synthesis and an increase in ^{14}C arachidonic acid. This indicates that acetaminophen inhibits PGE_2 synthesis at a step beyond arachidonic acid release. Acetaminophen inhibits both microsomal prostaglandin cyclooxygenase mediated synthesis of ^{14}C PGE_2 and co-oxidation of 1,3-diphenylisobenzafuran. Half maximal inhibition of ^{14}C PGE_2 synthesis has been observed at approximately 0.1 mM acetaminophen (Mattammal et al, 1979).

B. Hepatotoxicity: In adults liver damage may occur after ingestion of a single dose of 10 to 15 gm. (200 to 250 mg/kg). Nausea, vomiting, anorexia and abdominal pain occur during the initial 24 hours. Plasma transaminase and lactic dehydrogenase may be elevated but alkaline phosphatase activity and albumin concentrations are usually normal. Biopsy of the liver reveals centrilobular necrosis with sparing of peripheral areas (Mitchell et al, 1973 a,b). After 72 hours, the serum concentration of bilirubin is increased and prothrombin time is prolonged. Thus, A4AP hepatotoxicity may precipitate jaundice and coagulation

disorder and may progress to encephalopathy, coma and death. Azotemia, acute renal failure or hypoglycemia may also occur. Measurement of acetaminophen half-life in plasma during the first day of acute poisoning provides information about the severity of hepatic injury. Although acute adult toxicity usually requires a dose in excess of 10 to 15 gm, the clinical history is quite unreliable as the basis for assessing prognosis in a given case. Far more useful is the acetaminophen plasma level. When it exceeds 200 mg/L at 4 hours, 100 mg/L at 8 hours or 50 mg/L at 12 hours after ingestion, severe liver damage may occur (Rumack and Matthew, 1975). Acetaminophen does not appear to saturate the hepatic pathway for conjugation even when substantial doses are ingested in children (Peterson and Rumack, 1978). Kinetic values for elimination of acetaminophen after acute overdosage are difficult to interpret, because hepatic injury may occur with acetaminophen overdose and this could result in hepatic edema, leading to diminished hepatic blood flow and decreased acetaminophen metabolism without enzyme saturation (Peterson et al, 1984). Slattery and Levy (1979) suggested a kinetic model for acetaminophen elimination over a wide dose range, consisting of two saturable biotransformation pathways: 1. conjugation to glucuronic acid or sulfate by Michaelis-Menton kinetics as well as 2. first order kinetics linked with renal excretion of acetaminophen and formation of a hepatotoxic oxidative metabolite. Siegers has shown that hepatotoxic effects of acetaminophen were not correlated with prolongation of

half-life in rats and mice. Dithiocarb protected rats against acetaminophen liver damage but did not change acetaminophen half-life. Acetaminophen half-life was not influenced by a hepatotoxic dose of carbon tetrachloride (Siegers et al, 1978). This observation does not exclude the correlation between hepatotoxicity and half-life because it is not necessary that the kinetics of the drug are the same in humans and rats. Hepatic necrosis can be anticipated if the half-life is more than 4 hours and hepatic coma is likely if the half-life is greater than 12 hours. Hepatic damage is indicated by aspartate aminotransferase levels in excess of 80 I.U./L (Prescott and Wright, 1973). However, severe hepatic injury is usually accompanied by levels in excess of 1000 IU/L. Children are more dependent upon the sulfate pathway for elimination of acetaminophen and this pathway is limited in its capacity by the availability of sulfate as a substrate. A toxic oxidative metabolite rather than acetaminophen itself causes the hepato-cellular damage (Potter et al, 1974). This metabolite of acetaminophen becomes covalently bound to cells <u>in vivo.</u> There is a critical relationship between the extent of covalent binding of acetaminophen metabolites and the depletion of hepatic glutathione. (Potter et al., 1974). Arylation of hepatic macromolecules and hepatic necrosis caused by the reactive acetaminophen metabolites occur only when the dose of acetaminophen is large enough to produce sufficient toxic metabolite to exceed the availability of glutathione for detoxification. Acetaminophen is metabolized to its toxic

intermediate by the action of cytochrome P450 and this metabolism requires NADPH (Potter et al., 1973). Since a role for cytochrome P450 in the N hydroxylation of N-acetylarylamines was crucial to the hypothesis that the toxic metabolite of acetaminophen is produced by cytochrome P450 mixed function oxidase, the N-hydroxylation of N-acetylarylamines was further investigated, using 2-acetylaminofluorene as a model compound. Carbon monoxide-oxygen (90:10) atmosphere and specific antibody preparation against purified NADPH-cytochrome C reductase inhibited N-hydroxylation of 2-acetylaminofluorene. Furthermore, prior treatment of mice with cobaltous chloride for 3 days decreased both the amount of cytochrome P450 and the rate of N-hydroxylation of 2-acetylaminofluorene in liver microsomes by 55-60%. These results would be consistent with the hypothesis that acetaminophen can be N-hydroxylated by a cytochrome P450 dependent mixed function oxidase in liver microsomes (Thorgeirsson et al, 1972). The postulated iminoquinone would be generated by a two-electron oxidation of acetaminophen either chemically or electrochemically. This can be shown by using a porous flow-cell amperometric detector (Miner and Kissinger, 1979). The role of glutathione is to protect vital sites within hepatocytes from electrophilic attachments by arylating metabolites of acetaminophen (Mitchell et al., 1973, a,b). Pre-treatment of mice with phenobarbital, which induces liver microsomal enzymes, markedly increases acetaminophen-induced liver necrosis and acetaminophen metabolites' hepatic binding

(Jollow et al., 1973). Also, ethanol-fed hamsters exhibit an increased mortality rate after administration of A4AP as compared to control group (Rosen et al, 1983). It has been observed that hepatic necrosis due to A4AP intoxication was more severe in the group who are potentially induced due to their consumption during the previous three weeks of drugs likely to cause liver microsomal induction than in the "non-induced group" (Wright and Prescott, 1973). It has also been reported that a patient with anorexia nervosa who ingested 15 gm of acetaminophen and was treated with N-acetyl cysteine survived without evidence of liver damage and this was presumed to be due to depletion of cytochrome P450 in hepatocytes (Newman and Bargman, 1979). A similar mechanism may explain the idiosyncratic reactions (skin rash, aplastic anaemia) which may occur in humans (Spielberg, 1982). More suggestive evidence for this mechanism of toxicity has been provided by the demonstration that the amount of covalent binding of an oxidative metabolite of phenytoin (an arene oxide) to rat fetal macromolecules correlated with phenytoin-induced malformations (Martz et al., 1977).

Glutathione synthetase deficient lymphocytes are more susceptible to acetaminophen metabolite toxicity (Spielberg and Gordon, 1981a). Acetaminophen has been shown to reduce glutathione concentration in isolated rat hepatocytes and this depletion was correlated with its concentration. The depletion of glutathione from isolated hepatocytes by itself was shown not to injure cells, since diethylmaleate treated

isolated hepatocytes were not damaged. On the other hand, there was no depletion of glutathione in the absence of cytochrome P450. This demonstrates that acetaminophen requires metabolism to the postulated benzoquinone metabolite prior to conjugation to glutathione (Hogberg and Kristoferson, 1977).

Although acetaminophen mote often causes acute liver injury, prolonged use of acetaminophen within the therapeutic range may occasionally result in a chronic progressive process leading in some instances to cirrhosis, and liver biopsy shows either centrilobular necrosis or a picture suggestive of chronic hepatitis (Ockner, 1982). One study reported that 3 patients suffered severe hepatic damage from chronic abuse of acetaminophen. All the patients took rather large amounts of acetaminophen during long periods and they eventually developed biochemical and histologic evidence of toxic hepatitis that resolved when acetaminophen use was discontinued. One of these patients chronically abused alcohol, which induced cytochrome P450 mixed function oxidase; therefore he was perhaps more susceptible to liver injury. Another case had prolonged fever, low serum albumin and cachexia and this state may also have lowered her hepatic stores of glutathione. This study illustrates that acetaminophen toxicity can result from chronic abuse of the drug in metabolically susceptible patients (Barker et al, 1977). Another report has appeared of a patient who developed severe hepatic injury from chronic therapeutic

doses of acetaminophen. In this case, liver biopsy did not show changes consistent with viral hepatitis and the clinical course improved rapidly after cessation of the drug. In this patient also, his bad nutritional status may suggest depletion of hepatic glutathione (Ware et al, 1978). Additionally, an immunologic explanation for such hepatic injuries has not been excluded.

The human histopathology of acetaminophen overdosage includes a study of 14 fatal cases of acetaminophen overdosage. 1) In one case, dying before 12 hours post-ingestion, there was no abnormality; 2) Between 12 and 24 hours (1 case) the liver revealed centrilobular pallor, mild fatty change and mild to moderate hydropic vacuolation; 3) Approximately 24 hours after dosage (1 case) there was early centrilobular necrosis affecting 33% of the hepatocyte volume; 4) Between 28-48 hours (4 cases) there was extensive coagulative necrosis involving 56 to 94% of the hepatocyte volume with pyknotic nuclei and hydropic vacuolation and infiltration with polymorphs; 5) 48 to 72 hours after ingestion (3 cases) many of the necrotic cells became anuclear and changes typical of congestion and hypoxia appear; 6) Between 72 hours and 5 days (4 cases) there was increased phagocytic, mitotic and regenerative activity (Dixon, 1984). In a follow-up study of 50 non-fatal cases 17 cases showed interlobular bridging necrosis and 3 cases showed apparent micronodule formation but resolution was the rule as only one of the 17 survivors whose initial biopsy showed bridging necrosis had appreciable

residual fibrosis after one year (Portmann et al, 1975).

Treatment of Acetaminophen Toxicity

Hepatic toxicity of acetaminophen occurs in animals when there is 70% depletion of liver glutathione. The normal glutathione concentration in animals is 4 mmol/liter. If the liver of a standard 70 kg man is presumed to weigh 1.5 kg., it would be expected to contain 6 mmoles of glutathione of which 4.2 mmol (70%) must be depleted for acetaminophen to produce hepatic toxicity. If 4% of the acetaminophen metabolized enters the P450 pathway, which requires glutathione for detoxification, then about 15.876 gm of acetaminophen must be ingested by a 70 kg man to produce depletion of 70% of the stored glutathione and thereby induce hepatotoxicity (Rumack and Peterson, 1978). Antidotes to A4AP toxicity include cysteamine, methionine and acetylcysteine. In 1977 Prescott (Prescott et al, 1977) reported that I.V. acetylcysteine is less effective than I.V. cysteamine. In 1979 he reported that I.V. acetylcysteine is the drug of choice for acetaminophen overdosage (!) and; treatment with acetylcysteine was more effective than cysteamine and methionine and noticeably free of adverse effects (Prescott et al, 1979). Prescott reported that only one of 62 patients given I.V. acetylcysteine within 10 hours from ingestion developed severe liver damage while Rumack and Peterson (1978) reported that 17% of 49

patients given oral acetylcysteine within 10 hours from ingestion developed severe liver damage. Rumack and Peterson also reported that the intravenous use of either drug would not provide as high levels in the liver as oral absorption would and cysteamine is contraindicated to be given more than 10 hours after ingestion because of the increased incidence of encephalopathy. No such contraindication exists for acetylcysteine. Piperno (1976) also reported (Piperno and Berssenbruegge, 1976) that cysteamine is associated with gastrointestinal and central side effects and that it must be given early. In Britain I.V. acetylcysteine is used for treatment of acetaminophen overdosage while in North America the oral route is preferred. Recently it was reported that cimetidine could be used as an antidote for acetaminophen overdosage by preventing formation of the hepatotoxic oxidative metabolites while having no effect on conjugation of acetaminophen which yields non-toxic metabolites that are subsequently cleared from the body (Abernethy et al, 1982; Critchley et al, 1983).

N-Acetylcysteine, cysteamine and methionine act by serving as glutathione substitutes within the cell and detoxifying the oxidative metabolite. They must be given soon after the acetaminophen overdose in order to be effective. In those cases where intentional overdose of acetaminophen is not brought to medical attention within 10-16 hours, serious hepatic injury may occur despite the administration of the antidote. For this reason, an alternate approach has been

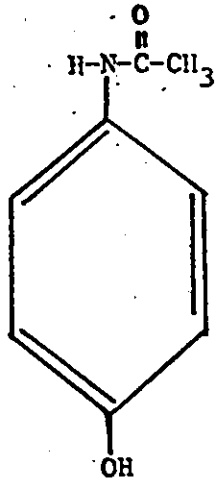
sought to the problem of acetaminophen hepatic injury.

Objective of this Study:

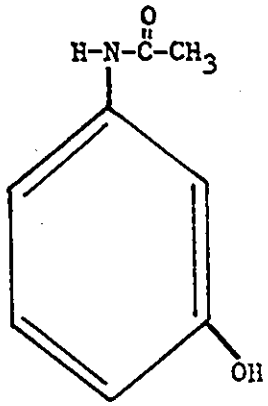
The original work reported in this dissertation concerns preliminary studies with two structural analogues of acetaminophen. Rather than search empirically for yet another antidote to acetaminophen toxicity, an evaluation of these two analogues has been undertaken in an attempt to find a compound which lacks the capability to directly injure cells. In order to demonstrate relevance to man, in vitro studies have been undertaken with human lymphocytes and phenobarbital induced mice hepatic microsomes as a source of cytochrome P450.

Acetaminophen Analogues

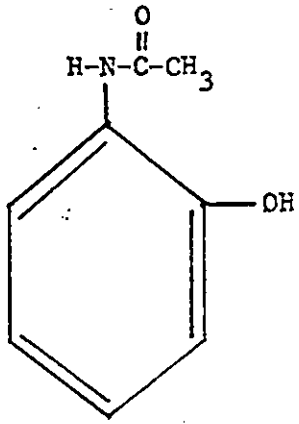
The two analogues studied differ from acetaminophen in the position of the hydroxyl group. They are N-Acetyl-meta-aminophenol (N-Acetyl-3-Aminophenol, A3AP) and N-Acetyl-ortho-aminophenol (N-Acetyl-2-Aminophenol, A2AP) (Fig. 2). The first report about A3AP analogue came from Dr. Peterson's laboratory. A3AP does not readily undergo oxidation in solution. In fact, an oxidized form of A3AP represents too high an energy state to exist in a biological system. A2AP has been briefly mentioned (Mitchell and



N-Acetyl-para-aminophenol
(N-Acetyl-4-Aminophenol, A4AP)



N-Acetyl-meta-aminophenol
(N-Acetyl-3-Aminophenol, A3AP)



N-Acetyl-ortho-aminophenol
(N-Acetyl-2-Aminophenol, A2AP)

Fig. 2. Acetaminophen analogues

Jollow, 1975) as a non-hepatotoxic substance in mice.

An in vivo study on the lack of hepatotoxicity of A3AP has already been reported (Dalpe-Scott et al, 1984). In this study A3AP was injected intraperitoneally into normal and phenobarbital induced CD-1 mice. Histological examination of livers from A4AP and A3AP treated mice was done. In addition, SGOT was measured as another criterion of hepatic injury. Plasma drug levels were determined at 0, 1, 4, 8 and 24 hours. Compared with A4AP, A3AP showed a substantially reduced potential for hepatotoxicity in both normal and phenobarbital induced mice (Dalpe-Scott et al, 1984). In fact, no hepatic injury could be detected with A3AP.

Other work on the effects of acetaminophen analogues on the ductus arteriosus of the chronically catheterized sheep fetus (Peterson et al, 1984) has shown that A2AP was as potent as A4AP in promoting constriction of the ductus arteriosus. This was shown by observing an increase in pressure in the fetal main pulmonary artery as compared to the fetal aorta. A3AP had no effect on the ductus arteriosus. (Peterson et al, 1984) In those experiments, catheters were placed in the sheep fetal main pulmonary artery, aorta, and inferior vena cava. A flow probe was chronically placed on the fetal left pulmonary artery. The drugs were injected directly into the inferior vena cava. Blood flow increased in the main pulmonary artery after infusion of both A4AP and A2AP and no increase was observed after A3AP infusion. Prostaglandin E_1

infused into the main pulmonary artery catheter of the fetus reverted all animals to the control state of pressure and flow.

Other studies in this laboratory have shown that A2AP inhibits prostaglandin synthesis in sheep seminal vesicles and in brain homogenates in vitro in a dose less than that of A4AP. A3AP inhibits the same systems but at higher doses. This supports the hypothesis that A3AP may have biological activity as an antipyretic, thus justifying this investigation of its potential toxicity.

A comparison of the hepatotoxicity of another structural analogue of acetaminophen has shown that compounds with a methyl group attached to the nitrogen of acetaminophen, such as N-methyl-P-hydroxy acetanilide do not produce hepatic damage (Mitchell and Jollow, 1975). Although this report did not mention anything about the methodology of this observation, it supports the hypothesis that analogues which cannot be metabolized to the intermediate active metabolite iminoquinone, do not have hepatotoxic effects.

Hepatotoxic Drugs

The introduction of a large number of new drugs during the past 3 decades has been paralleled by a striking increase in the number of instances of drug induced liver disease. Most

of these instances are the result of reactions to medicinal agents introduced during the past 25 years. It would be a mistake however to assume that long familiar drugs are necessarily free from suspicion in instances of hepatic injury. Several agents that have been in clinical use for many decades and had appeared to be free of potential for causing liver damage, have recently been recognized to cause hepatic injury. Even aspirin is now recognized to have the capacity to cause dose-related hepatotoxic effects in susceptible patients (Seaman et al, 1974). There are several reports about hepatotoxic effects of "therapeutic doses" of acetaminophen.

Two types of hepatic injury can result from adverse reactions to medicinal agents:

1. Acute hepatic injury
2. Chronic hepatic lesions

Age and sex appear to influence the vulnerability to drug induced hepatotoxicity. Children seem far less susceptible than adults to drug induced liver damage and males seem less likely than females to develop liver damage from drugs.

Acute Liver Damage

Drugs may lead to acute cytotoxic, cholestatic or mixed hepatic injury. Drugs which can lead to cytotoxic hepatic injury include carbon tetrachloride, halothane,

methoxyflurane (Peters et al, 1969) and floroxene (Reynolds et al, 1971). Cholestatic hepatic injury is produced by anabolic contraceptive steroids and erythromycin estolate. The mixed type of drug induced acute liver damage is induced by sulfonamides, para-aminosalicylic acid, phenylbutazone and several oral hypoglycemics. Drug induced hepatocellular jaundice is a serious lesion. The mortality ranges from 10 as high as 50%. The case fatality rate has been approximated as 50% in halothane related jaundice.

Chronic Hepatic Lesions

This type of hepatic damage is divided into many headings according to the type of injury.

Chronic Active Hepatitis:

This entity is characterized by both portal and peripheral inflammation. The inflamed exudate is composed of lymphocytes, plasma cells, eosinophils and fibrocytes. This necroinflammatory lesion is often accompanied by varying degrees of cirrhosis. This lesion has multiple causes, including a viral autoimmune etiology and drugs. Drugs which have been reported to induce this lesion include acetaminophen, oxyphenisatin, an ingredient of certain laxative preparations (Reynolds et al, 1971), alpha methyl dopa (Goldstein and Rothenberg, 1966), nitrofurantoin

(Klemola et al, 1975), isoniazid (Black et al, 1975), halothane (Thomas, 1974), propyl thiouracil (Fedotin and Lefer, 1975), and perhexiline (Paliard et al, 1978).

Drug-induced chronic active hepatitis is an important lesion. In any patient with the syndrome of chronic active hepatitis, the possibility of a drug as the cause should be a foremost consideration. No drug taken regularly, should be dismissed as a possible etiology without testing the effects of withdrawal and readministration (Zimmerman, 1979).

Chlorpromazine and halothane have been reported to cause chronic active hepatitis. This drug induced chronic active hepatitis presents a picture similar to that of the autoimmune type. Most patients had been taking the incriminated drug for months or even several years when hepatic injury was first recognized and withdrawal of the responsible drug usually leads to marked improvement or even apparent cure.

Subacute Hepatic Necrosis

This form of hepatic injury consists of varying degrees of confluent necrosis, fibrosis and regeneration. Subacute hepatic necrosis is a consequence of prolonged occupational exposure to tetrachlorethane, trinitrotoluene, chlorinated biphenyl chloronaphthalene mixtures and dinitrobenzene (Klatskin, 1975). This lesion also has been caused by a

number of drugs including cinchophen (Zimmerman, 1979) and isoniazid (Black et al, 1975).

Steatosis

Fatty liver is generally a relatively unimportant form of drug induced hepatic injury. Tetracycline toxicity is characterized by steatosis with tiny fat droplets in liver cells. Ethanol abuse is the most frequent cause of drug induced steatosis. Other drugs which can cause steatosis include glucocorticoid and antineoplastic agents such as methotrexate and L. asparaginase.

Phospholipidosis

A lesion first recognized in Japan in 1969 due to administration of the coronary vasodilator diethylaminoethoxyhexestrol (Coralgil). This lesion can lead to cirrhosis (Lullmann et al, 1975).

Peliosis Hepatis

This lesion consists of blood filled lacunae of varying sizes. Recent reports have shown the development of this lesion in patients taking anabolic or contraceptive steroids (Nadell and Kosck, 1977). This lesion also has been produced in experimental animals by oxazepam (Fox and Lachen, 1974).

Hepatic Vein Thrombosis:
Veno-Occlusive Disease and Budd Chiari Syndrome

The drug which has been incriminated most in the production of these lesions is the oral contraceptive preparation (Hoyumpa et al, 1971). A number of young women taking contraceptive steroids have been found to develop Budd Chiari syndrome secondary to thrombatic occlusion of hepatic vein by estrogenic steroid.

Hepatic Granuloma

Recently a number of drugs have now been shown to cause hepatic granulomatosis in addition to other forms of hepatic damage such as alpha-methyl dopa and isoniazid.

Post Necrotic Cirrhosis

Severe acute or subacute necrosis apparently can lead to collagenization of areas of collapse, nodular regeneration and cirrhosis. Post necrotic cirrhosis was reported following administration of cinchophen, acetohexamide (Goldstein and Rothenberg, 1966), iproniazid (Rosenblum et al, 1960), isoniazid and alpha-methyl dopa.

Fatty Cirrhosis

Methotrexate is now a known cause of steatosis and cirrhosis. The methotrexate induced cirrhosis somewhat resembles that of the alcoholic (Scheuer, 1975).

Chronic Intrahepatic Cholestasis

A syndrome that resembles primary biliary cirrhosis has resulted from the adverse reaction to a number of drugs. Many drugs have been incriminated in this disorder such as chlorpromazine and prochlorperazine (Ishak and Irey, 1972), tolbutamide (Gregory et al, 1967), thiabendazole (Russell et al, 1974) and methyltestosterone (Zimmerman, 1978). Each of these drugs can cause acute cholestasis.

Centrilobular Hepatic Fibrosis

This is another hepatic lesion that can lead to portal hypertension in the absence of cirrhosis. Chronic vitamin A intoxication can lead to this lesion. It results from intake of amounts that are more than 8 times the normal daily requirements for years (Munter, 1974). This abnormality tends to regress if vitamin A intake is discontinued.

Adenoma

There are many reports now that oral contraceptive steroids can cause hepatic adenoma (Edmondson et al, 1976, Sherlock, 1975). The risk of development of the tumor increases with increased duration of use of the oral contraceptives and with the increased hormonal potency of the preparation used.

Carcinoma

Contraceptives and anabolic steroids have been implicated as predisposing factors in hepatic carcinoma (Zimmerman, 1979). It should be emphasized however, that the small number of cases of the tumor that have been recorded represent but a tiny fraction of the millions of women who are using contraceptive pills.

1. Lymphocyte Toxicity

The methods used in the first part of this study are described under five headings:

1. Synthesis of A4AP, A3AP and A2AP
2. Preparation of microsomes.
3. Preparation of lymphocytes
4. Incubation conditions
5. Test for viability of cells

1. Synthesis of Acetaminophen and its Analogues

Acetaminophen and its analogues were synthesized according to the method of Fierz-David, and Kuster (Helv. Chim. Acta 22, 94, 1939 (Acylierungen mit Saure-anhydrid. Beispiel: O-Acetyl-Aminophenol). 22 gm of P-aminophenol were mixed with 14 gm of glacial acetic acid in 80 ml of distilled water at 80°C and then refluxed until the crystals of P-aminophenol were fully dissolved (10 minutes). 25 gm of acetic anhydride were then added drop-wise and the reaction maintained at 80°C for 30 minutes. The reaction mixture was cooled and the precipitate was collected. N-acetyl-p-aminophenol was recrystallized twice from hot ethanol. The final dry recrystallization product was checked for melting point (169-170.5°C). The same procedure was used to synthesize

A3AP and A2AP, but using m-aminophenol and o-aminophenol respectively. The melting point for A3AP was 146-149°C and the melting point for A2AP was 207-210°C.

Method of Recrystallization

The drug was solubilized in a minimal amount of hot ethanol (95%) and an equal amount of charcoal was added to the mixture. The solution was filtered and the drug was precipitated by cooling and adding distilled water drop-wise. A4AP crystals are plates in shape, A3AP are needles and A2AP are flakes.

2. Preparation of Microsomes

i. Induction of Cytochrome P450

Eight to 12 week old male C57Bl/6N mice were given 80 mg/kg of phenobarbital by intraperitoneal injection daily for 3 days. In order to assess if the cytochrome P-450 oxygenase system had been successfully induced, mice were tested on the 4th day of treatment by the hexobarbital hypnosis test (Conney et al, 1960). The duration of hexobarbital hypnosis was measured by determining the time required for the mice to regain their righting reflex after the intraperitoneal injection of 115 mg/kg hexobarbital. Pretreatment with phenobarbital shortened the duration of hexobarbital hypnosis

from 89 minutes or more (in control mice that received saline by intraperitoneal injection for 3 days) to 10-16 minutes. Mice that failed to wake up within 16 minutes of the hexobarbital injection were not used for the preparation of liver microsomes. Eight hours after the hexobarbital test, mice were given a last booster injection of phenobarbital consisting of 40 mg/kg by intraperitoneal injection. They were killed next day and their livers used as a source of microsomes.

ii. Preparation of Microsomes

Liver microsomes were prepared according to a modification of the method of Franklin and Estabrook (1971). 16 hours after the last booster injection of phenobarbital, mice were killed by decapitation and livers were removed and put in 0.25 M sucrose on ice. All subsequent procedures were carried out at 4°C. The livers were homogenized in 5 volumes (W/V) of 0.25 M sucrose in a Waring-Blender at low setting for 2 cycles each of 1 minute. The homogenate was then centrifuged at 8,000 x g for 15 minutes. The supernatant fraction was then further centrifuged for 15 minutes at 18,500 x g and the resulting supernatant at 105,000 x g for 60 minutes. The pellet of microsomal membranes was resuspended in 0.15M KCl and again spun at 105,000 x g for 60 minutes. Then the resultant pellet was finally resuspended in 0.15M KCl to yield a preparation of approximately 15 mg/ml protein. This suspension was stored in a number of small tubes at -80°C.

iii. Protein Assay: The protein content of the microsomes was assayed by the Coomassie Blue method (Bradford, 1976). This method is based upon the observation that maximum absorbance for an acidic solution of Coomassie Brilliant blue G-250 shifts from 465 to 595 nm when binding to protein occurs. The concentrated dye reagent was prepared as follows: Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml methanol. To this solution 100 mg 85% (W/V) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. One part of the dye reagent concentrate was diluted with four parts of distilled water and filtered on Whatman filters. 0.5 ml of the diluted dye reagent was added to 0.1 ml of each sample, (as well as standards and blanks) and each tube was well mixed on a vortex mixer and allowed to stand for five minutes in the spectrophotometer. The samples and standards were read against appropriate blanks in the spectrophotometer at 595 nm and a standard curve was plotted. Before use in the incubation medium, the amount of each microsome suspension which would produce 45-50% cell death of lymphocytes with 1500 µg/ml of A4AP was determined.

3. Preparation of Lymphocytes

Human lymphocytes were isolated from blood taken from 5 volunteers. They were 4 male and 1 female between 24 to 30 years old. The volunteers were healthy without any previous

history of blood or liver disease. They were not taking any medication. 20 ml of blood was drawn from each volunteer into heparinized syringes under aseptic conditions. The blood was diluted two-fold with sodium phosphate buffer 0.1 M, pH 7.4, 0.9% NaCl. 3 ml aliquots of diluted blood were layered onto 2 ml of Ficoll-Paque in 5 ml polystyrene tubes without mixing them and centrifuged at 1,000 x g for 15 minutes at 25°C. Differential migration during centrifugation resulted in the formation of layers containing different cell types. The bottom layer contains erythrocytes which have been aggregated by the Ficoll and therefore sediment completely through the Ficoll-Paque. The layer immediately above the erythrocyte layer contains mostly granulocytes, which at the osmotic pressure of the Ficoll-Paque solution attain a density great enough to migrate through the Ficoll-Paque layer. Because of their lower density, the lymphocytes settle together at the interface between the plasma and the Ficoll-Paque. Contamination with other slowly sedimenting particles (platelets and monocytes) is limited. These bands of lymphocytes were removed by drawing them into sterilized Pasteur pipettes and then poured into sterilized tubes containing 5 ml of Hepes buffer (15 mM Hepes, pH 7.4; NaCl 125 mM; KCl 6 mM; $MgS9_4$ 1.2 mM; NaH_2PO_4, 1mM; $CaCl_2$ 1mM and glucose 10 mM). Lymphocytes were suspended by gently drawing them in and out of Pasteur pipette and spun at 700 x g at 25°C for 5 minutes to get a pellet of cells at the bottom and washed once more with Hepes buffer and resuspended in the

same solution with sterilized Pasteur pipettes. Lymphocytes were repelleted again by spinning at 700 x g at 25°C and washed again with Hepes buffer and resuspended to yield from 2 to 4 x 10^6 cells/ml. The final number of suspended cells differs according to the source of the cells (between volunteers). The number of cells in each preparation was estimated in a Coulter Counter (Model A430).

4. Incubation Conditions

All manipulations for the preparation of the incubation material were carried out under strictly sterile conditions. The lymphocytes were incubated for 2 hours in 5 ml sterilized tubes. Each incubation tube contained a final volume of 1 ml including:

- 10^6 lymphocytes
- 0.5 mg to 1 mg of microsomal protein, depending on the batch of microsomes used, such that 1,500 ug/ml A4AP led to 45-50% cell death
- NADPH 0.6 mM. Other workers have used NADP and NADPH generating system (Glucose-6-phosphate, glucose-6-phosphate dehydrogenase). In preliminary experiments we found that addition of NADPH alone is equally effective.
- Incubations were carried out at 37°C for 2 hours in an atmosphere of 5% CO_2 in the presence of varying concentrations of A4AP, A3AP and A2AP.

After two hours cells were spun down at 700 x g for 5 minutes at 25°C. The cell pellets were resuspended in 1 ml of Hepes buffer containing 10% AB serum (AB serum was supplied in sterilized bags from the Red Cross and the complement was heat inactivated at 60°C for 1 hour prior to use in the incubation mixture). Penicillin (100 units/ml) and streptomycin (100 µg/ml) were used to prevent contamination of the lymphocyte culture by susceptible bacteria. Incubation was continued at 37°C for 16 hours under the same conditions as above.

The index of cell death was then obtained by the Trypan blue dye exclusion test. The stock solution of the various drugs that were tested were prepared in the following way:

- A4AP and A3AP were solubilized in a mixture of 50% polyethylene glycol M.W. 300, 50% distilled water and 0.2% sodium metabisulfite and the stock solution was kept at -20°C.
- A2AP was solubilized in a mixture of 50% ethanol (95%) and 50% polyethylene glycol M.W. 300. The solution of A2AP was prepared fresh for each experiment because it readily oxidizes in solution.

The following control tubes were run:

- Cells incubated with microsomes and NADPH in the absence of any drugs.
- Cells incubated with heat-inactivated microsomes and NADPH in the presence of the highest concentration of each of the three drugs.
- Cells incubated in Hepes buffer only with and without drugs.

- Cells incubated with NADPH and the highest concentration of each of the three drugs in absence of microsomes.
- Cells incubated with microsomes and the highest concentration of each of the three drugs in the absence of NADPH.
- Cells incubated in Hepes buffer and vehicle (50% polyethylene glycol MW 300, 50% distilled water, 0.2% sodium metabisulfite, and 50% polyethylene glycol MW 300 and 50% ethanol (95%)).
- Cells incubated under strict aseptic conditions without Penicillin and Streptomycin to see the effect of antibiotics on the viability of cells.

5. Viability of Cells

The dye exclusion test for cell viability depends upon the fact that viable cells do not take up certain dyes whereas nonviable cells do. In addition, it has been shown that non-viable cells which take up the dye by this technique do not respire, undergo glycolysis or extend cellular processes when replanted in a cell culture medium. Further, they are readily digested by dilute solution of trypsin (Phillips, 1973).

Numerous dyes have been used to differentially stain non-viable cells or tissues, but trypan blue is the most extensively used dye. Each dye however, has staining characteristics which preclude its use under certain conditions. In this study of drug induced lymphocyte

cytotoxicity, the use of trypan blue dye was appropriate. (Spielberg, 1980).

Stock Dye Solution

A 0.5% aqueous solution of trypan blue dye in distilled water was shaken until clear to dissolve all the dye. The solution was stable at room temperature.

Test for Viability

Cells in suspension can be stained directly for viability. The cells were dispersed 20 times by Pasteur pipette aspiration and two drops of the dye were added to 5 drops of the cell suspension in a new glass tube. The cells were then dispersed another 10 times with a Pasteur pipette. A drop of the suspension was placed on a glass slide and covered by a cover slip. Viability count was always made 2 minutes after dispersing the cells in the dye. The number of stained cells vs. non-stained cells from different areas of the glass slide were counted. At least 200 cells were counted from each glass slide. The fraction of dead cells was expressed as stained cells/total stained + non-stained cells.

Factors Affecting Accuracy of Test

The degree to which cells take up dye is pH dependent. Within a range of pH 6.6 to 7.6, trypan blue uptake occurs; maximally at pH 7.5 (Phillips, 1973). A major drawback for

trypan blue is that the dye has a greater affinity for protein in solution (serum proteins in cell culture medium) than it does for non-viable cells. This was first observed by Pappenheimer (1917) and recently verified by Phillips and Farr (1973) (Phillips, 1973). The number of cells in suspension stained by trypan blue decreases as the serum protein concentration is increased (Phillips, 1973). Therefore, the concentration of human serum (fresh frozen, type AB, Rh+) in the incubation medium was kept constant in all the experiments.

Application of the Same Technique in Other Experiments

The same method of measuring lymphocyte cytotoxicity by the trypan blue dye exclusion test has been used to assess in vitro the toxicity of the anticonvulsants: mephenytoin, phenacemide, phenytoin, phenobarbital and carbamazepine (Spielberg et al, 1981 a and b; Gerson et al, 1983) thalidomide teratogenesis (Gordon et al, 1981) and nitrofurantoin cytotoxicity (Spielberg and Gordon, 1981 b). Spielberg has also used this method to assess acetaminophen toxicity (Spielberg, 1980). It has been proposed that this method can be used for in vitro assessment of the toxicity of many drugs which require metabolic "activation" by microsomal enzymes.

2. The Electrochemical Cell

The second part of this study includes using an electrochemical detector (Bioanalytical Systems, Model 2A, Lafayette, Ind.) and HPLC (Water's Associates, Model 45 pump, U6K injector, C18 microbondapak reverse phase column) to study the susceptibility of each drug to oxidation in vitro. We connected the detector cell to the effluent of the HPLC. The use of electrochemical oxidation avoids the need for addition and subsequent removal of oxidizing agents. By using electrochemical oxidation the reactive metabolite of acetaminophen (N-acetyl-p-quinoneimine) is generated. The cell is composed of a working electrode which is connected to a voltage convertor amplifier at adjustable potential (usually 0.8v), a reference electrode detects the current flowing through the solution and serves as the exit for effluent from the detector (Fig. 3). This cell is electrically connected to an instrumentation amplifier which is connected to the chart recorder. The injection volume was 3 ul of varying concentrations of 1, 5, 10, 20 µg/ml of A4AP, A3AP and A2AP. Because of the resistance of A3AP to oxidation, a very high concentration was used at 100 µg/ml. For the three drugs the mobile phase in the chromatography consisted of 20% methanol, 80% distilled water, 0.01M ammonium phosphate monobasic adjusted to pH 3.0 with metaphosphoric acid. The difference in voltage between the working and auxillary electrodes was adjusted to 0.8 V and the reference electrode gave a signal which was quantitated

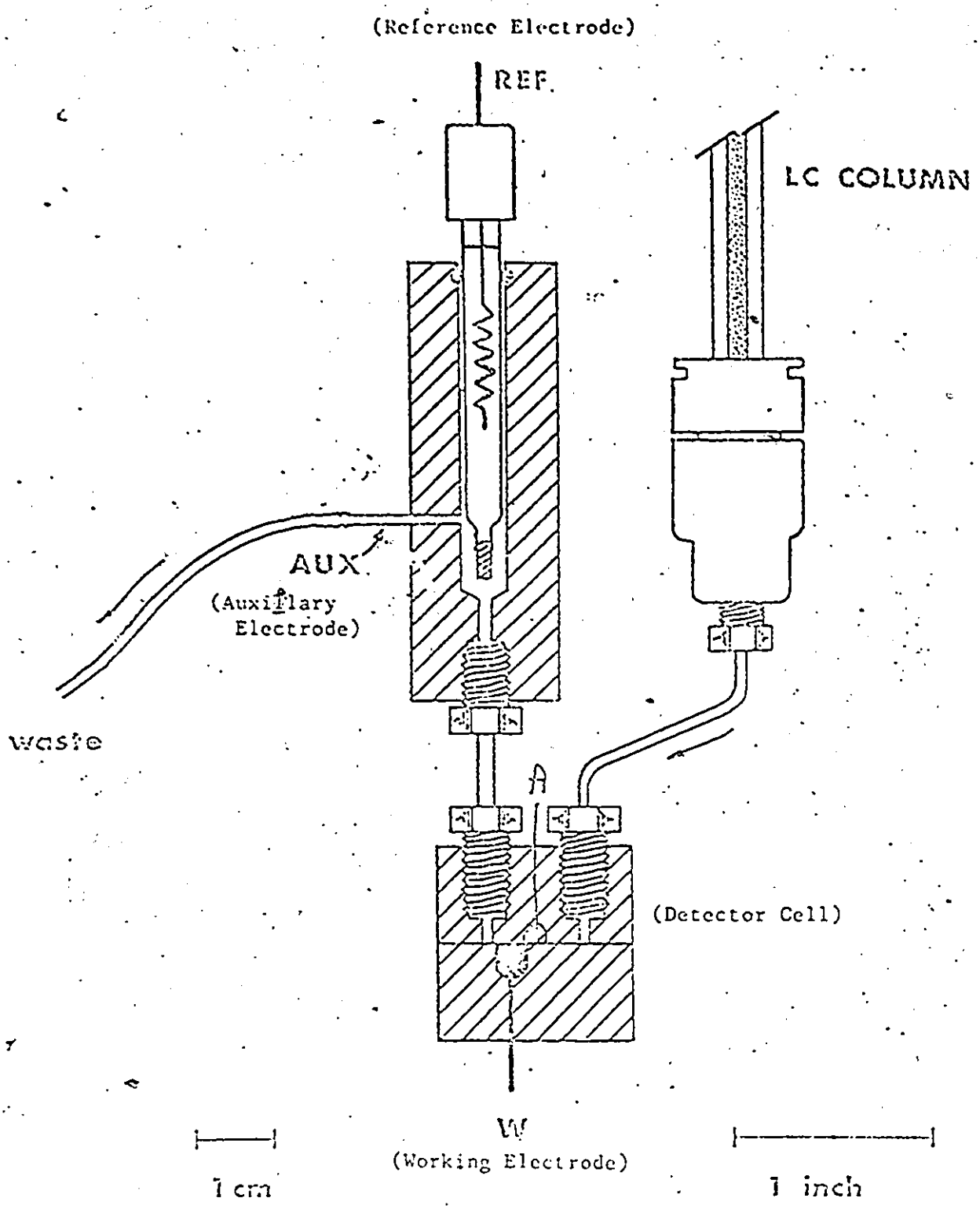


Figure 3. Complete Electrochemical Cell.

by peak height on the chart recorder. At the same time the effluent from the electrochemical cell passed through a U.V. detector (254 nm) and the concentration of the solution was recorded on a second chart recorder. The electrochemical cell was used to detect the electrons released in the process of oxidation of acetaminophen and its analogues. Thus, a single sample could be analyzed simultaneously for electrochemical signal and ultraviolet absorption.

Materials

- Glucose 50% sterile, phenobarbital and heparin were supplied from Abbott Laboratory, Montreal, Canada.
- P-aminophenol was supplied from Aldrich Chemical Company Inc., Milwaukee, Wisconsin, U.S.A.
- Charcoal was supplied from BDH Chemicals Canada Ltd., Toronto, Canada
- Glacial acetic acid, acetic anhydride, ethanol, sucrose, potassium chloride, sodium chloride, sodium phosphate monobasic monohydrate, sodium phosphate dibasic anhydrous, magnesium sulfate, calcium chloride, methanol, sodium meta-bisulfite, orthophosphoric acid were supplied from Fisher Scientific Company, Fair Lawn, New Jersey, U.S.A.
- Penicillin and Streptomycin were supplied from Gibco Canada Inc., Toronto, Ontario.
- Hexobarbital was supplied from May and Baker Canada Ltd., Montreal, Canada.

- Ficoll Paque was supplied from Pharmacia Fine Chemicals, Piscataway, New Jersey, U.S.A.
- Glucose-6-Phosphate dehydrogenase (Type XV), Hepes, NADP, NADPH, Polyethylene glycol (M.W. 300), Bovine serum albumin (R.I.A. grade), Coomassie Brilliant Blue-G250 and Trypan blue dye was supplied from Sigma Chemical Co., St. Louis, MO.

Results of lymphocyte toxicity study

Control conditions for the lymphocyte toxicity study are described in Table 1. From these preliminary studies we were able to optimize the incubation conditions for cell survival. Using optimal conditions, the percentage of cell deaths after 16 hours' incubation in Hepes buffer was very low. The metabolism of acetaminophen by hepatic microsomes was found to be essential for expressing its cytotoxicity and the drug had no cytotoxic effects in the absence of the microsomal preparation or the presence of heat inactivated microsomes. The tubes containing A4AP or A3AP in the absence of NADPH or the microsomes had very minimal cell death, whereas the tubes containing A2AP lacking NADPH or microsomes showed approximately 42% cell death. Also, I observed that the colour changed to orange in the tubes containing A2AP. This orange colour deepened in the presence of the microsomal system (microsomes + NADPH). This colour change was probably due to oxidation products derived from A2AP in solution and the oxidation was enhanced in the presence of cytochrome P450 mixed function oxidase .

Toxicity to the cells was manifested by increasing percentage of cells failing to exclude dye. Toxicity was particularly marked at concentrations of A4AP associated with depletion of

Table 1. Control conditions for lymphocyte cytotoxicity method

Date	NADPH	NADPH Generating System	Hepes Buffer	A4AP 1500 µg/ml	A3AP 1500 µg/ml	A2AP 1500 µg/ml	Microsomes	Penicillin + Streptomycin	% of Cell Death
6/2/1984	-	-	+	-	-	-	-	-	0% 1%
2/2/1984	-	+	+	-	-	-	+	-	0% 3%
2/2/1984	+	-	+	-	-	-	+	-	1% 1%
2/2/1984	-	-	+	+	-	-	+	-	1% 0%
2/2/1984	-	+	+	+	-	-	-	-	0% 0%
2/2/1984	+	-	+	+	-	-	-	-	0% 1%
13/2/1984	+	-	+	+	-	-	heat inactivated	+	0% 1%
12/3/1984	+	-	+	-	+	-	-	+	3% 1%
29/2/1984	+	-	+	-	-	+	-	+	43% 41%

glutathione (Spielberg, 1980). This toxicity was apparent some time after drug challenge. With the test procedure used no evidence of toxicity to cells was apparent immediately after challenge, whereas cell damage was evident 16 hours, later. In the microsomal system there was no difference between preparations using NADPH and those using an NADPH generating system (Table 2). NADPH was simpler to use and less expensive than the NADPH generating system (Glucose-6-P + Glucose-6-Phosphate dehydrogenase + NADP). Although in his work, Spielberg (1980) made no mention of the danger of contamination of the incubation culture or of the necessity to include an antibiotic in the culture, he reported 10% cell death in his controls and although under the most careful sterile conditions in the absence of antibiotics the percentage of cell death both in our control tubes and in the tubes containing various concentrations of drugs was not different from the results obtained in the presence of antibiotics, in order to prevent microbial contamination in routine assays, antibiotics were routinely added. In the presence of antibiotics cell death remained below 2%.

When the full microsomal drug metabolizing enzyme system was included in the incubation mixture, A4AP and A2AP caused a dose-dependent increase in cell death. In the absence of the drugs, the microsomes and NADPH alone had no effect on the viability of the cells. Table 3 shows the percentage of cell death at various drug concentrations for all experiments. Table 4 and Figure 4 show the results of the lymphocyte

Table 2. Effect of using NADPH instead of NADPH generating system (Glucose-6-phosphate + Glucose-6-phosphate dehydrogenase + NADP)

	% of cell death in tubes containing NADPH	% of all death in tubes containing NADPH generating system*
500 µg/ml of A4AP	8 11	7 11
1000 µg/ml of A4AP	10 20	10 19
1500 µg/ml of A4AP	38 43	28 39

*NADP, glucose-6-phosphate dehydrogenase, glucose-6-phosphate

Table 3. % of Cell Death at Different Concentrations of the Drugs in All Experiments

Volunteer	Date	A_4AP (ug/ml)				A_3AP (ug/ml)				A_2AP (ug/ml)			
		100	500	1000	1500	100	500	1000	1500	100	500	1000	1500
Individual I	15.03.84	2	3	29	50	0	1	1	16	7	68	93	80
	15.03.84	0	1	27	25	2	5	0	3	24	77	66	69
	09.04.84	1	5	27	45	1	1	8	11	0	25	80	87
	09.04.84	0	2	25	68	0	4	1	1	3	22	49	91
	07.05.84	3	4	16	35	0	2	2	7	2	30	50	84
	07.05.84	1	1	19	40	3	4	1	5	3	24	44	88
Individual II	08.02.84	–	–	–	25	–	–	–	1	–	–	–	–
	08.02.84	–	–	–	26	–	–	–	2	–	–	–	–
	20.03.84	0	2	7	29	3	1	1	0	1	22	59	76
	20.03.84	0	1	6	27	1	1	0	2	5	54	69	92
	04.05.84	3	2	7	24	1	2	1	6	0	22	44	91
	04.05.84	2	0	8	28	0	1	2	4	0	26	50	89
	15.05.84	1	2	6	16	0	3	3	3	1	10	68	78
	15.05.84	3	1	10	26	1	0	0	2	4	12	60	75
Individual III	22.02.84	–	–	–	–	–	–	–	–	–	–	80	89
	22.02.84	–	–	–	–	–	–	–	–	–	–	81	94
	27.03.84	3	5	13	52	1	1	7	6	1	35	39	52
	27.03.84	0	6	19	46	0	4	6	3	2	20	34	50
Individual IV	12.03.84	2	5	22	52	0	1	0	2	0	27	58	77
	12.03.84	1	6	10	48	0	1	0	3	2	41	63	80
	11.04.84	2	4	12	31	1	1	1	0	1	10	49	73
	11.04.84	2	6	11	29	0	1	1	6	0	5	56	77
	07.05.84	6	4	25	53	1	2	9	7	1	10	40	78
	07.05.84	8	10	12	56	1	3	8	6	0	25	62	82

Table 3. % of Cell Death at Different Concentrations of the Drugs in All Experiments

		A_4AP (ug/ml)				A_3AP (ug/ml)				A_2AP (ug/ml)			
Volunteer	Date	100	500	1000	1500	100	500	1000	1500	100	500	1000	1500
Individual V	02.04.84	-	5	-	-	-	-	-	-	-	-	-	-
	02.02.84	-	11	-	-	-	-	-	-	-	-	-	-
	13.02.84	3	-	-	46	2	-	-	-	-	37	-	90
	13.02.84	2	-	-	36	2	-	-	-	-	50	-	69
	29.03.84	2	8	17	33	7	4	3	3	4	27	61	80
	29.03.84	1	7	19	24	2	2	3	5	2	31	47	74
	09.05.84	2	8	17	28	1	2	2	0	3	20	55	77
	09.05.84	1	10	18	21	2	0	2	3	2	35	40	80
	09.05.84	0	7	10	35	2	0	2	3	0	18	67	89
	09.05.84	1	12	20	38	2	0	2	3	0	27	68	85

Table 4. Toxicity of Acetaminophen and Its Analogues to Human Lymphocytes

Concentration ug/ml	A_4AP	A_3AP	A_2AP
0	[a]1.1±[b]0.2	1.1±0.2	1.1±0.2
100	1.8±0.3	1.3±0.3	2.6±0.9
500	4.9±0.6	1.8±0.3	28.9±3.2
1000	15.8±1.4	2.5±0.5	58.3±2.7
1500	36.4±2.3	4.0±0.6	79.9±1.9

a) Mean for 26 to 30 determinations of 5 volunteer cells

b) SE of the Mean

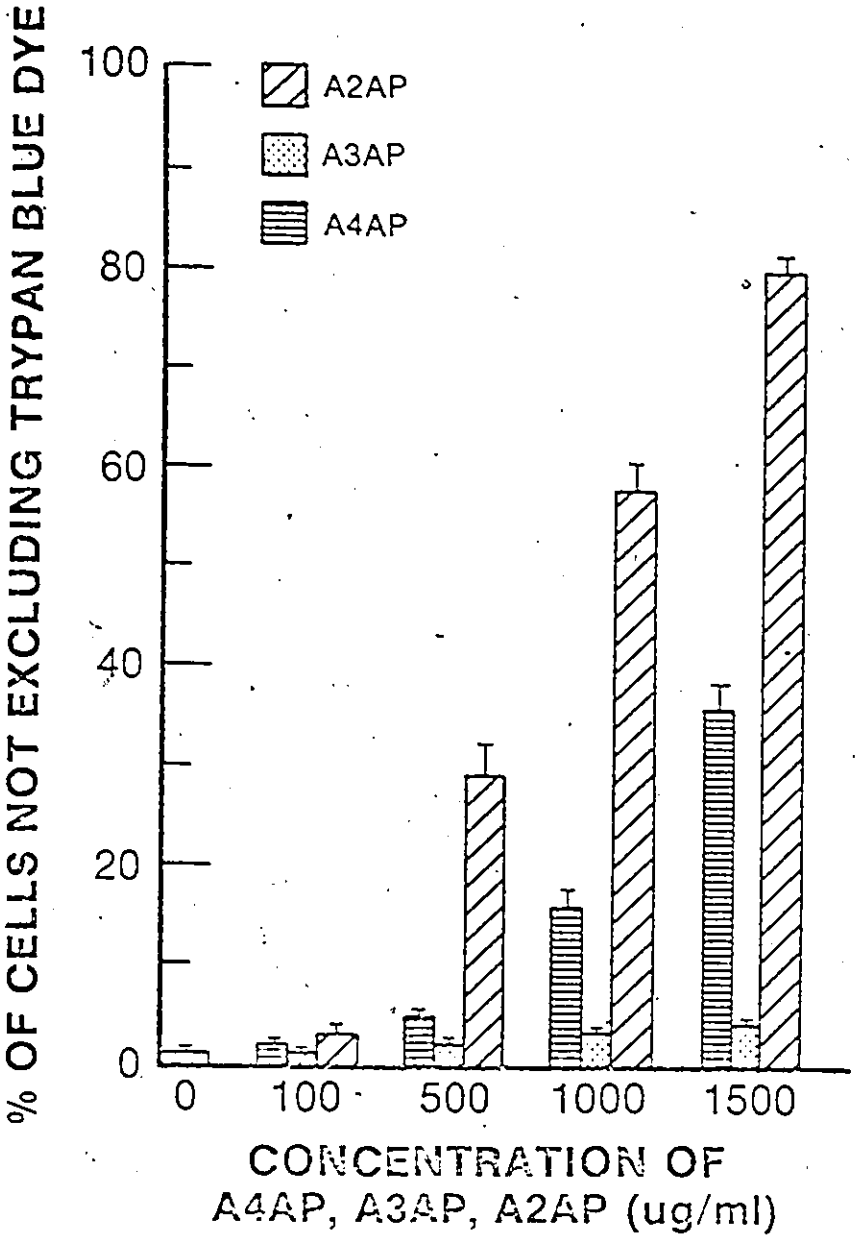


Figure 4. Effect of A4AP, A3AP and A2AP on lymphocyte trypan blue dye exclusion. Values represent means of 26 to 30 determinations for each drug using 16 cell lines.

trypan blue exclusion test expressed as mean ± SEM for 26 to 30 determinations on cells from 5 volunteers. At a concentration of 100 µg/ml, all three drugs caused minimal cell death, which was similar to controls. This could be explained by the fact that these cells should be depleted of glutathione before the drugs manifest their cytotoxicity and the dose of drug used was insufficient to deplete the cells of glutathione. The variation among the different volunteers in the percentage of cell death could be explained by the fact that human cells differ in their content of glutathione.

In this study one way analysis of variance (ANOVA) was followed by 2 multiple comparison tests. The first multiple comparison test used Tukey's method (1953), (Kushner and De Maio, 1980), often referred to as the HSD test (for "honestly significant difference") . The difference between two means is "honestly significant" at a given alpha level (.05 or .01) if it equals or exceeds the HSD. Table 5 shows the difference between the means for the 3 drugs at the following concentrations 100, 500, 1000, 1500 µg/ml. From that table we can see that there was no significant difference between the means obtained for the 3 drugs when used at a concentration of 100 µg/ml while at 500 µg/ml there was significant difference between the means for A2AP vs A4AP and A2AP vs A3AP at a level of .01 alpha. At 1000 µg/ml concentration there was significant difference between the means of the three drugs at alpha level of 0.05, while there was significant difference between the means of A4AP vs A2AP

TABLE 5: Differences Between the Means of the Three Drugs at Every Concentration

100 ug/ml

HSD=1.914 at alpha=0.05

	A4AP	A3AP	A2AP
A4AP	-	0.5	0.8
A3AP	-	-	-
A2AP	-	1.3	-

500 ug/ml

HSD=6.46 at alpha=0.05
HSD=15.44 at alpha=0.01

	A4AP	A3AP	A2AP
A4AP	-	3.1	24*
A3AP	-	-	-
A2AP	-	27.1*	-

1000 ug/ml

HSD=12.03 at alpha=0.05
HSD=15.14 at alpha=0.01

	A4AP	A3AP	A2AP
A4AP	-	13.3*	42.5*
A3AP	-	-	-
A2AP	-	55.8*	-

1500 ug/ml

HSD=10.73 at alpha=0.05
HSD=13.50 at alpha=0.01

	A4AP	A3AP	A2AP
A4AP	-	32.4*	43.5*
A3AP	-	-	-
A2AP	-	75.9*	-

*P < .05

and A2AP vs A3AP at a level of 0.01 alpha. At the 1500 μg/ml concentration there were significant differences between the means of the 3 drugs, also at a level of 0.01 alpha. Table 6 shows the differences in the means of the various concentrations of the same drug. Increasing the dose resulted in statistically significant increases in cytotoxicity for both A4AP and A2AP such that the 1500 μg/ml concentration gave 36 and 80 percent cell deaths respectively. Other experiments were done using concentrations other than 100, 500, 1000 and 1500 μg/ml for A2AP and A4AP. For A2AP we used 200, 400, 600 μg/ml, which gave 14, 21, 30 percent cell death respectively. For A4AP we went to a very high concentration, which was 2000 μg/ml, and got 64% cell death. Because only a few tubes were used for these determinations, we have not included them in the summaries of data; however, the results are consistent with the dose-response observed. For A3AP there was a significant difference between the cytotoxicity of 1500 μg/ml and all other concentrations at a level of 0.01 alpha. At the maximum concentration of A3AP (1500 μg/ml), there was only 4% cell death. Thus, the differences detected by statistical analysis of A3AP data represent the striking reproducibility of the method, allowing for a difference between 2.5% and 4% cell death to be detected. In biological terms, such cytotoxicity is unimportant. For A2AP there was a statistical difference between the means of 100 μg/ml and 500 μg/ml and all other different concentrations at a level of 0.01 alpha. There was a significant difference between the

TABLE 6: Differences Between the Means of the Different Concentrations for Every Drug

A4AP

HSD=7.54 at alpha=0.05
HSD=9.23 at alpha=0.01

ug/ml	100 ug/ml	500 ug/ml	1000 ug/ml	1500 ug/ml
100	-	3.1	14*	34.6*
500	-	-	-	31.5*
1000	-	10.9*	-	20.6*
1500	-	-	-	-

A3AP

HSD=.803 at alpha=0.05
HSD=.981 at alpha=0.01

ug/ml	100 ug/ml	500 ug/ml	1000 ug/ml	1500 ug/ml
100	-	0.5	1.2*	2.7*
500	-	-	-	2.2*
1000	-	0.7	-	1.5*
1500	-	-	-	-

A2AP

HSD=20.687 at alpha=0.05
HSD=25.294 at alpha=0.01

ug/ml	100 ug/ml	500 ug/ml	1000 ug/ml	1500 ug/ml
100	-	26.3*	55.7*	77.3*
500	-	-	-	51.0*
1000	-	29.4*	-	21.6+
1500	-	-	-	-

* $p < .01$
+ $p < .05$

means of A2AP at 1000 µg/ml and 1500 µg/ml at a level of 0.05 alpha. A2AP was far more toxic to the cells than A4AP, while A3AP had very minimal toxicity on the cells. There was little change in the dose response to A3AP, while A2AP and A4AP had steep dose response curves.

The other multiple comparison test used in this study was the Student-Newman-Keul (SNK) test. Tables 7 - 13 demonstrate the statistical analysis of the results using SNK multiple comparison and this test confirmed the previous analysis using the Tukey statistical test. While Tukey's and SNK are widely accepted statistical tests, the SNK is slightly more conservative than Tukey's method.

Other, experiments were done to demonstrate the effect of N-acetylcysteine (the most widely used antidote for acetaminophen overdosage) in preventing the cytotoxicity of the three drugs. N-acetylcysteine was added to the incubation media, at a concentration of 9.93 mM, an amount equimolar to the maximum concentration of the test drug. N-acetylcysteine was very effective in preventing the A4AP cytotoxicity. It had no effect on A2AP cytotoxicity at 1500 µg/ml but had some effect at 1000 µg/ml. The relatively small effect of N-acetylcysteine (9.93 mM) on A2AP at 1000 µg/ml (6.62 mM) could be due to its higher concentration (on a mole to mole basis) in relation to A2AP. A3AP of course had no cytotoxicity, therefore, N-acetylcysteine did not affect the percentage of cell death for this drug. Table 14

Table 7.- Anova and SNK multiple comparisons for A4AP vs A3AP vs A2AP at 100 ug/ml

	GROUP SIZE	MEAN % DEATH	VARIANCE	STD. DEV.	SEM
A4AP	28	1.85714	3.2381	1.79947	.340068
A3AP	28	1.28571	2.13757	1.46204	.2763
A2AP	26	2.61538	22.2462	4.71658	.924998
GRAND MEAN	1.90244				

ANALYSIS OF VARIANCE TABLE:

	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE
BETWEEN GROUPS	23.9228	2	11.9614
WITHIN GROUPS	701.297	79	8.87717
TOTAL	725.22	81	

F 1.34743 , DF (2 / 79), CUM PROB .734162

Anova was non significant- therefore multiple comparison test was not allowed

Table 8.. Anova and SNK multiple comparisons for A4AP vs A3AP vs A2AP at 500 ug/ml

	GROUP SIZE	MEAN % death	VARIANCE	STD. DEV.	SEM
A4AP	28	4.92857	10.9577	3.31024	.625576
A3AP	26	1.80769	2.08154	1.44275	.282947
A2AP	28	28.9286	282.661	16.8125	3.17727

GRAND MEAN 12.1341

ANALYSIS OF VARIANCE TABLE:

	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE
BETWEEN GROUPS	12123.8	2	6061.89
WITHIN GROUPS	7979.75	79	101.01
TOTAL	20103.5	81	

F 60.013 , DF (2 / 79), CUM PROB 1

SNK MULTIPLE COMPARISON TABLE:

	A4AP	A3AP	A2AP
A3AP	3.12088		
A2AP	-24 [*]	-27.1209 [*]	

[*] = P < 0.05

Table 9. Anova and SNK multiple comparisons for A4AP vs A3AP vs A2AP at 1000 ug/ml.

	GROUP SIZE	MEAN % DEATH	VARIANCE	STD. DEV.	SEM
A4AP	26	15.8462	50.2954	7.09193	1.39084
A3AP	26	2.53846	7.37846	2.71633	.532717
A2AP	28	58.2857	208.952	14.4552	2.73177

GRAND MEAN 26.375

ANALYSIS OF VARIANCE TABLE:

	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE
BETWEEN GROUPS	46167.2	2	23083.6
WITHIN GROUPS	7083.56	77	91.9942
TOTAL	53250.8	79	

F 250.924 , DF (2 / 77), CUM PROB 1

SNK MULTIPLE COMPARISON TABLE:

	A4AP	A3AP	A2AP
A3AP	13.3077 [*]		
A2AP	-42.4396 [*]	-55.7473 [*]	

[*] = P < 0.05

Table 10. Anova and SNK multiple comparisons for A4AP vs A3AP vs A2AP at 1500 ug/ml.

	GROUP SIZE	MEAN % DEATH	VARIANCE	STD. DEV.	SEM
A4AP	30	36.4	157.214	12.5385	2.2892
A3AP	28	4.03571	11.8135	3.43708	.649546
A2AP	30	79.8667	109.292	10.4543	1.90868

GRAND MEAN 40.9205

ANALYSIS OF VARIANCE TABLE:

	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE
BETWEEN GROUPS	84210.8	2	42105.4
WITHIN GROUPS	8047.63	85	94.6779
TOTAL	92258.4	87	

F 444.722 , DF (2 / 85), CUM PROB 1

SNK MULTIPLE COMPARISON TABLE:

	A4AP	A3AP	A2AP
A3AP	32.3643 [*]		
A2AP	-43.4667 [*]	-75.831 [*]	

[*] = P < 0.05

Table 11. Anova and SNK multiple comparison for A4AP at all concentrations

	GROUP SIZE	MEAN % DEATH	VARIANCE	STD. DEV.	SEM
100	28	1.85714	3.2381	1.79947	.340068
500	28	4.92857	10.9577	3.31024	.625576
1000	26	15.8462	50.2954	7.09193	1.39084
1500	30	36.4	157.214	12.5385	2.2892

GRAND MEAN 15.125

ANALYSIS OF VARIANCE TABLE:

	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE
BETWEEN GROUPS	21432.4	3	7144.13
WITHIN GROUPS	6199.87	108	57.4062
TOTAL	27632.3	111	

F 124.449 , DF (3 / 108), CUM PROB 1

SNK MULTIPLE COMPARISON TABLE:

	100	500	1000	1500
500	-3.07143			
1000	-13.989 [*]	-10.9176 [*]		
1500	-34.5429 [*]	-31.4714 [*]	-20.5538 [*]	

[*] = P < 0.05

Table 12. Anova and SNK multiple comparison for A3AP at all concentrations

	GROUP SIZE	MEAN % DEATH	VARIANCE	STD. DEV.	SEM
100	28	1.28571	2.13757	1.46204	.2763
500	26	1.80769	2.08154	1.44275	.282947
1000	26	2.53846	7.37846	2.71633	.532717
1500	28	4.03571	11.8135	3.43708	.649546

GRAND MEAN 2.42593

ANALYSIS OF VARIANCE TABLE:

	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE
BETWEEN GROUPS	119.229	3	39.743
WITHIN GROUPS	613.179	104	5.89595
TOTAL	732.407	107	

F 6.74073 , DF (3 / 104), CUM PROB .999665

SNK MULTIPLE COMPARISON TABLE:

	100	500	1000	1500
500	-.521978			
1000	-1.25275	-.730769		
1500	-2.75 [*]	-2.22802 [*]	-1.49725 [*]	

[*] = P < 0.05

Table 13 . Anova and SNK multiple comparisons for A2AP at all concentrations.

	GROUP SIZE	MEAN % DEATH	VARIANCE	STD. DEV.	SEM
100	26	2.61538	22.2462	4.71658	.924998
500	28	28.9286	282.661	16.8125	3.17727
1000	28	58.2857	208.952	14.4552	2.73177
1500	30	79.8667	109.292	10.4543	1.90868
GRAND MEAN	43.8036				

ANALYSIS OF VARIANCE TABLE:

	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE
BETWEEN GROUPS	95192.5	3	31730.8
WITHIN GROUPS	16999.2	108	157.4
TOTAL	112192	111	

F 201.594 , DF (3 / 108.), CUM PROB 1

SNK MULTIPLE COMPARISON TABLE:

	100	500	1000	1500
500	-26.3132 [*]			
1000	-55.6703 [*]	-29.3571 [*]		
1500	-77.2513 [*]	-50.9381 [*]	-21.581 [*]	

[*] = P < 0.05

Table 14. Effect of N-acetylcysteine on lymphocyte cytotoxicity

	% Cells Not Excluding Trypan Blue Dye	
	without NAC	with NAC
1500 ug/ml	70	8
A4AP	43	1
1500 ug/ml	3	0
A3AP	1	3
1500 ug/ml	83	84
A2AP	77	83
1000 ug/ml	44	10
A2AP		
No Drug	0	0

affect the percentage of cell death for this drug. Table 14 summarizes the effect of N-acetylcysteine on the cytotoxicity of the three analogues.

Result of the Electrochemical Cell Study

The oxidizability of acetaminophen and its analogues was evaluated using an electrochemical method (Miner and Kissinger, 1979). Figures 5 and 6 demonstrate that the amperometric signal was proportional to the concentration of A4AP and A2AP. This signal was quantitated by peak height measurement and represented a two electron oxidation of each drug within the electrochemical cell. For A3AP there were peak heights corresponding to the concentrations of the drug using an ultraviolet detector (254 nm); however, there was no signal recorded with the electrochemical cell (Fig. 7). Thus, A4AP and A2AP were readily oxidized by the electrochemical cell while A3AP was not, even when very high concentrations of A3AP (100 μg/ml) were studied.

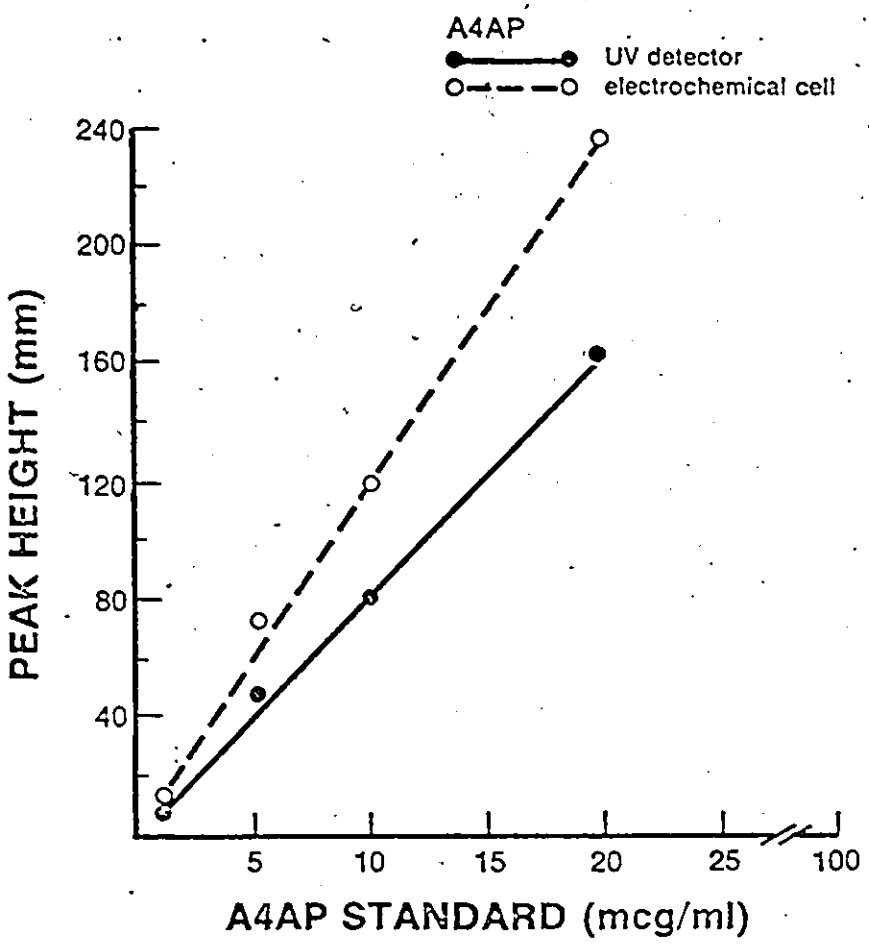


Figure 5. Relationship between the concentration of A4AP and the peak height recorded by both U.V. detector and electrochemical cell.

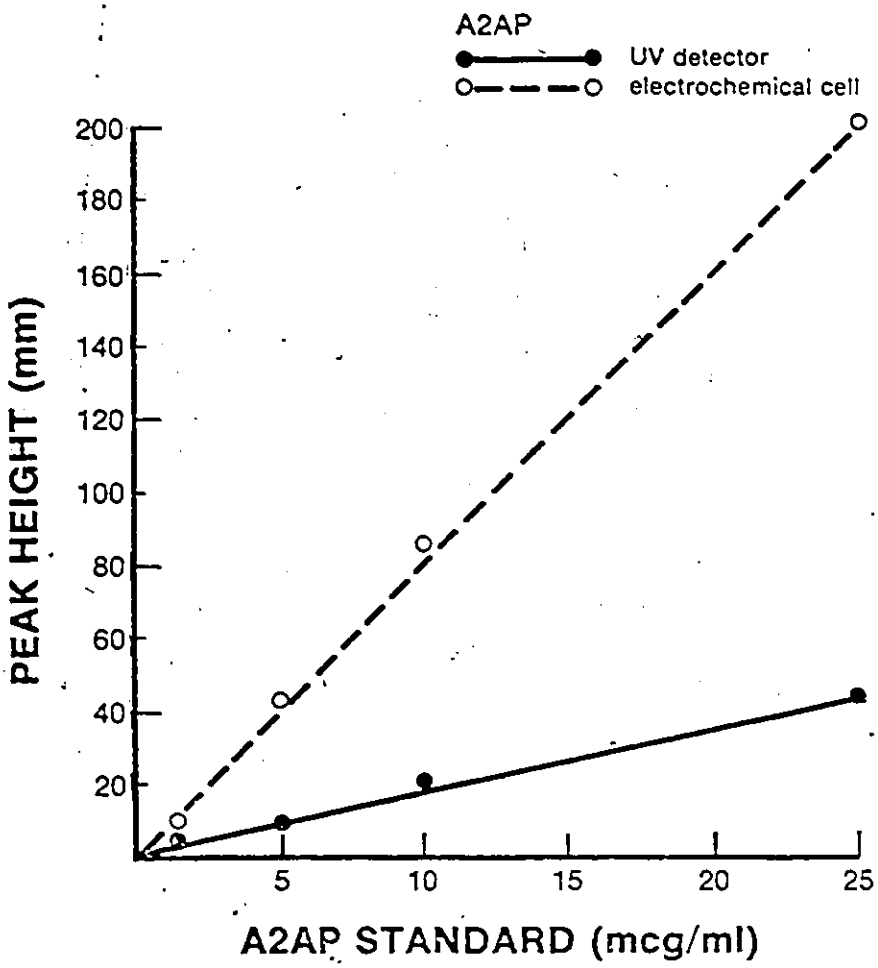


Figure 6. Relationship between the concentration of A2AP and the peak height recorded by both U.V. detector and electrochemical cell.

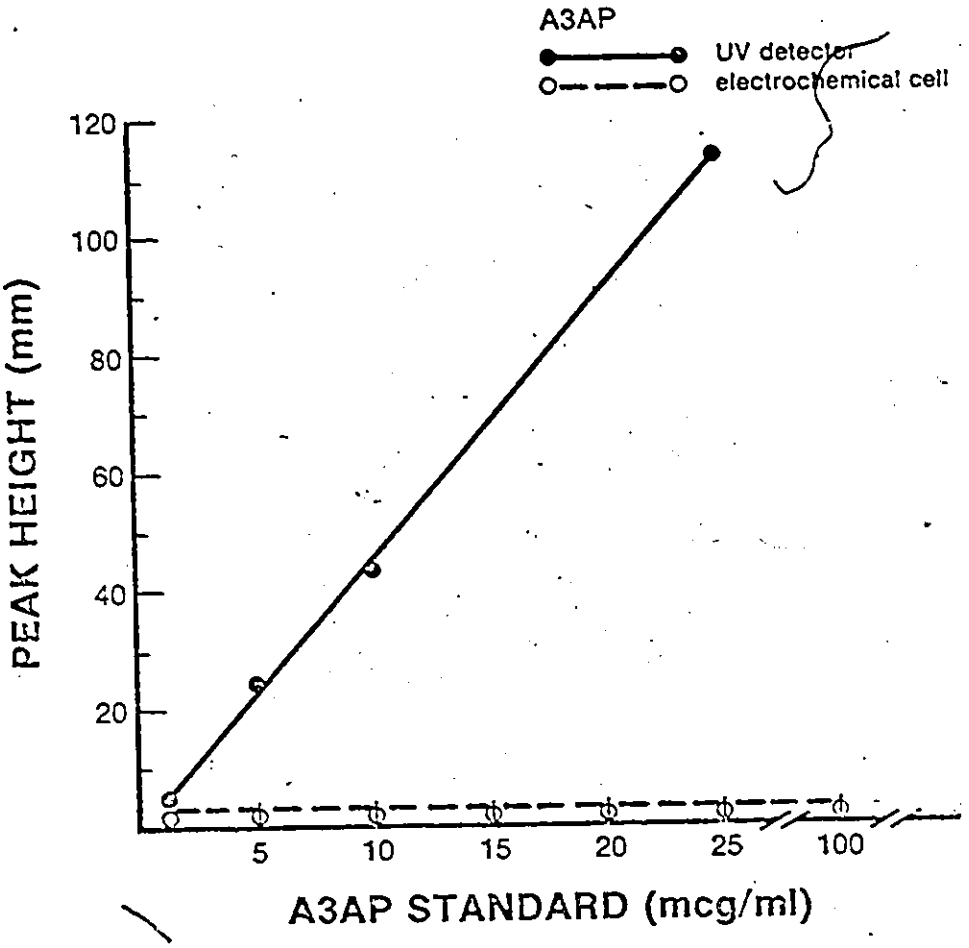


Figure 7. Relation between the concentration of A3AP and the peak height recorded by both U.V. detector and electrochemical cell.

Discussion

Self-poisoning from many causes is now an extremely common problem which may account for over 10% of acute medical admissions to hospital (Matthew and Lawson, 1975). Very few self-poisonings, however, result in death and the overall mortality rate from medicines and other noxious substances either in hospital or out of hospital is less than 3 per 100 self-poisonings. It has been estimated that poisoning is now the cause of over 100,000 admissions in England and Wales (Volans, 1976).

Acetaminophen is a very useful and effective drug but carries the disadvantage of toxicity to the liver on acute overdosage or chronic abuse. Acetaminophen overdosage was first recorded as a cause of death due to hepatic failure by Davidson and Eastham (1966) and Boyd and Bereczky (1966).

The rise in popularity of acetaminophen as an analgesic has resulted in an increased number of hospital admissions and deaths related to overdose of this drug and this has coincided with a fall in hospital admissions and deaths due to salicylate. Death from overdosage and toxic reaction after acetaminophen in England and Wales increased from 7 in 1968 to 61 in 1974 and deaths from acetaminophen in analgesic mixtures in England and Wales increased from 3 in 1968 to 71 in 1974. Consumption of acetaminophen in England and Wales

increased from 1,000 million tablets in 1966 to 2,000 million tablets in 1969 and 3,000 million tablets in 1974. There were 7,000 admissions to hospital for toxic reactions to acetaminophen in England and Wales in 1973. In the United States, acetaminophen overdose cases increased from 482 cases in 1973 to 747 cases in 1974 to 1,181 cases in 1975 (Rumack and Peterson, 1978).

Gazzard in 1976 reported on a questionnaire for one hundred and seven patients admitted to hospitals because of acetaminophen overdosage between the years of 1973 and 1974 in England. The reasons for taking acetaminophen were as follows (Gazzard et al, 1976) (1) 68 patients had pre-existing psychiatric diagnoses and depression, sixteen of this group had previously attempted to take their own lives and 11 were actually psychiatric in-patients who had smuggled acetaminophen tablets into the hospital (2). In the other 39, the suicide had been a much more impulsive attempt, often following a breakdown in interpersonal relationships. About half of the patients had bought acetaminophen specifically with suicide in mind. Most of these patients said that they had chosen acetaminophen because it was freely available and easily seen in the shop. The remaining 55 used acetaminophen as a stock analgesic and they said they took the tablets because they were the only thing, when they decided to commit suicide, that was readily available in the cabinet in the bathroom and only 5 from this group had obtained the tablets with a prescription.

Restriction of acetaminophen to prescription only would probably mean that patients would merely choose to take aspirin rather than acetaminophen. This would serve only to increase the number of people taking aspirin overdoses, in whom there is a higher overall mortality rate than in those taking acetaminophen (Wright, 1974). Restriction of aspirin to prescription only would greatly increase the work load of general practitioners and probably have little effect on the incidence of toxicity. Limiting the sale of acetaminophen to pharmacy-only would not be very effective as most of the patients bought their tablets in pharmacies anyway. It would be possible to limit the number of tablets in a pack, but many of the patients had bought packs in several different pharmacies to avoid suspicion. To decrease the number of suicidal attempts with acetaminophen, Gazzard et al,(1976) suggested putting a carefully worded warning on the tablet bottles that there would be an interval of several days before coma ensued.

The study described in this thesis was undertaken in an attempt to assess the difference in toxicity between acetaminophen and its analogues in the hope of finding another answer to the problem of acetaminophen overdosage. A study of the cytotoxicity of acetaminophen analogues in humans was crucial to determining whether or not they would be less toxic than acetaminophen. The fact that analgesics are very popular drugs and that acetaminophen is one of the most commonly used analgesics, due to the public knowledge

that salicylate upsets the stomach, made the problem of acetaminophen overdosage worthy of a serious study.

Dalpe-Scott's demonstration that in vivo A3AP has substantially diminished potential for hepatotoxicity in mice was relevant to this study. In her experiment she gave A3AP by intraperitoneal injection (500 and 750 mg/kg) to both normal and phenobarbital treated CD-1 mice. She found that A3AP produced no hepatic damage. On the other hand, when she performed the same experiment with A4AP, the drug produced centrilobular hepatic necrosis. In the latter experiment, A4AP caused an increase in the serum glutamic oxaloacetic transaminase (SGOT, aspartate aminotransferase) to 2,578 IU/L following an intraperitoneal dose of 500 mg/kg and 14,583 IU/L following a dose of 750 mg/kg in normal CD-1 mice while the level of this enzyme was much less increased, to 420 and 490 IU/L, following intraperitoneal injection of A3AP of 500 and 750 mg/kg (respectively).

Very recently Dalpe-Scott and Peterson (1984) reported that A3AP inhibited PGE synthesis in brain homogenates. The concentration of A3AP for 50% inhibition of PGE_2 synthesis was 250 μM, while it was 75 μM and 50 μM for A4AP and acetylsalicylic acid respectively. Since the first report of Flower and Vane in 1972 that inhibition of PGE synthesis is the mechanism of action of all antipyretics, all subsequent reports have confirmed this statement. Therefore we can expect that A3AP may well have antipyretic properties at

doses higher than that for A4AP. In this regard A3AP did not show any cytotoxicity even at high concentrations (1500 μg/ml).

During the last few years, suspensions of freshly isolated mammalian cells including hepatocytes and kidney epithelial cells (Moldeus et al, 1978) and intestinal epithelial cells have become increasingly used as an experimental model for studies on the metabolism of various drugs and other xenobiotics and on their toxicity. The viability of the freshly isolated cells is usually excellent and they catalyze the various phase I and phase II reactions involved in drug biotransformation at rates comparable to those observed in vivo or with isolated perfused organs (Billings et al, 1977).

Possibly the major advantage of the isolated-cell model system resides, however, in its accessibility to studies of sequences of reactions. The activating role of cytochrome P-450 and the protective role of the various conjugation reactions, especially conjugation with glutathione, can therefore be studied simultaneously in isolated cells making them extremely useful in studies on drug toxicity.

In our study acetaminophen metabolites generated by a mouse hepatic microsomal system were targeted at human lymphocytes and individual differences in susceptibility to toxicity were examined. Lymphocytes were chosen because they are readily obtainable human cells that contain glutathione, and thus

have some resistance against electrophilic metabolites. The cells do not produce significant quantities of reactive metabolites, but are capable of detoxification at least by virtue of their glutathione content. In this study we showed that A3AP was not toxic to human lymphocytes whereas the same concentrations of A4AP were toxic. The meta position of the hydroxyl group in A3AP makes it resistant to oxidation to the intermediate toxic metabolites. In the light of Spielberg's demonstration (1980) of a correlation between lymphocyte cytotoxicity and hepatotoxicity, A3AP would be expected to be less toxic to the liver than A4AP at the same dosage. Our observation that A2AP was more toxic than A4AP could be ascribed to the fact that A2AP does not need activation by cytochrome P450 to yield an intermediate toxic metabolite i.e. A2AP gives rise to the active metabolite in solution spontaneously therefore it was used freshly made up in each experiment. It is difficult to explain Mitchell and Jollow (1975) report that A2AP lacks hepatotoxicity. However, he did not indicate how he arrived at this conclusion. We believe that Mitchell and Jollow made this observation in vivo and that his failure to observe hepatotoxicity was due to rapidity of metabolism and/or extrahepatic excretion of A2AP so that the drug did not have sufficient contact with the liver cells for an adequate length of time to induce hepatotoxicity.

We also think that using N-acetylcysteine in the incubation culture to examine its efficacy as an antidote for A4AP and

A2AP is a good model for comparing other drug antidotes which act by binding to the toxic metabolites of the drug.

The conclusions from our lymphocyte study are supported by the results of the studies with the electrochemical cell which showed that A4AP and A2AP were oxidized electrochemically while A3AP was resistant to oxidation by the cell at the same voltage. A3AP showed no evidence of oxidation by the electrochemical cell even at very high concentrations of 100 mcg/ml. The difference between A4AP and A3AP in their susceptibilities for oxidation are in conformity with the lymphocyte cytotoxicity study, which indicated that A3AP was much less toxic than A4AP to human cells

Summary and Conclusion

The toxicities of N-acetyl-p-aminophenol (A4AP), N-acetyl-m-aminophenol (A3AP) and N-acetyl-o-aminophenol (A2AP) on human lymphocytes in culture have been studied by a modification of Spielberg's method (1980). Toxicity was determined by estimations of cell death as evidenced by the trypan blue exclusion technique. In order to duplicate the hepatic oxidation of N-acetylaminophenols, known to occur *in vivo*, a mouse liver microsome preparation and NADPH were added to the lymphocyte culture medium.

A3AP was found to have barely detectable toxicity, while A2AP was highly toxic. The addition of N-acetylcysteine to the culture medium completely protected the cells against the toxic effects of A4AP but only partially protected against the toxicity of A2AP. Since A4AP is readily oxidized to toxic metabolites by liver cells, we therefore examined the susceptibility of these compounds to oxidation in an electrochemical cell. We found that, whereas A4AP and A2AP were readily oxidized in this cell, A3AP resisted oxidation.

It is concluded that A3AP is a potentially much less hepatotoxic drug than A2AP or A4AP. Other studies in our laboratory have shown that A3AP inhibits PGE_2 synthesis in brain homogenates. The two properties of A3AP: (1) lack of cytotoxicity; and, (2) inhibition of brain PGE_2 synthesis

suggest the value of further investigation of A3AP as a possible antipyretic or analgesic.

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