2. LITERATURE REVIEW

2.1 Paracetamol

Paracetamol (Acetaminophen, *N*-acetyl-p-aminophenol is a derivative of para-aminophenol. It was first used in medicine by Von Mering in 1893. However, it has been gained popularity since 1949, after it was recognized that the major active metabolite of both acetanilide and phenacetin were proved to be excessively toxic. Although paracetamol is a metabolite of both phenacetin and acetanilide but it dose not share the renal or hematologic toxicity of its precursors.

The molecular weight of paracetamol is 151.2. It is a moderately water and lipid-soluble, weak organic acid with a pK_a of 9.5. Thus, it is unionized over the physiological range of pH.

Phenol has appreciable water solubility and apparently form strong hydrogen bond. Introduction of other radicals into the hydroxy group of p-aminophenol and into the free amino group of aniline reduces toxicity without lose of antipyretic action (Roberts and Morrow, 2001).
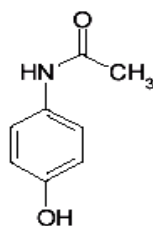

Figure 1 Molecular structure of paracetamol

2.1.1 Pharmacological effects and mechanism of action of paracetamol

Paracetamol has antipyretic and analgesic actions, but no significant anti-inflammatory effects (Clissold, 1986). Paracetamol has analgesic and

antipyretic properties, because it is more effective against enzyme for prostaglandins biosynthesis in the central nervous system than those in the periphery.

2.1.2 Pharmacokinetic properties of paracetamol

2.1.2.1 Absorption

Paracetamol absorption appears to be negligible from stomach but very rapid from small intestine. The mean absorption rates of paracetamol were

similar in proximal and distal parts of small intestine (Gramatte and Richter, 1993). In rats, absorption does occur slowly from stomach and colon, and is most rapid from small intestine, with 70% of the drug being absorbed within 30 minutes (Bagnall *et al.*, 1979). Absorption is by passive transport with first-order kinetics. The gastric emptying is the rate-limiting step in the absorption of paracetamol (Clements *et al.*, 1978).

Although paracetamol is rapidly absorbed from gastrointestinal tract, however, it is incompletely available to the systemic circulation after oral administration, due to first-pass metabolism (Rawlins *et al.*, 1977; Perucca Richens, 1979 and Hirate *et al.*, 1990). The concentration reaches a peak in 30 to 60 minutes and the plasma half-life is about 2 hours after therapeutic doses (Roberts and Morrow, 2001). The usual therapeutic doses produce plasma concentration of 5 to 20 µg/ml. After 8 hours, only small amount of unchanged paracetamol is detectable in plasma (Clissold, 1986).

As paracetamol absorption seems to be predominantly dependent on the rate of gastric emptying, any drug, disease, or other condition which alters the rate of gastric emptying may influence the rate of paracetamol absorption.

2.1.2.2 Distribution

Paracetamol distributes throughout most tissues and fluids, reaching a

tissue : plasma concentration ratio of about unity in all tissues except fat and cerebrospinal fluid. With normal therapeutic dose, paracetamol is slightly bound to plasma proteins (William *et al.*, 2001), only 20 to 50% may be bound at the concentrations encountered during acute intoxication (Roberts and Morrow, 2001). Generally, the apparent volume of distribution of paracetamol is about 1 l/kg. The distribution volume is similar in healthy subjects, children and the elderly (Briant *et al.*, 1976; Peterson and Rumack, 1978), in patients with epilepsy (Perucca and Richens, 1979), Gilbert's syndrome (Douglas *et al.*, 1978) and in anephric patients (Lowenthal *et al.*, 1976).

Divoll *et al.* (1982) demonstrated that the volume of distribution of paracetamol (corrected for weight) was larger in men than in woman (0.99 and 0.86 l/kg) and declined with age in both sexes. The authors explained that the reduction in volume of distribution of paracetamol in women and elderly might be due to increasing fat per kilogram body weight and incomplete distribution of nonlipophilic property of paracetamol into body fat.

Beaulac-Baillargeon and Rocheleau (1994) found an inverse correlation (r=0.85) between maximal plasma paracetamol concentration and the weight of the pregnant women (P<0.01) but not with the weight of the control women. The authors suggested that weight gain in pregnant women due to the expansion of total body water caused by an increase in the plasma volume, extracellular fluid and amniotic fluid. So that increasing in volume of distribution of paracetamol relates to lower in maximal plasma paracetamol concentration.

2.1.2.3 Metabolism

Paracetamol is metabolized extensively in the liver and a minor extent the intestine (Josting *et al.*, 1976, Mitchell *et al.*, 1977) (Figure 2).
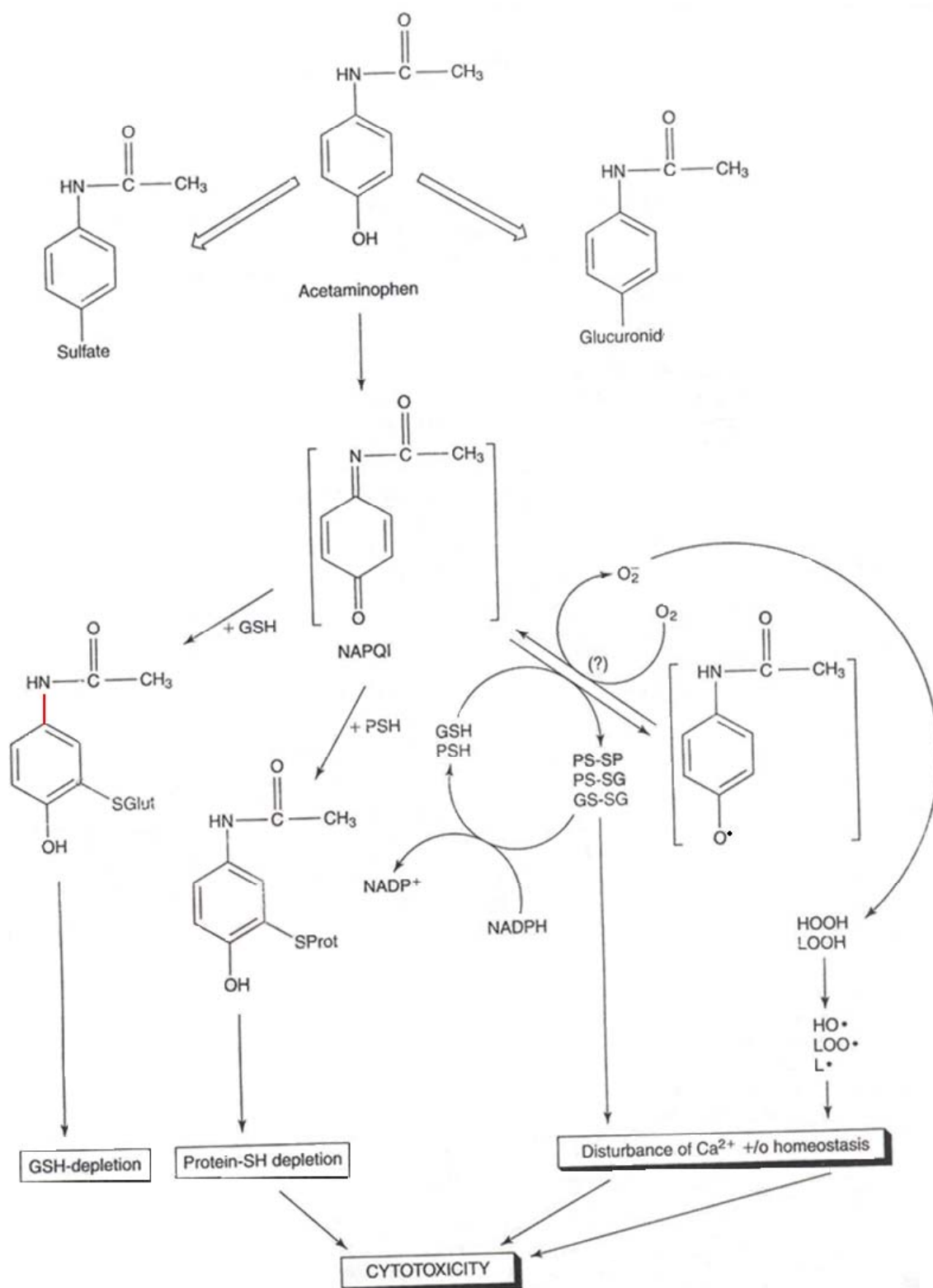
Figure 2 Pathways of paracetamol metabolism. (Source: Dahm and Jones, 1996)

It is metabolized predominantly by glucuronide conjugation (approximately 60%) and sulfate conjugation (approximately 35%). Glucuronidation and sulfation require UDP-glucuronyltransferase and sulfotransferase which are located in the endoplasmic reticulum and cytoplasmic compartments of the cell, respectively.

In addition, both glucuronide and sulfate conjugations are capacity-limited (saturable) processes (Galinsky and Levy,1981.; Levy and Yamada, 1971) that may be due to cosubstrate depletion, i.e. uridine diphospho glucuronic acid (UDPGA) and $3^/$-phosphoadenosine-$5^/$-phosphosufate (PAPS) concentrations in the liver for glucuronidation and sulfation respectively. It appears that the formation of paracetamol glucuronide and sulfate conjugate in man become capacity limited upon administration of the 2 g dose of paracetamol (Levy and Yamada, 1971). In general, both conjugates are biologically inert metabolites and excreted mainly in the urine (Rumore and Blaiklock, 1992). A minor fraction of paracetamol is probably metabolized via oxidation by cytochrome P450. Two possible products of CYP-mediated paracetamol oxygenation are known, *N-acetyl-p*-benzoquinoneimine (NAPQI) and 3-hydroxyparacetamol, the latter compound being considered nontoxic (Chen *et. al*., 1998).

The formation of NAPQI is dependent on some cytochromes P450, identified in liver microsomes, in rats as CYP1A1, CYP1A2, and CYP2E1 (Patten *et al*., 1993); in mice as CYP1A2 and CYP3A4 (Zaher *et al*., 1998); and in humans as CYP2E1, CYP1A2 and CYP3A4 (Raucy *et al*., 1989; Thummel *et al*., 1993). However, recent pharmacokinetics studies in human volunteers have demonstrated that involvement of CYP1A2 and CYP3A4 in NAPQI formation *in vivo* is much less than that of CYP2E1, as omeprazole (CYP1A2 inducer) and rifampicin (CYP3A4 inducer) treatment have no effect on the

formation of NAPQI from paracetamol, whereas disulfiram (CYP2E1 inhibitor) treatment decreases NAPQI formation (Sarich *et al.*, 1997; Manyike *et al.*, 2000). Studies using CYP2E1 and CYP1A2 knockout mice have shown that the former, but not the latter, are more resistant to paracetamol-induced hepatotoxicity than wild-type animals (Lee *et al.*, 1996; Tonge *et al* .,1998; Zaher *et al.*, 1998), indicating that CYP2E1 is a primary contributor to paracetamol biotransformation among CYPs. Concerning the other CYP enzymes responsible for bioactivation of paracetamol, Hazai *et al.* (2002) recently reported that selective inhibition of CYP2A6 as well as CYP2E1 significantly decreased NAPQI formation in human liver microsomes, whereas CYP1A2 and CYP3A4 inhibition did not affect NAPQI production, suggesting that CYP2A6 may also be responsible for paracetamol bioactivation. .The CYP2E1 and 2A6 were significantly different in the ratio of NAPQI to 3-hydrpxyparacetamol produced. CYP2E1 produces products in the ratio of 6:1, NAPQI vs 3-hydroxyparacetamol, while for 2A6 the ratio is 1:3 (Chen *et. al.*, 1998). It is not known whether the oxidation of paracetamol carried out by CYP generates a transient radical species, or if a concerted two-electron oxidation occurs.

The toxic intermediate, *N-acetyl-p*-benzoquinoneimine (NAPQI), is produced through the *N*-oxidation of paracetamol to *N*-hydroxyparacetamol, followed by dehydration, equation 1 (Vries, 1981).

$$\text{(1)}$$

paracetamol   *N*-hydroxyparacetamol   NAPQI
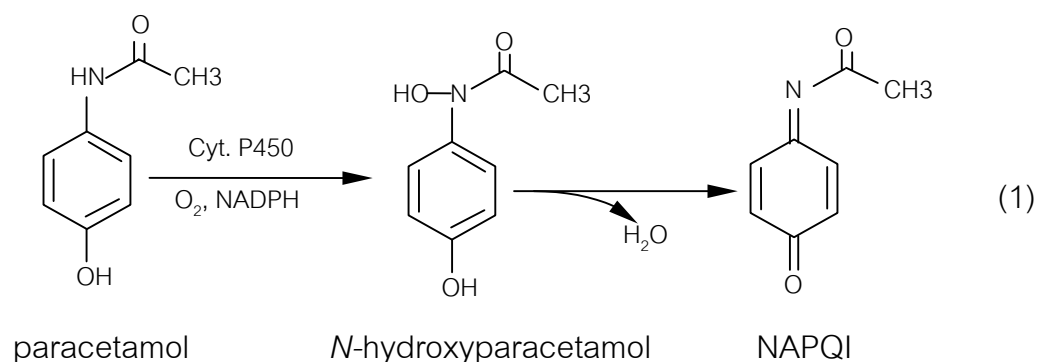
However, many studies proposed that *N*-hydroxyparacetamol is not formed as intermediate, instead paracetamol undergoes one or two electron oxidation to quinoneimine reactive species, equation 2 (Vries, 1981).

(2)

paracetamol   NAPSQI   NAPQI
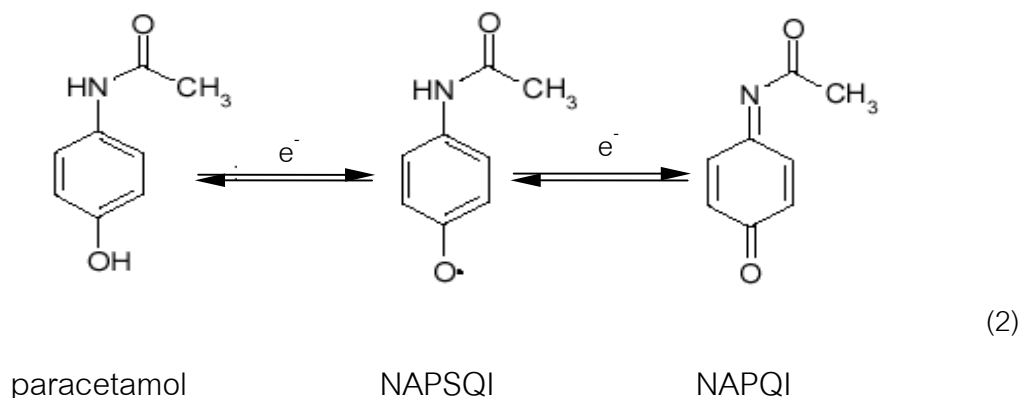
The semiquinone radical, *N*-acetyl-*p*-benzosemiquinone (NAPSQI), intermediate of paracetamol might undergo a cyclic oxidation-reduction process consisting of the oxidation of the semiquinone to the quinoneimine by molecular oxygen, with generation of superoxide, followed by the reformation of the simiquinone by microsomal NADPH-cytochrome *c* reductase (figure 3). The hepatotoxicity of paracetamol might be due to production of the semiquinone free radical (the semiquinone is far more reactive than the quinoneimine) and/or active oxygen species, such as H_2O_2 and $O_2^{\cdot-}$. Both the intermediate and active oxygen can bind and inactivate intracellular proteins (Hinson *et al*., 1981; Vries, 1981)
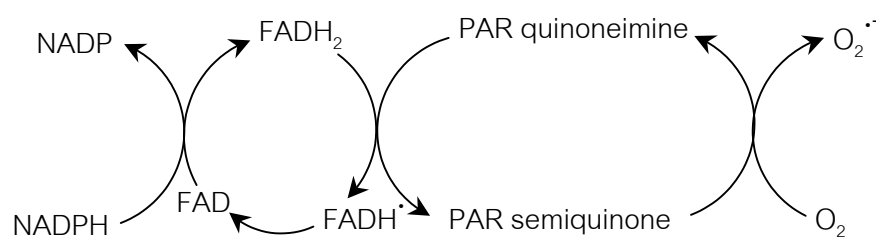

Figure 3 Proposed oxidation-reduction cycle for PAR semiquinone and associated production of superoxide (Vries, 1981).

NAPQI formed after the ingestion of a therapeutic dose of paracetamol is promptly detoxified by conjugation with glutathione. Glutathione is a sulfhydryl compound that plays an extremely important role in protecting liver, renal, and other organ damage from reactive metabolite of paracetamol, NAPQI. Firstly, glutathione attacks NAPQI with the glutathione-s-transferase. Then, the glutamate and glycine portions are split off by gamma-glutamyl transpeptidase and cysteinyl glycinase, respectively. The free cysteine-drug conjugate is finally acetylated by a cytoplasmic acetyltransferase to form a mercapturic acid conjugate. The cysteine and mercapturic acid conjugates are nontoxic metabolites and excreted in the urine (Rumore and Blaiklock, 1992).

When a toxic dose of paracetamol is ingested, the glucuronidation and sulfation pathways become saturated, shifting the primary metabolic process to the cytochrome P450 isoenzyme system and produced large amount of NAPQI (Peter and Edward, 1999). As a result, cellular glutathione stores is depleted faster than it can be regenerated, accumulation of toxic metabolite occurs. In the absence of intracellular glutathione, this reactive metabolite formed covalent binding to cellular macromolecules, especially proteins essential for cellular homeostasis. Moreover the production of oxidative stress within the hepatocyte may result in hepatic necrosis in humans (Golden *et al*., 1981), rats, mice and hamsters (Mitchell *et al*.,1973a).

The main region of paracetamol-induced hepatic damage is the centrilobular zone because centrilobular cells contain the largest concentration of cytochrome P450 in the liver. Similarly, the renal bioconversion of paracetamol in excess dose within the kidneys may result in a nephrotoxic intermediate metabolite, which produces tubular or cortical necrosis due to depletion of glutathione. However, nephrotoxicity is not necessary be

associated with hepatic necrosis (Kritharides *et al*., 1988).

2.1.2.4 Elimination

In young healthy subjects approximately 85-95% of therapeutic dose is excreted in urine within 24 hours, as unchanged paracetamol (4%), glucuronide (55%), sulphate (30%), cysteine (4%) and mercapturic acid conjugates (4%) (Forrest *et al*., 1979; Prescott, 1980). However, neonates and children aged 3 to 10 years excreted significantly less glucuronide and more sulfate conjugate than children aged 12 years and adults. In rats, sulfate conjugate and mercapturic acid are primarily excreted at low dose of the drug whereas at higher dose, glucuronide conjugate is more dominant.

Other minor metabolites have been described, each accounting for 1% or less of a therapeutic dose. These include sulphate and glucuronide conjugates of 3-methoxy-paracetamol, 3-hydroxy-paracetamol (Andrews *et al*., 1976) and 3-methyl-thioparacetamol (Klutch *et al*., 1978).

As a moderately lipid-soluble, weak organic acid, paracetamol undergoes considerable filtration with subsequent extensive tubular reabsorpion. However, excretion of paracetamol is independent of urinary pH but appears to be weakly correlated with urine flow rate (Morris and Levy, 1984; Prescott, 1980). The highly polar sulphate and glucuronide conjugates of paracetamol are apparently active secreted by the tubules as indicated by their respective renal clearance rates of approximately 170 and 130 ml/min, and there is no correlation with urine flow or pH. The renal clearance of the sulphate conjugate of paracetamol is concentration dependent (Morris and Levy, 1984).

2.1.3 Adverse Effect of paracetamol

With normal therapeutic doses, paracetamol is virtually free of any significant adverse effects. Skin rash and other allergic reactions occur

occasionally. The rash is usually erythematous or urticarial, but sometimes it is more serious and may be accompanied by drug fever and mucosal lesions (Clissold, 1986; David and Edward, 1994; Mycek *et al*., 1997). Patients who show hypersensitivity reactions to the salicylates only rarely exhibit sensitivity to paracetamol and related drugs (Stevenson and Lewis, 1987). There may be minor alterations in leukocyte count, but these are generally transient (Mycek *et al.*, 1997). In a few isolated cases, the use of paracetamol has been associated with agranulocytosis, neutropenia, thrombocytopenia, and pancytopenia (Roberts and Morrow, 2001). Paracetamol causes methaemoglobinemia and oxidative hemolysis in dogs, pigs and cats but not normally in humans, even after over dosage (Henne-Bruns *et a*l., 1988). In chronic toxicity studies, paracetamol is less potential for nephrotoxicity (renal papillary necrosis) than aspirin and other non-steroidal anti-inflammatory analgesics (Golden *et al*., 1981). Strain-dependent cataract formation and other ocular abnormalities have been described in induced mice (Zhao and Shichi, 1998) and in one study, paracetamol produced a high incidence of liver cell tumors in 1F mice (Flaks, 1983). High doses of paracetamol given chronically to animals may cause testicular atrophy and inhibition of spermatogenesis (Wiger *et al*., 1995).

2.1.4 Hepatotoxicity

The hepatotoxicity of paracetamol is generally accepted by the formation of NAPQI, a metabolite formed by cytochrome P450. The quantitatively most significant of these is CYP2E1 (Raucy *et al*.,1989).

A number of recent reports indicate that massive overdose of paracetamol can produce a fulminate acute centrilobular hepatic necrosis in humans (Golden *et al*., 1981; McJunkin *et al*., 1976) and experimental animals (Lim *et al*., 1994). There are considerable species differences in susceptibility.

The acute hepatotoxic doses in hamsters, mice, and rats are about 150 mg/kg, 300 mg/kg and 3,000 mg/kg, respectively. In adults, hepatotoxicity may occur after ingestion of a single dose of 10 to 15 g (150 to 250 mg/kg) of paracetamol (Barker *et al*., 1977; Bonkowsky *et al*., 1978; Clissold, 1986; Hamlyn *et al*., 1978). The doses of 20 to 25 g or more are potentially fatal and death caused by severe hepatotoxicity with centrilobular necrosis, sometimes associated with acute renal tubular necrosis. Symptoms occur in the initial 24 hours and may persist for a week or more. Clinical indication of hepatic damage become manifest within 2 to 4 days of ingestion of toxic doses. Initially, plasma transaminases are elevated, and the concentration of bilirubin in plasma may be increased; in addition, the prothrombin time is prolonged. Perhaps 10% of poisoned patients who do not receive specific treatment develop severe liver damage; of these, 10 to 20% eventually die of hepatic failure. Acute renal failure also occurs in some patients.

Severe liver damage (with levels of aspartate aminotransferase activity on excess of 1,000 I.U. per liter of plasma) occurs in 90% of patients with plasma concentrations of paracetamol greater than 300 µg/ml at 4 hours or 45 µg/ml at 15 hours after the ingestion of the drug (Roberts and Morrow, 2001; Prescott *et al*., 1979). Minimal hepatic damage can be anticipated when the drug concentration is less than 120 µg/ml at 4 hours or 30 µg/ml at 12 hours after ingestion (Roberts and Morrow, 2001). The potential severity of hepatic necrosis can also be predicted from the half-life of paracetamol observed in the patient; values greater than 4 hours imply that necrosis will occur, while values greater than 12 hours suggest that hepatic coma is likely (Gazzard *et al*., 1977; Prescott *et al*., 1971). In patients with severe liver damage there may be a progressive increase in the plasma half-life.   Prolongation of the plasma paracetamol half-life is associated with a marked increase in ratio of

the plasma concentrations of unchanged to conjugated drug, and in patients with fatal hepatic necrosis the conjugation of paracetamol may virtually cease.

In children, risk is defined as doses > 150 mg/kg. Maximum liver damage occurs 2-4 days after ingestion, and elevated levels of bilirubin in serum and jaundice may appear 2-6 days after ingestion. Symptoms include vomiting, anorexia, and epigastric pain. Hepatic encephalopathy may develop, even in relatively mild cases. Paracetamol levels in serum associated with hepatotoxicity are usually > 300 µg/ml at 4 hours or > 50 µg/ml at 12 hours postingestion. Hepatotoxicity is usually not seen at levels < 120 or < 50 µg/ml at 4 hours and 12 hours, respectively (Adamson, 1988; Clissold, 1986; Miners *et al.*, 1988).

2.1.5 Mechanisms of toxicity

The precise mechanism by which paracetamol causes cell death remains unknown, although there are two prevailing theories that are controversial. According to the first theory, there are biochemical reactions between the reactive metabolites and macromolecular cell components (proteins. lipids, DNA). The second theory, the oxidative stress theory, which believed that paracetamol metabolites cause oxidative stress in the cell and ultimately leading to its demise (Gibson *et al.*, 1996).

The first theory stated that NAPQI can oxidize cysteine groups in proteins (Albano *et al.*,1985; Kyle *et al.*, 1990; Birge *et al.*,1991) by covalent binding to this amino acid in proteins and leading to protein arylation *in vitro* and *in vivo* (Hoffmann *et al.*,1985). In the 1970s, covalent binding of radiolabeled paracetamol to proteins was described and suggested to play an important role in the toxicity mechanism of paracetamol (Potter *et al.*,1973 ; Potter *et al.*,1974).

Studies in mice have shown that paracetamol metabolites form adducts to hepatic proteins of 44, 55, and 58 kilodaltons (kd) with high

selectively. These proteins have been identified as a selenium binding protein, the function of which has not been established. A similar 58 kd hepatic protein was targeted in a human paracetamol overdose (Bartolone *et al.*,1992). Thus, the 58 kd cytosolic protein may be a critical target in paracetamol hepatotxicity.

However, the mechanistic importance of covalent adduct formation to cellular injury has been challenged on the basis of two lines of evidence. First, a number of compounds, that experimentally prevent hepatotoxicity from paracetamol, including *N*-acetylcysteine, cimetidine, and antioxidants do so without significant reducing the overall extent of covalent adduct formation (Vermeulen *et al.*, 1992). Second, specific derivatives of paracetamol, such as $3^{/}$ hydrocyacetanilide and 3,5–dimethyl paracetamol produce covalent binding to hepatic proteins without causing hepatotoxicity or induce toxicity without the formation of covalent protein adducts.

The second theory is the oxidative stress theory. Recent studies (Arnaiz *et al.*, 1995; Jaeschke *et al.*, 2003) support a major role for oxidative stress produced by NAPQI in hepatotoxicity of paracetamol. NAPQI can oxidize GSH, thereby lowering the GSH/GSSG status, and it can oxidize protein SH group, leading to the formation of interstrand disulfide bridges, of interprotein crosslinking or of mixed disulfides (between protein and glutathione).

Another hypothesis is that oxidative stress, lipid peroxidation (LPO), caused by a redox cycling metabolite of paracetamol leading to lipid peroxidation. NAPQI was suggested to give rise to futile cycling of P450, using reducing equivalents of NADPH with concomitant reduction of molecular oxygen to the superoxide anion radical ($O_2^{\bullet -}$). The superoxide anion radical is enzymatically reduced to hydrogen peroxide (H_2O_2), which in turn may lead to hydroxyl free radical ($OH^{\bullet}$) formation in the presence of traces of metal ions in

the Fenton reaction (Goeptar *et al.*,1995). When reacting with lipids, these very reactive hydroxyl free radicals may initiate lipid peroxidation, but it is a late event and appears to be the result rather than the cause of cellular necrosis (Vermeulen *et al.*,1992; Kamiyama *et al.*,1993).

The key event in the production of cell death from paracetamol appears to be the disruption of cellular Ca^{2+} homeostasis. Activation of Ca^{2+} dependent proteases, phospholipases, and endonucleases produces catastrophic cellular damage (Shen *et al.*, 1992). The hepatotoxicity can vary up or down, depends on the rates of either or both of the two critical processes such as microsomal production of toxic intermediates and their detoxification by glutathione, each process is influenced, in turn, by several factors. For example, the rate of formation of NAPQI is not simply a function of drug dose or bioavailability, but also depends on the activity of the glucuronide and sulfate conjugation pathway, the availability of glucoronic acid and sulfate, and the activity of the cytochrome P450 pathway. Prior administration of other drugs (e.g., phenobarbital and ethanol) that induce the microsomal cytochrome P450 pathway predisposes to paracetamol hepatotoxicity (Vermeulen *et al,*. 1992), whereas inhibitors of the pathway, including cimetidine is protective. The increased susceptibility to paracetamol hepatotoxicity caused by prior chronic use of ethanol, barbiturate, and other inducers.

2.1.6 Treatment of paracetamol poisoning

When the formation of NAPQI is sufficient, glutathione stores will be below a critical level (about 20 to 30 percent of normal) that is no longer adequate to sustain detoxification of NAPQI. At this point, the disruption of cellular structure and function occurs mainly as a result of two processes

dependent upon the electrophilic and oxidative properties of NAPQI and was enhanced by the loss of the protective reducing properties of glutathione.

In the treatment of paracetamol overdoses, administration of sulfhydryl compounds such as *N*-acetylcysteine (NAC) and methionine increase synthesis of glutathione or restoring intracellular glutathione (Lauterburg *et al*., 1983). *N*-acetylcysteine can directly conjugate with NAPQI (Buckpitt *et al*., 1979; Miners *et al*., 1984). In addition, *N*-acetylcysteine may repair of oxidative damage by generating cysteine or glutathione (Boobis *et al*., 1986). Various cysteine prodrugs have been reported to protect the liver from paracetamol-induced hepatotoxicity in experiments using animals. The mechanism responsible for this protection may be metabolism of these prodrugs to L-cysteine, which is incorporated into hepatic glutathione synthesis (Lauterburg *et al*., 1983; Hazelton *et al*., 1986a; Roberts *et al*., 1987; Kitamura *et al*., 1989; Wlodek and Rommelspacher, 1997; Srinivasan *et al*., 2001).

Several studies have shown that inhibitors of cytochrome P450 oxidation are effective in decreasing paracetamol-induced hepatotoxicity (Mitchell *et al*., 1973a). Selective inhibition of metabolic oxidation by cimetidine has also been shown to reduce paracetamol-induced hepatotoxicity in animals (Prescott and Critchely, 1983). However, cimetidine has not been proven to affect the metabolism of paracetamol in humans, since an oxidative inhibitor has to be given before paracetamol overdose (Abernethy *et al*., 1983). Recently, combined treatment of intoxicated mice with NAC and cimetidine at 2 hours after paracetamol administration provided 100% survival rate and a marked reduction in plasma AST and ALT level. In comparison, single administration of either NAC or cimetidine caused only a partial improvement in these parameters (Al-Mustafa *et al*., 1997).

Moreover, physiological antioxidant defences have been found to

protect against paracetamol-induced liver damage by inhibition of lipid peroxidation. However, covalent binding of NAPQI to intracellular macromolecules might also exist (Tatsuya *et al.*, 1995).

2.1.7 Protective mechanism

Prevention of GSH depletion and induction of glutathione transferase and glutathione reductase are the most efficient ways of direct protection against paracetamol hepatotoxicity (Vermeulen *et al.*, 1992). Glutathione plays an important role in the detoxification of electeophilic metabolite of acetaminophen by trapping the reactive metabolite and then it prevents the covalent binding to tissue macromolecules and its hepatotoxicity (Mitchell *et al.*, 1973b).

Glutathione S-transferases are a group of emzymes represent some 10% and 3% of the hepatic cytoplasmic protein in rat and man, respectively (Jakoby,1978). According to hepatic levels, reduced glutathione are normally quite high and at these levels, cytosol enzymes may be of little importance in detoxification, only when the level of glutathione has become depleted would the cytosolic enzymes play an important role in protecting the hepatocyte from paracetamol-induced necrosis (Jakoby,1978., Coles and Ketterer, 1990). As an important characteristic, glutathione S-transferases localized within the membranes of the endoplasmic reticulum would affect the steady level of lipophilic reactive metabolites close to their site of xenobiotic generation including paracetamol. Although the conjugation of NAPQI to electrophilic amino acids, such as cysteine, is not thought to require enzymatic catalysis, the reaction of NAPQI with GSH can occur with or whithout the mediation of GSH-conjugating enzymes such as glutathione S-transferases (GST) (Henderson *et al.*, 2000). The glutathione S-conjugates are metabolized by the same degradative enzyme that metabolized glutathione. The breakdown

products of the glutathione S-conjugates are glutamate, glycine and cysteine-conjugates, which can also be reabsorbed into the cell. The glutamate and glycine may then be used for glutathione synthesis, whereas the cysteine S-conjugates can be acetylated on the amino group of the cysteinyl residue by intracellular *N*-acetyltransferases to form the corresponding mercapturic acids (*N*-acetylcysteine S-conjugates). Mercapturic acids are released into the circulation or bile (Hinchman *et al*., 1991).

Glutathione depletion reflected a loss of total glutathione and no conversion to oxidized glutathione, since hepatic concentration of the latter was always less than 0.15 mM (reduced glutathione + oxidized glutathione is in range of 4 to 5 mM). Furthermore, the rate of releasing of glutathione in the reduced form was about 10-fold higher (11.8 nmole GSH/min/gm liver) than in the oxidized form (1.0 nmole/min/gm liver) (Gianna and Helmut, 1978). Conjugation with GSH is an essential aspect of both xenobiotic and normal physiological metabolism (Strange *et al*., 2000). Formation of conjugates can result in depletion of GSH and has been used as a tool to study the role of GSH in detoxification.

Treatment with exogenous antioxidant may appear to be helpful in conditions related to oxidative stress. It is anticipated that an antioxidant would be useful as a therapeutic agent if it is easily available and nontoxic.

2.2 Reactive oxygen species (ROS)

Cell damage is induced by reactive oxygen species (ROS). ROS are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. Examples are hydroxyl radical, superoxide, hydrogen peroxide, and peroxynitrite. The main source of ROS *in vivo* is aerobic respiration, although ROS are also produced by cytochrome P450

metabolism of xenobiotic compounds, stimulation of phagocytosis by pathogens, arginine metabolism, and tissue specific enzymes (Nicholls and Budd, 2000).

Radical reactions are generally chain reactions. The radicals are generated in a step or steps call "initiation", they participate in a sequence of "propagation" reactions in which their number is conserved, finally they are destroyed in a "termination" process or processes (Roberfroid and Colderon, 1995).

In initiation of radical chain reaction, oxygen (O_2), hydrogen peroxide (H_2O_2), water (H_2O) and polyunsaturated fatty acids appear to play an essential role as the major substrates for these events. O_2 does so because it serves as an acceptor for single electron arising in cells either via the one-electron enzymatic reduction of endogenous and exogenous organic molecules or as a side product of incomplete electron transport in mitochondria, endoplasmic reticulum or nuclear membranes. Monoelectronic reduction of O_2 produces superoxide anion ($O_2^{\bullet-}$). H_2O_2, the product of $O_2^{\bullet-}$ dismutation, plays a role mainly because it is the substrate of mental in catalyzed decomposition to yield the hydroxyl radical ($HO^{\bullet}$). Polyunsaturated fatty acids occupy a key position in radical chain reaction, not because they are direct substrates of major initiation processes, but because they can easily be transformed in alkyl hydroperoxides (LOOH). The alkyl hydroperoxides are transformed to either alkoxyl ($LO^{\bullet}$) or alkylperoxyl ($LOO^{\bullet}$) radical as a result of a molecule assisted homolysis.

Propagation of radical chain reaction: The hydroxyl radical ($HO^{\bullet}$) plays a major role, but hydroperoxide (LOOH) as well as alkoxyl or alkylperoxyl ($LO^{\bullet}$, $LOO^{\bullet}$) radicals of polyunsaturated fatty acids are still key targets and/or key intermediates. H-atom transfer, electron transfer, as well as addition are the

major events in biologically relevant radical propagation.

Termination of radical chain reaction: Both dismutation and reduction involve electron transfer, either by electron exchange between identical molecular entities or by electron capture in a classic oxidoreduction process. Scavenging involves the formation of a radical that is less likely to propagate the radical but, rather, is likely to homolink. The most important homolinking reaction in biological system is that involving 2 glutathione radicals (GS$^\bullet$) to form the oxidized glutathione (GSSG).

2.2.1 The production of reactive oxygen species

2.2.1.1 The superoxide radical ($O_2^{\bullet-}$) is derived from molecular oxygen by the addition of a single electron (Yu, 1994). Reactions that produce superoxide radicals occur in many organelles of cell. For example, oxyhemoglobin in cytosol of erythrocytes can decompose and release $O_2^{\bullet-}$ and methemogolbin. Some phagocytes such as neutrophils and macrophages possess a reduced nicotinamide adeninedinucleotide phosphate oxidase (NADPH oxidase) that produce $O_2^{\bullet-}$ when the cells are activated by a component of the bactericidal armamentarium (Babior, 1978).

An electron is transported from a donor, X such as hemoglobin, cytochrome, quinone, thiol or redox metal to oxygen molecule to form superoxide and oxidized donor, X^+. The dismutation of superoxide requires two protons to form hydrogen peroxide. Superoxide dismutase (SOD) enzyme is responsible for its detoxification. The fundamental reaction of producing $O_2^{\bullet-}$ is as follow :

$$X + O_2 \longrightarrow X^+ + O_2^{\bullet-}$$

$$O_2^{\bullet-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$
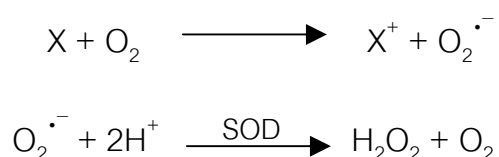
2.2.1.2 Hydrogen peroxide (H_2O_2) is a non radical and can be generated from several enzymes including xantine oxidase urate oxidase and D-amino acid oxidase. In addition, any biological system that generates $O_2^{\bullet-}$ will also produces H_2O_2 by $O_2^{\bullet-}$ dismutation. H_2O_2 can cross cell membrane rapidly and can probably react with ferrous ion (Fe^{2+}), and possibly copper ions (Cu^{2+}) to generate $HO^{\bullet}$ and OH^-. This reaction is called Fenton reaction (Young and Woodside, 2001).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + OH^-$$

In addition, it can degrade heme proteins including myoglobin, hemoglobin and cytochrome *c* to release irons (Gutteridge, 1986). Trace of Fe^{3+} might be able to react further with H_2O_2, although this is slower than the reaction of H_2O_2 with Fe^{2+} at physiological pH.

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + O_2^{\bullet-} + 2H^+$$

2.2.1.3 Hydroxyl radical ($HO^{\bullet}$) may be formed by the generation $O_2^{\bullet-}$ in the presence of redox active iron and hydrogen peroxide (Halliwell and Gutteridge, 1992).

$$O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + OH^- + HO^{\bullet}$$
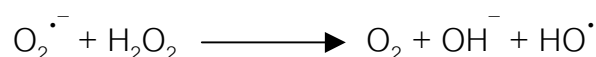
This reaction is called Haber-Weiss reaction. Hydroxyl radical can attack all classes of biological macromolecules. It can depolymerize polysaccharide (McCord, 1974), cause DNA strand breaks (Halliwell and Gutteridge, 1992), inactivate enzymes (Zhang *et al*., 1990), and initiate lipid

peroxidation (Gutteridge *et al*., 1982).

        2.2.1.4 Peroxyl radical (LOO$^\bullet$) and alkoxyl radical (LO$^\bullet$) are good oxidizing agents. They are generated from hydroxyl radical (HO$^\bullet$) or decomposition of organic peroxides (LOOH) by heating or by the addition of transition metal ions e.g. iron. These reactions stimulate lipid peroxidation in biological systems

$$HO^\bullet + LH \xrightarrow{\quad H_2O \quad} LO^\bullet \xrightarrow{\quad O_2 \quad} LOO^\bullet \xrightarrow{\quad LH \quad} LOOH + LO^\bullet$$

$$LOOH + Fe^{3+} \longrightarrow LOO^\bullet + Fe^{2+} + H^+$$

$$LOOH + Fe^{2+} \longrightarrow LO^\bullet + OH^- + Fe^{3+}$$
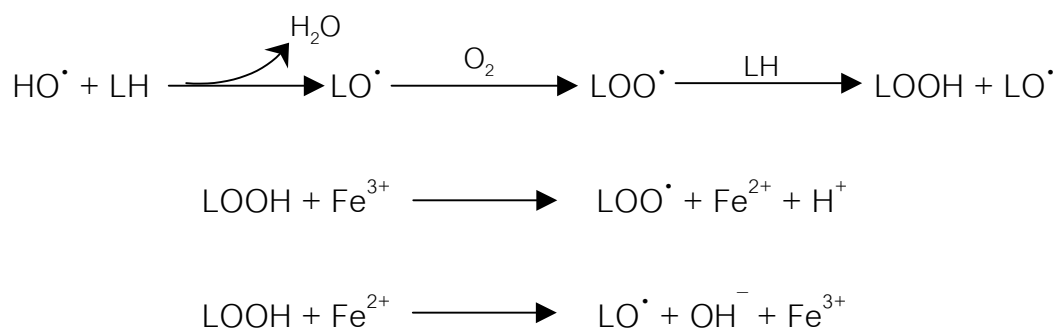
        2.2.1.5 Peroxynitrite (ONOO$^-$) is a reactive species, generated by reaction of superoxide (O$_2^{\bullet-}$) with nitric oxide (NO$^\bullet$).

$$NO^\bullet + O_2^{\bullet-} \longrightarrow ONOO^-$$
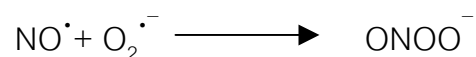
        Production of peroxynitrite depends on NO$^\bullet$ and O$_2^{\bullet-}$ concentrations, which are regulated mainly by nitric oxide synthase (NOS) and superoxide dismutase (SOD). Peroxynitrite is involved in oxidation of thiols *in vivo* and *in vitro*. One of the most important targets is glutathione (Bonini and Augusto, 2001). Peroxynitrite was shown to be involved into pathogenesis of many diseases, including acute and chronic inflammatory processes, atherosclerosis and neurodegenerative disorders.

        2.2.2 Oxidative stress

        The oxidative stress is the set of intracellular or extracellular condition

that leads to the chemical or metabolic generation of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2) or related species. It can result from diminished antioxidants and/or increased production of ROS.

The oxidative stress results in adaptation and cell injury (Halliwell and Gutteridge, 1998a). Mild oxidative stress results in up regulation of the synthesis of antioxidant defense systems in an attempt to restore the oxidant/ antioxidant balance. If the oxidative stress increases in the cell, it can damage all type of biomolecules including double stranded DNA, proteins and lipids. The primary cellular target of the oxidative stress can vary depending on the cell, the type of stress imposed and severity of stress.

Secondary damage occurs when oxidative stress produces rise in free intracellular metal ions, such as Ca^{2+}, Cu^{2+}, and Fe^{2+}. Ca^{2+} can stimulate proteases and nucleases, damaging both DNA and the cytoskeleton as well as increasing nitric oxide radical ($NO^{\bullet}$) synthesis; excess $NO^{\bullet}$ can inhibit mitochondrial energy generation and may lead to production of cytotoxic peroxynitrite ($ONOO^-$). Figure 4 shows the summary of oxidative stress that can produce cell injury by multiple pathways.

All aerobic organisms are physiological oxidative stress as a consequence of aerobic metabolism. The intermediates that are formed, including superoxide and hydrogen peroxide, lead to the further production of toxic oxygen radicals that can cause lipid peroxidation.

2.2.3 Lipid peroxidation

Lipid peroxidation is a chain reaction process that involves the participation and the production of free radical species. It is a continuous physiological process occurring in cell membranes (Loeckie *et al*., 1999), which defined as the oxidative deterioration of polyunsaturated fats.
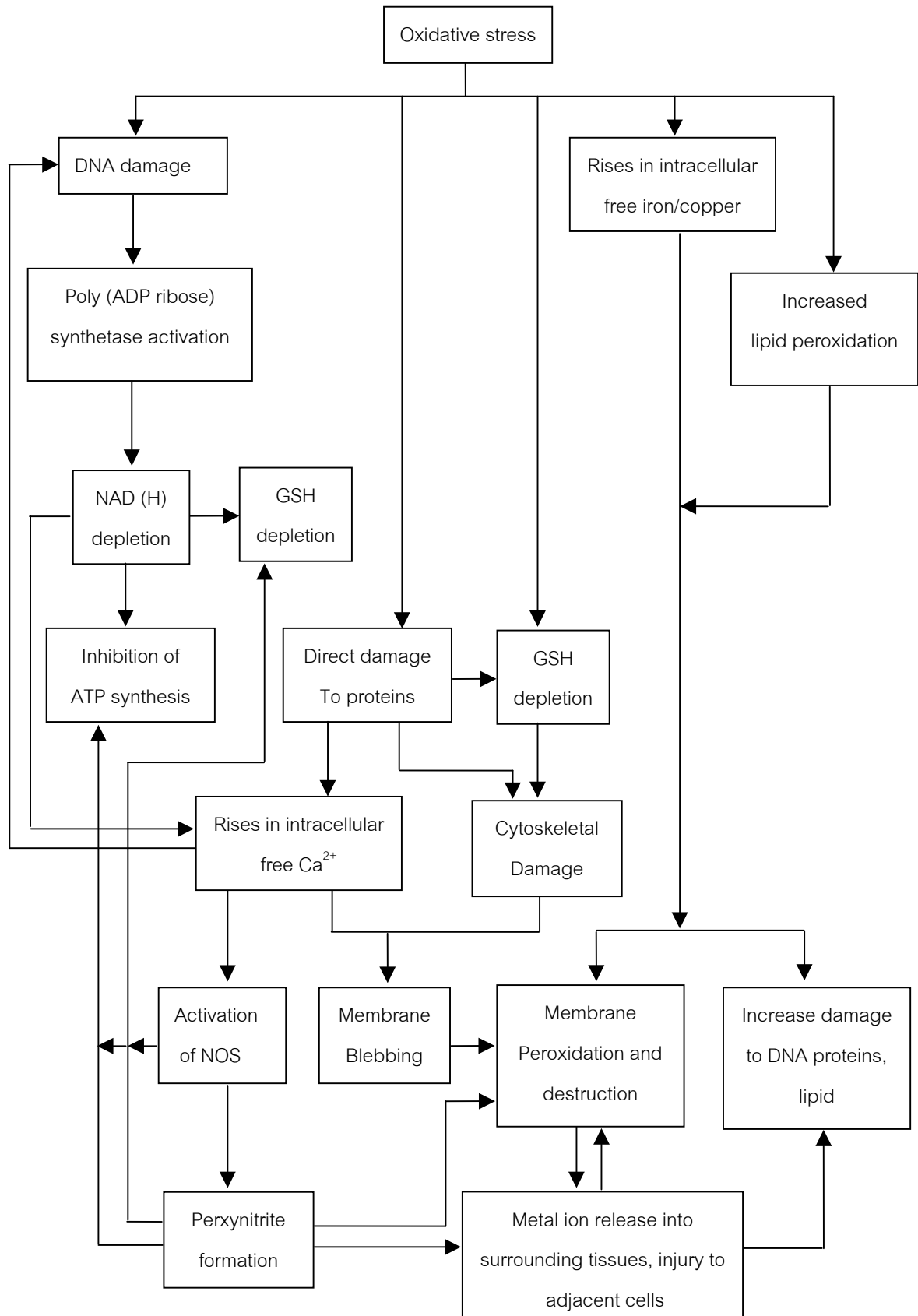
Figure 4 Summary of oxidative stress that can produce cell injury by multiple pathways (Halliwell and Gutteridge, 1998a).

The membrane of mammalian cells contains large amount of polyunsaturated fatty acids which are especially susceptible to lipid peroxidation. Lipid peroxidation of cell membranes results in decreased membrane fluidity, inability to maintain ionic gradients, cellular swelling, and tissue inflammation. It involved the reaction of oxygen and polyunsaturated lipids to form lipid free radicals and semistable hydroperoxides, which in turn promote free radicals chain reaction.

A schematic representation of the well-established sequence of reactions involved in the peroxidation of polyunsaturated fatty acids is demonstrated. Lipid peroxidation proceeds in three distinct steps: Initiation, propagation and termination (Noguchi and Niki, 1999) as shown in Figure 5.

The initial step of lipid peroxidation occurs when radical species, such as the hydroxyl radical ($OH^{\bullet}$), removes allylic hydrogen from a polyunsaturated fatty acid (PUFA). The removal of the allylic hydrogen from a polyunsaturated fatty acid forms a lipid redical ($L^{\bullet}$). An immediate rearrangement occurs by forming a more stable lipid radical, whose dienes are conjugated. In an aerobic conditions this radical reacts with oxygen, giving rise to a lipid peroxyl radical ($LOO^{\bullet}$).

In propagation reactions, the lipid peroxyl radical abstracts an allylic hydrogen atom from another adjacent PUFA and results in a lipid hydroperoxide (LOOH) and a second lipid radical ($L^{\bullet}$). This second lipid radical can proceed through the same reactions as the first, generating additional lipid hydroperoxides (Halliwell and Gutteridge, 1998a). These hydroperoxides can undergo decomposition to numerous aldehyde products of different chain lengths. The malonyldialdehyde (MDA) is a frequently used as an indicator of lipid peroxidation in biological tissue (Young and McEneny, 2001).
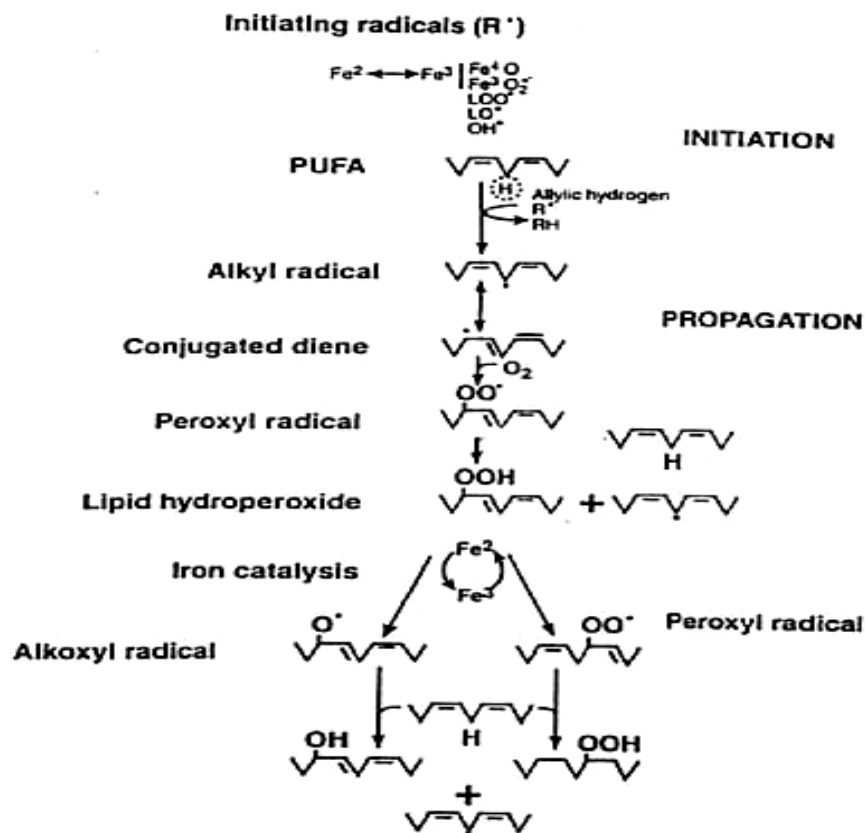
Figure 5 Schematic process of lipid peroxidation, chain reactions resulting
in the formation of many lipid peroxide radicals (Halliwell and
Gutteridge,1998a)

The chain propagation does not continue forever, but the chain
oxidation is terminated when lipid radical or lipid peroxyl radical is
scavenged by an antioxidant such as vitamin E and C or when two lipid
peroxyl radicals react to give non radical products such as ketones and
alcohols. If an attempt is made to evoke a causative role of peroxidation, it
must be shown that the peroxidation precedes or accompanies the cell
damages. Thus, the prevention of peroxidation by antioxidants prevents the
cell damages. Measurement of lipid peroxidation may therefore be an
excellent marker of tissue damages (Gutteridge, 1995).

2.3. Antioxidant defense systems.

When the protective and controlling mechanisms can not work or are imbalance, the excess of radicals will cause pathophysiological processes. Thus, agents which act as antioxidants are useful for treatment of diseases relating to radicals. Antioxidants can act at different levels in the oxidative sequence. The antioxidants are classified according to solubility as lipophilic, such as vitamin E, coenzyme Q_{10} (ubiquinone), and carotenoids, or hydrophilic, such as ascorbate and uric acid. In addition, antioxidants contain glutathione system consists of reduced and oxidized glutathione molecules and the enzymes glutathione peroxidase and glutathione reductase. The enzymatic primary antioxidant defense system included superoxide dismutase and catalase (Grune et al., 2000).

2.3.1 Superoxide dismutase (SOD)

Superoxide dismutase catalyzes the reduction of superoxide anions to produced hydrogen peroxide and oxygen.

$$O_2^{\bullet -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$
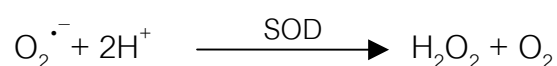
The enzyme superoxide dismutases are classified into three distinct classes depending on the metal ion component, Cu/ZnSOD, MnSOD and FeSOD. In rat hepatocytes, 70% of SOD is found in the cytosolic compartment. The activity of SOD varies among the tissues. The highest levels are seen in the liver, adrenal gland, kidney and spleen. The activity of SOD is regulated through biosynthesis, which is sensitive to tissue oxygenation (Halliwell and Gutteridge, 1998b).

2.3.2 Catalase

This enzyme is a major primary antioxidant defense component that

primarily works to catalyze the decomposition of H_2O_2 to ground state oxygen (O_2) molecule and H_2O (Halliwell and Gutteridge, 1998b). In animals, it is present in all major body organs especially in liver and is location in organelles such as peroxisomes, mitochondria (in liver), and endoplasmic reticulum. Animal catalase consists of four protein subunits, each of which contains a ferric (Fe^{3+}) heme group bound to its active site (Lockitch, 1989).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$
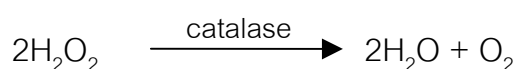
2.3.3 Vitamin E (α-tocopherol)

Vitamin E is the generic term used to describe a group of at least eight compounds that exhibit the biological activity of α-tocopherol. α-tocopherol, major constituent and the most active form of the fat soluble vitamin E, is an essential factor in the cellular antioxidant defense system within membranes and lipoproteins. α-tocopherol has been demonstrated (Andreas,1999). The α-tocopherol inhibits lipid peroxidation by scavenging lipid peroxyl (LOO$^{\bullet}$) or alkoxyl (LO$^{\bullet}$) radicals, which are intermediates in the chain reaction (Noguchi, and Niki, 1999).

$$\alpha TH + LOO^{\bullet} \longrightarrow \alpha T^{\bullet} + LOOH$$
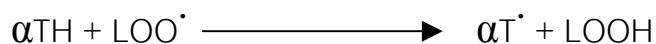
The α-tocopherol radical ($\alpha T^{\bullet}$), although not completely unreactive, is less efficient at abstracting hydrogen than are peroxyl radicals, so the chain reaction of peroxidation is slowed. Several biological mechanisms may exist for recycling $\alpha T^{\bullet}$ back to α-tocopherol, although none of them had yet been proven vigorously to operate *in vivo* in humans. Likely mechanisms include the reaction of $\alpha T^{\bullet}$ with ascorbic acid at the surface of membranes and

lipoproteins (Halliwell and Gutteridge, 1998b)

$$\alpha T^{\bullet} + \text{ascorbate} \longrightarrow \alpha TH + \text{ascorbate}^{\bullet}$$
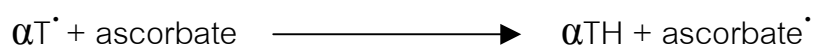
The primary dietary source of vitamin E is vegetable oil, secondary source includes liver, egg, cereals, and legumes (Bonorden and Pariza, 1994). Vitamin E and other antioxidant prevent or minimize oxidative damage in biological systems.

2.3.4 Vitamin C

Ascorbic acid (vitamin C), a common constituent in fruits and vegetable, is regarded as the most efficient water soluble free radical chain breaking antioxidant in human tissue and plasma (Bonorden and Pariza, 1994). It exerts antioxidant properties by direct reacting with superoxide anion radical, hydroxyl radical and reacts with singlet oxygen (Halliwell and Gutteridge, 1998b). Vitamin C can function synergistically with vitamin E in preventing membrane lipid peroxidation via recycling of vitamin E from its free radical as shown in Figure 6 (Packer *et al*., 1979).
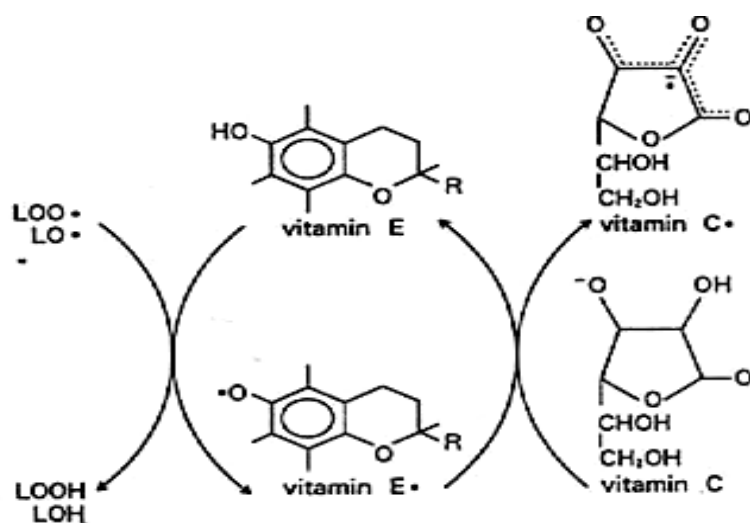

Figure 6 Chain reaction of vitamin E with lipid radicals and vitamin C (Halliwell

and Gutteridge,1998a)

Besides of antioxidant, ascorbic acid can act as a prooxidant (Herman, 1979.; Combs and Gerald, 1998). In the presence of transition metal ions (e.g., Fe^{3+}, Cu^{2+}), high concentrations of vitamin C can act as a reducing agent to generate $O_2^{\cdot -}$, H_2O_2, and $OH^{\cdot}$. However, such metal ions are normally available in very limited amounts *in vivo*, the antioxidant properties of ascorbate is predominant.

2.3.5 Vitamin A

Carotenoids have been long considered antioxidants because of their capacity to scavenge free radicals (Krinsky, 1979). Carotenoids protect lipid against peroxidation by quenching free radicals and other reactive oxygen species, notably singlet oxygen. β -carotene displays an efficient biological radical trapping antioxidant activity through its inhibition of lipid peroxidation induced by the xanthine oxidase system. β-carotene, like vitamin C, appears to function as both an antioxidant and a prooxidant (Halliwell and Gutteridge, 1998b).

2.3.6 Glutathione.

Glutathione is a tripeptide composed of glutamate, glycine and cysteine, and its active group is represented by the thiol (-SH) of cysteine residue. Glutathione is a ubiquitous molecule that is produced in all organs, especially in the liver (Lauterburg *et al*., 1984) and occurring at intracellulary concentration of 5 to 10 millimolar. Plasma and urine contain lower level of glutathione (Halliwell and Gutteridge, 1998b).

In cells, total glutathione can be free or bound to proteins. Free glutathione is present mainly in its reduced form, which can be converted to the oxidized form during oxidative stress, and can be reverted to the reduced form by the action of the enzyme glutathione reductase. The redox status depends on the relative amounts of the reduced and oxidized forms of

glutathione (GSH/GSSG) and appears to be a critical determinant in cell.

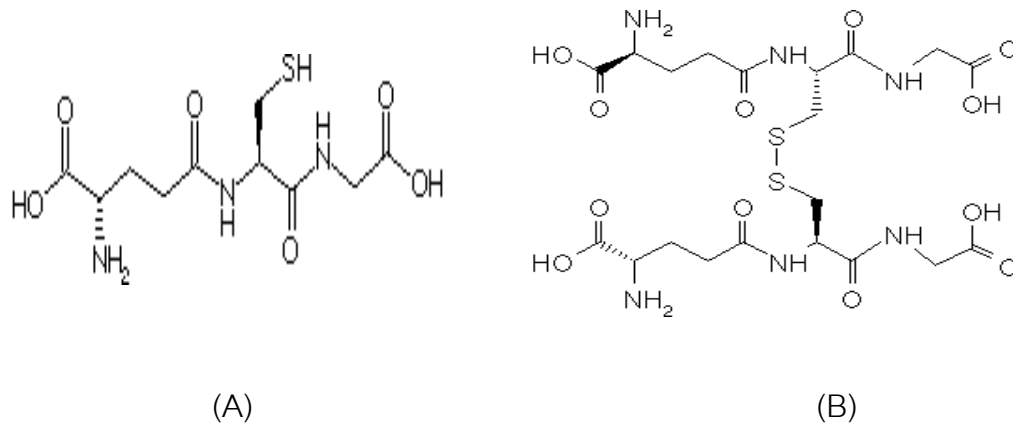(A)   (B)

Figure 7 Structure of glutathione; (A) reduced form and (B) oxidized form

The synthesis of glutathione from its three amino acid precursors, glutamate, cysteine and glycine takes place in the cytosol. Cell can make the necessary cysteine from the essential amino acid methionine. Glutathione is synthesized within the cell in two steps. The first step, cysteine and glutamate are combined by the enzyme γ-glutamylcysteine synthetase (Whitcomb and Block, 1994).   The γ-glutamylcysteine synthetase is controlled by negative feedback from its end product, GSH. When glutathione is consumed and feedback inhibition is lost, the availability of cysteine as a precursor can become the limiting factor (Richman and Meister, 1975; Guoyao *et al*., 2004). The second step, adding glycine to the γ-glutamylcysteine dipeptide, is catalyzed by glutathione synthetase.

Although glutathione is synthesized inside the cell, its biodegradation occurs outside of cells.   Glutathione can be break down by γ-glutamyltranspeptidase, which located on extracellular membrane to glutamate residue to formation of γ-glutamyl amino acids and cysteinylglycine. The latter is cleaved by dipeptidase to form cysteine and glycine.   The

breakdown products (glutamate, glycine and cysteine) can be reabsorbed into the cell for GSH synthesis (Figure 8).

Glutamate

methioine

ATP

ADP + PI

Cysteine

Gly + Cys

Gammaglutamylcysteinyl synthetase

γ- Glutamylcysteine

Dipeptidase

ATP

ADP + PI

Glycine

Glu + GlyCys

Glutathione synthetase

Cys

Glu   SH

O   CH$_2$ O   Gly   Feedback inhibition

C—NH—CH—C—NH—CH$_2$

CH$_2$ COO$^-$

CH$_2$

CH—NH$_3^+$ carboxyl peptide linkage

COO$^-$ **Glutathione** - - - - - - - - - - - -

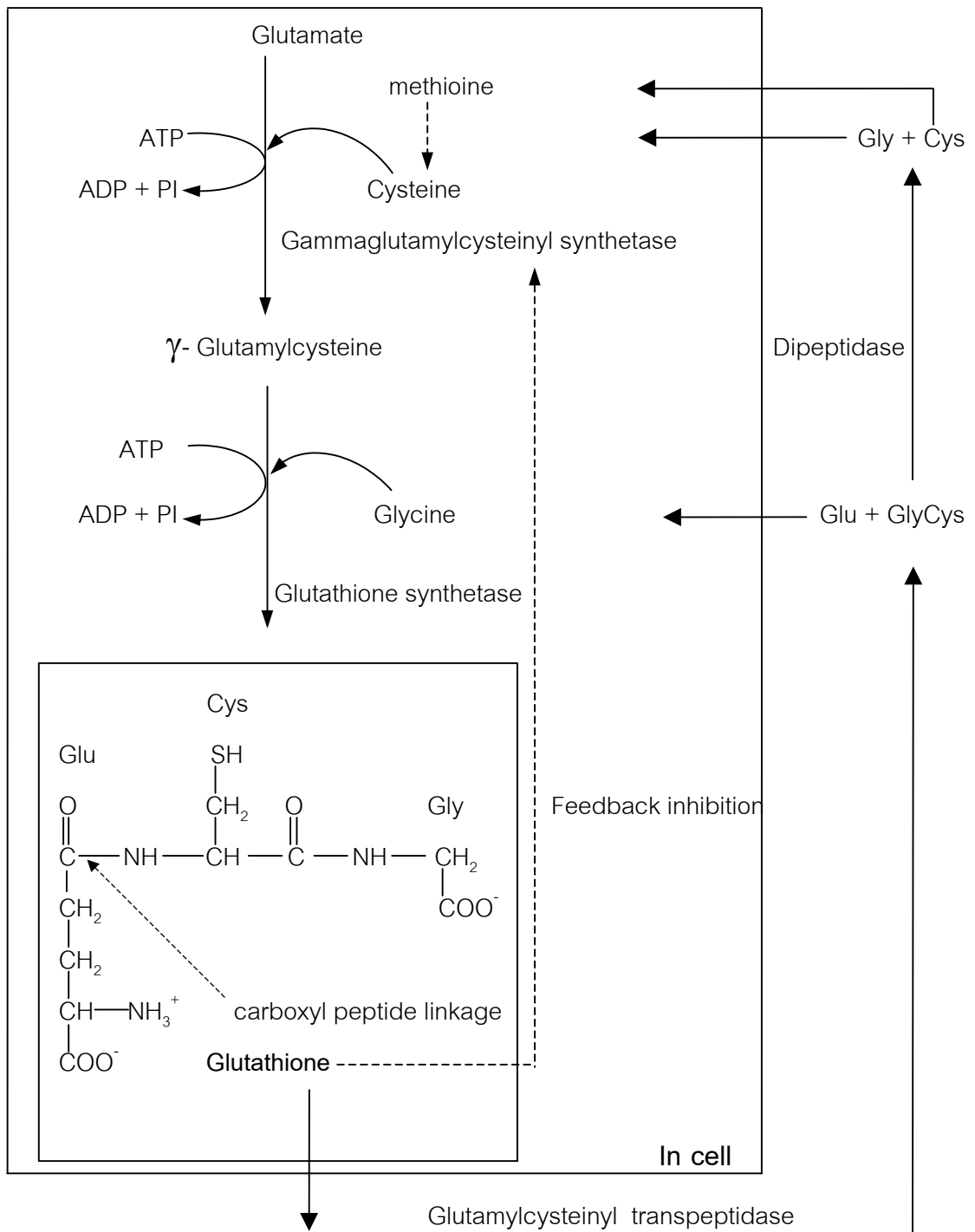**In cell**

Glutamylcysteinyl transpeptidase

Figure 8 Glutathione synthesis and metabolism. (Glu = glutamate, Cys =
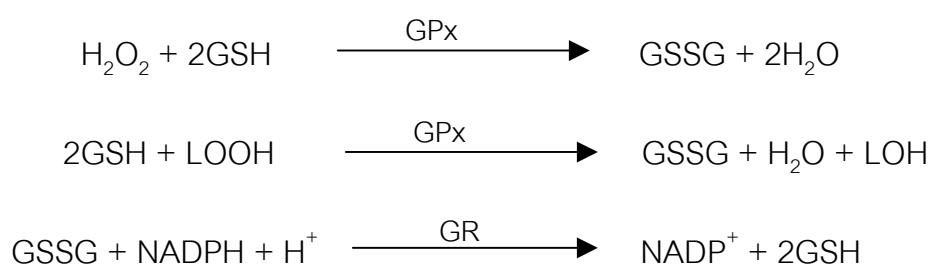
cysteine, Gly = glycine)

Glutathione (GSH) performs a variety of important physiological and metabolic functions in all mammalian cells, including the detoxification of free radicals, metals, and other electrophilic compounds. One important detoxification mechanism involves the binding of GSH to electrophilic chemicals and the export of the resulting GSH S-conjugates from the cell. These conjugation reactions have been extensively characterized for a multitude of foreign chemicals, but they are also critical for the metabolism of endogenous reactive intermediates and for the formation of specific biological mediators.

The glutathione S-transferases catalyses the conjugation of GSH with several compounds produced *in vivo* during oxidative stress. Glutathione forms conjugates with a great variety of eletrophilic compounds nonenzymatically, when the eletrophile is very reactive, or more often through the action of glutathione S-transferase (GST).

The glutathione S-conjugates are metabolized by the same degradative enzymes that metabolize glutathione. The breakdown products of the glutathione S-conjugates are glutamate, glycine, and cysteine, which can also be reabsorbed into the cell. The glutamate and gylcine may then be used for glutathione synthesis, whereas the cysteine S-conjugates can be acetylated on the amino group of the cysteinyl residue by intracellular *N*-acetyltransferases to form the corresponding mercapturic acid. Mercapturic acids are released into the circulation or bile, some are eventually excreted in urine, and some may undergo further metabolism (Hinchman *et al.*, 1991).

The antioxidant function of GSH depends primarily on its role as a component of the enzymatic pathway that cells developed against ROS, consisting of glutathione peroxidase (GPx) and glutathione reductase (GR). Endogenously produced hydrogen peroxide or organic hydroperoxide are

reduced by glutathione in presence of glutathione peroxidase. As a consequence, GSH is oxidized to GSSG, which in turn is rapidly reduced back to GSH by glutathione reductase at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The reduction of organic hydroperoxides by GSH may be catalyzed by either this glutathione peroxidase or by glutathione S-transferases (GST).

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O$$

$$2GSH + LOOH \xrightarrow{GPx} GSSG + H_2O + LOH$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

During the course of the reaction catalyzed by glutathione peroxidase, glutathione is recycled. In contrast, glutathione is consumed during the generation of glutathione S-conjugates by glutathione S-transferase. Both processes lower the level of total intracellular glutathione.

Therefore, in order to maintain a constant intracellular glutathione concentration, the glutathione consumed has to be replaced by resynthesis from its constituent amino acids (Cohen and Hochstein, 1963; Brigelius-Flohe, 1999; Hinchman and Ballatori, 1994). Conjugation with GSH is an essential aspect of both xenobiotic and normal physiological metabolism (Strange *et al*., 2000; Eaton and Bammler, 1999). Formation of conjugates can result in depletion of GSH and has been used as a tool to study the role of GSH in antioxidant defense.

Treatment with exogenous antioxidant may appear to be helpful in conditions related to oxidative stress. The recent study, *Parkia speciosa* seeds were found to have antioxidant activity by measuring superoxide

scavenging activity and DPPH free radical scavenging assay be using Trolox (Trolox Equivalent Antioxidant Concentration = TEAC) as a reference (Prasatthong, 2001).

2.4. *Parkia speciosa* Hassk

Parkia speciosa (sator) is a tropical legumimous tree in the family of Leguminosae, sub-family Mimosaceae. It is found growing naturally in the rainforests of southern Thailand, Malaysia and Indonesia In Thailand, it is known as "sator" and in Malaysia, it is called peteh or petai (Smitinand, 1980). It is commonly found in many rural areas in southern Thailand.

2.4.1 General description

It is a large, evergreen tree that can grow up to 15-35 m in height. Leaves are twice pinnate, with 14-18 pairs of side branches bearing very small, dark green leaflets. Each leaflet is oblong with a blunt end and an asymmetric base. The inflorescence resembles a drumstick as it has along stalk carrying a large globular head of close-packed, creamy white-colour flowers at the end. The flowers produce a great deal of nectar and have a strong, somewhat sickly smell. They are pollinated by bats and only the apical flowers develop fruits. Six to ten fruits develop in each inflorescence. The pods are green at first, becoming dark brown or blackish brown when ripe. When the tree is fruiting, the groups of young, light green pods give it a distinctive appearance easily visible from a great distance. Pods are large, 36-45 cm long and 3-5 cm wide. Each pod contains 10-18 large seeds (Smitinand, 1980)

2.4.2 Variety of *P. speciosa*

There are many known varieties of *P. speciosa*, but only three varieties are common in southern Thailand. Many other varieties are cultivated elsewhere, but they are poorly documented at present. The three varieties of

southern Thailand are described by Bamroongrugsa and Yaacob (1990) as :

1. **Sator kow or rice sator**. This is the most popular variety of sator in the local markets. It has many small seeds in the pod. The seeds have a strong odour and quite sweet. This variety is suitable for consumption. It can produce fruits at 4-5 years after planting and is also classified as an early maturing variety.

2. **Sator darn**. This variety has larger pods and seeds than those of the sator kow, but it produces fewer pods per tree. In addition, its stem canopy is larger and taller than that of the sator kow. In this variety the first flowering can be seen at 6-7 years after planting. As the sator darn had harder seeds, a stronger odour, and better taste than sator kow, it is more popular.

3. **Sator tea**. This variety has very hard pods and seeds, so it is not suitable for consumption.
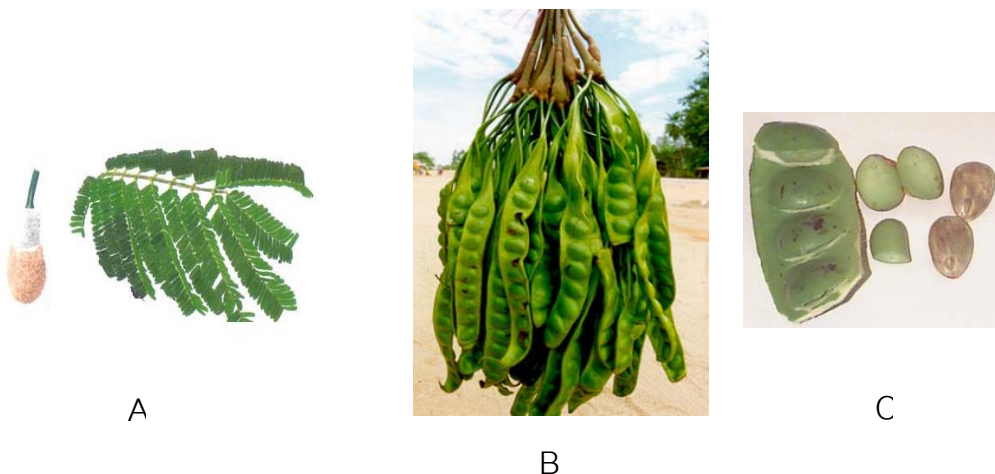

A

B

C

Figure 9 Photograph of *P. speciosa* (sator). (A) leaves and inflorescence

(B) pods and seeds (C) seeds

2.4.3 Uses of *P. speciosa*

P. speciosa is grown for its edible seeds.   The seeds contain high

nutritional value and are served as a local vegetable in many dishes of southern Thailand. The composition per 100 g edible seeds is carbohydrates 15.5 g, protein 8.0 g, fat 4.0 g, calcium 76 mg, phosphorous 83 mg, iron 0.7 mg, vitamin A 9 IU, vitamin B_1 0.11 mg, vitamin B_2 0.01 mg, vitamin C 6.0 mg, and niacin 1 mg. The immature seeds have been eaten as food either cooked or raw. Young leaves and fresh part of the flower stalks can be eaten raw. Half ripe pods are pickled in salt.

2.4.4 Chemistry and pharmacology of *P. speciosa*

P. speciosa seeds have a distinctive smell, suggesting the presence of sulphur compounds. Gmelin *et al* (1981) found that *P. speciosa* seeds contain antibacterial cyclic polysulphides (1,2,4-trithiolane, 1,2,4,6-tetrathiopane, 1,2,3,5,6-pentathiepane, 1,2,4,5,7,8-hexathionane, 1,2,4,6,7-pentathiocane) which are also responsible for their strong pungent, mushroom like flavors; with djenkolic acid and dichrostachinic acid, thought, to be the precursors of these cyclic polysulphides (Susilo and Gmelin, 1982). The thiol content in the seeds determined previously was high. The sulphur containing compounds in the seeds could be cysteine and their derivatives such as glutathione and thiazolidine-4-carboxylic acid (TCA) which is often called thioproline (Suvachittanont *et al*., 1996).

TCA is a cyclic sulphur containing amino acid, which is a condensation product of formaldehyde and cysteine (Schubert, 1936.; Ratner and Clarke, 1937). The fresh *P. speciosa* seeds contain substantial amounts of formaldehyde and thiol compounds of which the amounts decreased after boiling. The decrease in thiol content after boiling may result from the formation of thioproline. So thioproline in fresh *P. speciosa* was undetectable but increased markedly after boiling (Suvachittanont *et al*., 1996). TCA can protected mice against paracetamol induced hepatotoxicity (Nagassawa *et al*,

1984). Endogenous formation of TCA had been considered as a detoxification pathway of formaldehyde (Debey *et al*, 1958). Oshima *et al.* (1983) suggested that the *N*-nitrosated derivative of TCA, *N*-nitrosothiazolidine-4-carboxylic acid (nitrosothioproline), is commonly excreted in human urine (Figure 10). Furthermore, TCA is an effective nitrite-trapping agent in the human body, thereby inhibiting the endogenous formation of carcinogenic *N*-nitroso compounds (Tsuda and Kurashima, 1991). An inhibitory effect of TCA on carcinogenesis by co-administration with *N*-benzyl methylamine and nitrite has also been reported (Tahira *et al.*, 1988)
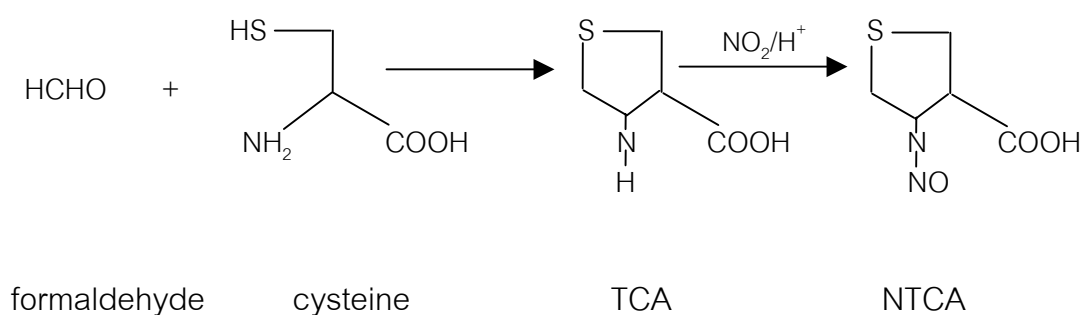

Figure 10 Formation of thiazolidine-4-carboxylic acid (TCA) and *N*-nitrosothiazolidine-4-carboxylic acid (NTCA)

The protein content in *P. speciosa* seeds is 8% of the fresh weight. When protein extracts of *P. speciosa* seeds were fed to alloxan induced diabetic rats daily for two weeks, blood glucose was about 25% lower than that of unfed animals. Laxative effect of the seeds is evident from two observations. Firstly, rats fed with homogenized seeds have softer stool than those of control. Secondly, proteins extracted from the seeds stimulate rat duodenum contraction *in vitro* (Suvachittanont and Pothirakit, 1988).

P. speciosa seed lectin have been purified and characterized from a

crude extract. This lectin was found to be a glycoprotein containing 1.5% carbohydrate with a high percentage of glycine, aspartic acid, isoleucine and serine, but low in cysteine and methionine. The purified lectin contained trace amounts of Mg^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} in the range of 0.03-0.06 mole per mole of lectin, but there was no Ca^{2+} and Mn^{2+}. Biological activities of the lectin found is that it agglutinated rat erythrocytes, but not those of humans (Suvachittanont and Pentpaiboon, 1992) and it possess mitogenic activity, as it increased the incorporation of [^3H] thymidine into the DNA of human lymphocytes (Suvachittanont and Jaranchavanapet, 2000).

The green pericarp may be eaten together with the seeds and is also believed to have antidiabetic activity. Chloroform extracts of *P. speciosa* empty pods fed to alloxan induced diabetic rats produced a significant reduction in blood glucose levels. A hypoglycemic assay guided extraction isolation and structure elucidation gave stigmast-4-en-3-one, that is the biologically active compound (Jamaluddin, 1995).