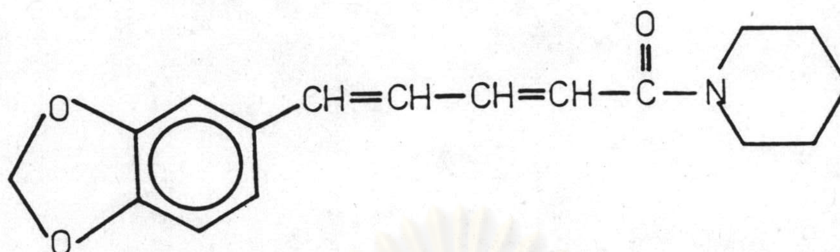
CHAPTER I

GENERAL REVIEW OF THE LITERATURE

INTRODUCTION

Pepper is one of the oldest and most important member of the spices, and is still used universally as a food flavoring agent. It was used by the ancient Greeks and was highly valued by the Romans. The search for a trade route by sea to India was to some extent instigated by the European demand for pepper which, during the Middle Ages, was of great economic importance in Western Europe. It is still used extensively as a condiment and flavoring for all types of savory dishes, for preserving and pickling, and in the manufacture of sauces, ketchups, brandy, etc. (Pruthi, 1980). The two forms of the spice, black pepper and white pepper, are obtained from the fruits and seeds of Piper nigrum, black pepper consisting of the dried ground fruits (peppercorn) and white pepper consisting of the dried ground seeds. The pungency of pepper is due to the presence in the fruit of various resins and a yellow crystalline alkaloid, piperine, which is present to the extent of 4.5-8% (Govindarajan, 1977).



Chemical Structure of Piperine

Piperine, the most common alkaloid in the Piper species of Piperaceae family, which has the composition $C_{17}H_{19}O_3N$, molecular weight 285.16, occurs in the kernel of the ripe fruit (white pepper) and in the unripe fruit (black pepper) (Lewis, 1977). It is also present in the leaves of Rhododendron fauriae (Ericaceae) (Kawaguchi et al, 1942) and in the fruits of Xylopia brasilliensis (Lewis, 1977). It may be hydrolyzed to piperidine and piperic acid (Glasby, 1976). Piperine is a neutral or feebly alkaline, crystalline substance, insoluble in water but readily soluble in alcohol and when pure is colorless, and without taste or smell. Among the Piper species, black pepper (Piper nigrum Linn.) and long pepper (Piper longum Linn.) are commonly used in many regions of the world (Govidarajan, 1977).

Pepper is an aromatic carminative stimulant, and is also supposed to possess febrifuge properties. Its action as a stimulant is more especially evident on the mucous membranes of the rectum and urinary organs (Bose, 1928). Externally applied,

it is rubefacient. It is regarded as a useful remedy in haemorrhoidal affections, and in relaxed conditions of the rectum attended with prolapsus, it is also sometimes employed as a substitute for cubebs in gonorrhoea. It is likewise given in combination with aperients to facilitate its action and prevent griping. As a masticatory, or when locally applied as a gargle, pepper has been found useful in relaxed uvula, paralysis of the tongue, and in other affections of the mouth or throat. The use of piperine in higher concentrations produces a strong sensation of heat which increases to a sensation of burning and of severe pain without inflammation (Cole, 1985). Piperine also has respiratory stimulant and convulsant properties in various laboratory animals (Kulshrestha et. al. 1969, 1971; Singh et. al. 1973)). The central seat of action of piperine was ascertained to be in the brain stem. It has also been used as an insecticide and antihelmintic agents (Synerholm et. al., 1945; Harville, 1948; Harville et. al. 1977.; Choudhury and Das, 1983; Matsubara and Tanimura, 1966; Su, 1977; Su and Sondengam, 1980)

ศูนย์วิทยทรัพยากร

PHARMACOLOGY OF PIPERINE

จุฬาลงกรณ์มหาวิทยาลัย

Despite its long use, there have been scant published data on the biological activity of piperine and the crude extract from plants (Toh et. al., 1955; Neogi et. al., 1972; Lee et. al., 1984). Piperine is considered to be the active principle of various Piper species which are commonly used for a condiment and employed in folklore medicine for treatment of asthma, bronchitis, pyrexia, insomnia and abdominal disorders (Atal et.

al. 1975; Perry, 1980; Chopra and Chopra, 1955; Kirtikar and Basu, 1944). Piperine was recently found to possess central nervous system (CNS) depressant properties (Woo et. al., 1979; Pei, 1983). Pharmacological studies have indicated that piperine and several of its derivatives protect rats and mice against various kinds of experimental convulsions, including those induced by maximal electroshock, leptazol, picrotoxin and strychnine and potentiate the sedative effect of depressant agents (Pei, 1979, 1983; Pei and Xie; 1980; Woo et. al., 1979). In addition, it was found that piperine, beside CNS depressant activity, had various pharmacological activities such as antipyretic, analgesic and anti-inflammatory activities (Lee et. al., 1984). According to folklore medicine of many Asian countries including Thailand, Piper of different species have been used in indigenous drug preparations for inducing menstruation and termination of early pregnancy (Chandhoke et. al., 1978; Piyachaturawat et. al., 1982). Recently, it was reported that piperine inhibited implantation, produced abortion and delayed labor in mice (Chailurkit, 1984). Antifertility activity of the crude extract as well as the natural amides of the plants in some studies was also observed (casey, 1960; Kholkute et. al., 1979).

In some experiments, it has been found that repeated administration of pharmacologically effective doses of piperine caused a significant induction of hepatic mixed function oxidase system (Shin and Woo, 1980, 1981, 1984, 1985). On the other hand, it was described that addition of long pepper to vasaka

015931

leaves (Adhatoda vasica) increased the antiasthmatic properties of the latter (Atal et. al., 1981). It had been observed that long pepper or piperine enhanced the bioavailability of test drugs in experimental animals (Atal et. al., 1980). Moreover, recent study has also shown the in vivo and in vitro effect of piperine on the hepatic drug-metabolizing enzyme and demonstrated that piperine is a potent nonspecific inhibitor of drug metabolism (Atal et. al., 1985). More recently, it was found that piperine possesses inhibitory action to liver mitochondrial bioenergetics (Reanmongkol et. al., 1988).

TOXICOLOGY OF PIPERINE

Acute toxicity of piperine was investigated in different species of animals including mouse, rat and hamster (Piyachaturawat et. al., 1983). All animals, after receiving a fetal dose of piperine intraperitoneally, immediately exhibited an increase in locomotor activity. The activity was progressively increased from running, jumping to convulsion and finally the death was occurred with whole body muscle spasm. There was a brief state of depression before convulsion and death were occurred. The cause of death may possibly be the certain types of neurotoxicity and respiratory paralysis. The report on the acute toxicity of piperine was carried out in mice, rats, and dogs with intraperitoneal and intragastric LD_{50} values ranged from 21.5 to 59.6 mg/kg body wt. Recent study had reported the intravenous LD_{50} value of 15.1 mg/kg body wt for mice and noted that the intragastric lethal dose was relatively high

(Piyachaturawat et. al., 1983). The daily intake of piperine in adult Indian population, as estimated from the curry powder consumption, was approximately 17 mg/kg body wt (Srinivasan and Satyanarayana, 1981). However, there has been no evident report of piperine toxicity in human. Preliminary in vitro study had also demonstrated certain degree of cytotoxicity of piperine to, at least, neuronal cells in culture (Unchern and Fukuda, unpublished observation). It is quite interesting that many of our traditional spices, e.g. mustard, chilli, pepper, contain compounds with remarkable pharmacological effects.

MONOAMINE OXIDASES

Monoamine oxidases are the intrinsic protein of the outer mitochondrial membrane and have been known to exist in at least two functionally and structurally distinct forms, MAO-A and MAO-B (Tipton, 1967; Borges and D'Iorio, 1972; Student and Edwards, 1977; Schnaitman et. al., 1967; Schurr, 1982; Chau and Hackenbrock, 1975; Houslay and Tipton, 1976; Leung et. al., 1982; Pearce and Roth, 1984; Sandler and Youdim, 1972). They oxidatively deaminate amine transmitters in the nervous system and biogenic amines throughout the body. Two types of MAO activity have been identified which differ in specificity for substrates and sensitivity to inhibitors (Ekstedt, 1976). Type A activity preferentially deaminates serotonin (5-HT), norepinephrine (NE) and is more sensitive than type B activity to inhibition by clorgyline (Garrick and Murphy, 1982; Fowler et. al., 1980, 1982). Type B activity preferentially deaminates B-phenylethylamine and benzylamine and is more sensitive than type

A activity to inhibition by deprenyl (Fuller, 1972; Murphy, 1978). Dopamine and tryptamine are metabolized by both forms of the enzyme. Furthermore, pargyline and l-deprenyl are so potent as inhibitors for the two forms of MAO. Pargyline binds specifically and irreversibly to the flavin adenine dinucleotide (FAD) cofactor of MAO, and the FAD cofactor is covalently bound, in turn, to the MAO apoenzyme (Parkinson and Callingham, 1980). Inactivation of MAO in vivo with irreversible MAO inhibitors leads to elevation of brain levels of these amines with a concomittant decrease in levels of their deaminated metabolites (Tipton and Mantle, 1977). Animal behavioral studies have shown that inhibition of MAO can result in overt behavioral changes (Tipton and Mantle, 1977; Sandler and Youdim, 1972). Thus a regulatory role has been assigned to MAO in the brain. The antidepressant activity of drugs that inhibit MAO was described for a number of reasons, e.g., potentiation of indirectly acting amines, the usefulness of MAO inhibitors as drugs has been limited. The interest in both MAO and its inhibitors, however, has been maintained through the years. The reasons for this are: first, our knowledge of the enzyme has been enriched by its purification and characterization; second, *in vitro* and *in vivo* tests have shown the enzyme to exist in multiple forms with different substrate specificity and inhibitor sensitivity; third, the development of selective inhibitors with an activity for each enzyme form have raised the hopes of developing new compounds with greater therapeutic usefulness; and finally, a better understanding has emerged of the molecular mechanism of

interaction of MAO inhibitors with the enzyme active site (Oguchi et. al., 1982; Mantle et. al., 1976; Harris and Cooper, 1982).

The mechanism and kinetics of the interaction between the inhibitors and MAO has been studied by a variety of methods, employing radioactively labelled inhibitors, such as covalent binding of the inhibitor to enzyme has been demonstrated with [^{14}-C]pargyline, which correlated with the degree of inhibition of the enzyme activity (Harris and Cooper, 1982).

Synthesis of Monoamine Oxidase, a Flavoprotein.

The monoamine oxidase (MAO) activity of mammalian tissues ultimately depends upon the availability of riboflavin in the diet (Sagara and Ito, 1982; Tipton, 1975). Rats fed a diet deficient in this vitamin have a reduced amount of MAO in the liver and other tissues, and this reduction parallels the lowering of tissue content of noncovalently bound flavins. Riboflavin-deficient rats had less than half as much free flavins as their controls, the largest decrease being accounted for by the loss of FAD. This is of immediate importance to the relationship to MAO, for FAD is very likely the intermediate in the formation of covalently bound flavin. There are fewer of these flavin esters in the brain, but their content in that organ is much more resistant to dietary insult than that in the liver.

Riboflavin deficiency has long been known to increase the sensitivity of MAO to inhibitors (Smith and Reid, 1978). The oxidation of the MAO-B substrate phenylethylamine by the liver become more sensitive to clorgyline in the course of riboflavin

deficiency. FAD also serves as coenzyme for a number of acyl-coenzyme A dehydrogenases, and the withdrawal of the vitamin from the diet leads to very rapid changes in fat metabolism. As early as 1 day after weanling rats are given a riboflavin-deficient diet to consume, their tissues display a decline in the rate of oxidation of palmitoyl-L-carnitine and of hexanoate. Later on in the course of the deficiency there are important changes in the lipid composition of hepatic mitochondria and microsomes. The loss of MAO-A and MAO-B activities during the course of vitamin deprivation, the B-form declines somewhat more slowly than the MAO-A. This difference may be secondary to changes in the composition of the lipid in which the enzyme is embedded.

Hormones also play a role in the regulation of MAO activity (Gabay et. al., 1976). The most extensively studied endocrine influence is that of the thyroid, which has a special bearing on MAO for the reason that hypothyroidism resembles riboflavin deficiency in some respects. The effect of the thyroid state on liver MAO, much of which has been devoted to trying to explain the fact that administration of thyroid hormone to animals seems to lower the hepatic MAO activity. This unusual effect occurs even though thyroid hormone increases the level of incorporation of ^{14}C-labeled riboflavin into covalently bound flavins of rat liver and brain. Actually, thyroid administration results in an increase of MAO of skeletal muscle, another organ whose mitochondria contain MAO in the outer membrane.

FAD occurs in MAO in two forms. Several investigators have found dissociable FAD in brain MAO but rat and bovine liver MAO contain the covalently bound form (Houslay and Tipton, 1973, 1974). The first step in the formation of FAD in either case is the phosphorylation of riboflavin to form FMN. The flavokinase catalyzing this reaction is sensitive to riboflavin deficiency, and it decreases in activity in the liver unless the vitamin is supplied. FMN then reacts with ATP to form the dinucleotide FAD. Unlike the flavokinase, the FAD synthetase (or pyrophosphorylase) increases in activity during riboflavin deficiency. The synthesis of MAO containing covalently bound FAD requires transformation of the dinucleotide, and one can postulate two further enzymes for this process. The first of these would be a FAD hydroxylase catalyzing the formation of a modified FAD and thus preparing it for condensation with a suitable functional groups derived from the peptide chain of the apoenzyme.

The postulated FAD hydroxylase might have a metal cofactor. Indeed, the lowering of hepatic MAO activity as a result of simple nutritional iron deficiency may actually be a second-order effect, derived from an iron requirement at the stage indicated above. The second enzyme needed for covalent binding of FAD is a conjugase; this would catalyze the formation of the thioether link of modified FAD with the peptide chain of the apoenzyme (apo-MAO-SH, apo-MAO). The apoenzyme contains the sequence: serine-glycine-glycine-cysteine-tyrosine and it is through the sulhydryl group of cysteine residue that the modified FAD is bound, i.e. as a thioether. This conjugation reaction is

หอสมุดกลาง สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

analogous to the laboratory synthesis of 8-(S-cysteinyl) riboflavin, in which the amino acid is reacted with the 8-bromo derivative of the vitamin. How this is achieved in the case of MAO is not yet known, but a beginning has been made with another yellow enzyme 6-hydroxy-D-nicotine oxidase, found in Arthrobacter oxidans. The FAD is covalently bound here through a histidyl-N-residue, and this binding has been achieved *in vitro* with polysomes prepared from the bacterium. The covalent incorporation of flavin into proteins of mitochondrial outer membranes is blocked by the administration of cycloheximide to rats; the inhibition takes effect in about 2 hours. The mode of formation of one identified protein of the outer mitochondrial membrane of rat liver, the MAO apoprotein would be synthesized in the cytosol and then inserted into the outer membrane.

Irreversible Inhibition of MAO.

MAO is irreversibly inhibited by a number of compounds (Fuller, 1972; Neff and Yang, 1971). Historically, phenylhydrazine was the first of these inhibitors. The hydrazines and the acetylenics inhibit MAO by the formation of adducts with the covalently bound FAD. Alpha-hydroxybutynoic acid is an exception in that it combines with free FAD. Allenic compounds are tautomeric with acetylenics and, although both inhibit MAO irreversibly, the later do so by forming a N-5-flavocyanine, whereas the allenics do not; the reason for this is not known. There are two types of inhibitory process to be distinguished: a slowly developing, temperature-sensitive inhibition, and a rapid, temperature-insensitive one; in the case of phenylhydrazine both

have been shown to be involved oxidative process. The inhibition proceeds initially by anaerobic reduction of the flavin, with dehydrogenation of the phenylhydrazine to phenyldiazene (phenyldiimide). The phenyldiazene is then the immediate inhibitor, adding to the reoxidised flavin.

In regard to the acetylenic inhibitors, pargyline and deprenyl are bound to MAO stoichiometrically, whereas clorgyline combines in excess (Fuller, 1972). This nonspecific binding of clorgyline can also be observed with high concentrations of pargyline, but at low concentrations of the inhibitor only the MAO subunit is attacked and corresponds to a single electrophoretic band. The labelled enzyme contains 1 mol of inhibitor residue per equivalent of 109,000 daltons, and the binding occurs only with MAO, among four flavoenzymes tested. This specificity is also encountered *in vivo*. Pargyline binding is prevented by prior treatment of MAO with tranylcypromine, iproniazid and modaline.

Structure-activity relationships among the propragyl derivatives demonstrate the importance of lipophilicity of the molecule. There is a very high correlation between the inhibitory potency of these compounds and their hydrophobicity. The aromatic portion of the molecule is the least specific in endowing inhibitory potency; the length of the chain extending from the aromatic nucleus to the N atom is more important. This relationship signifies that the ability of the inhibitor to disolve in and penetrate the lipid around the enzyme may be an

important factor determining its efficacy. The mobility of the inhibitor in the lipid mesh of the membrane may lie at the basis of selectivity for the different forms of the enzyme.

Adduct formation with the so-called suicide inhibitors is not reversible and, as already discussed, the covalent linkage of modified FAD to the cysteine residue of MAO is probably hydrolized in metabolism. This means that new molecules of the enzyme must be resynthesized, and the inhibited ones removed from the mitochondrial membrane (Tipton and Mantle, 1977).

The structure-activity relationships of the acetylenic inhibitors show the importance of lipids of the mitochondrial membrane for MAO inhibition. This has also been demonstrated in regard to MAO activity. Extraction of lipids from mitochondrial preparations results in loss of nearly all MAO-A activity, but little of MAO-B. The substitution of purified lipids may restore activity or flavor the binding of the enzyme to the lipid-depleted mitochondrial residues. The properties of MAO are different after extraction, presumably as the result of altered conformation of the protein in the water-soluble state, as distinct from that in the membrane (Tipton and Youdim, 1976).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Lipids and MAO Function.

Some investigators have speculated that the lipids play a role in determining substrate and inhibitor specificity of MAO, in particular the A and B types of activity. There was some expectation that if there were a differential role of lipids in the two types of kineticaaly defined activity this might become apparent through thermodynamic measurements.

Both MAO-A and MAO-B activities are influenced by lipid phase transitions, but these transitions do not characterize either type specifically (Achee and Gabay, 1981). A particular type of MAO deaminates various substrates with different energies of activation for each of several substrates, and conversely that the energy of activation estimated with a given substrate is fairly constant for enzyme sources containing only MAO-A or MAO-B, or for others containing a mixture of the two types. The lipophilic character of MAO substrates is significant factor in determining the energy of activation, and this property does not distinguish MAO-A from MAO-B.

Multiple Forms of Monoamine Oxidase.

The concept of MAO multiplicity with different substrate specificity and inhibitor sensitivity arose from studies of temperature and pH stabilities and inhibitor sensitivity towards different amine substrates (Borges and D'Iorio, 1972). It was shown that at least two deaminating systems exist in rat liver and brain mitochondrial preparations. Validation of these findings, however, had to await further evidence from

electrophoretic studies with solubilized partially purified oxidase preparations and use of selective inhibitors (Tipton and Youdim, 1976). The electrophoretic studies complicated the situation in that up to five bands of MAO activity could be separated. The suggestion has been made that these electrophoretically separable forms of the enzyme may be artifacts of of the solubilization procedure used and not true isoenzymes and that they are caused by the attachment of MAO to mitochondrial membrane phopholipid structures which influence its physicochemical properties. It is now becoming apparent that the immediate lipid environment of MAO exerts certain influences on enzyme properties and characteristics. Whether the lipid environment plays a direct role in enzyme multiplicity and substrate specificity is far from clear and remains to be resolved.

The Concept of MAO Type A and Type B. (see table IX in Appendix)

The development of the irreversible selective inhibitor clorgyline, now considered to be a "suicide" propragyl-containing inactivator enabled investigators to classify two types of MAO activity in mitochondrial preparations from a number of sources including the brain (Fowler et. al., 1982; Hall et. al., 1982). With 5-HT as substrate, much lower clorgyline concentrations were required to inactivate MAO than with benzylamine as substrate. When tyramine was used as the substrate and rat brain as enzyme source, half the activity was inhibited at concentrations of clorgyline capable of inhibiting 5-HT deamination fully. Tipton

(1975) formulated the concept of two MAOs, type A being active towards 5-HT and selectively inactivated by clorgyline and type B being relatively resistant to inactivation by clorgyline and showing higher affinity towards benzylamine than to 5-HT. Tyramine and dopamine were considered substrates for both forms. Subsequently, another propragyl "suicide" inhibitor, deprenyl, was described (Fuller, 1972) which selectively inhibits the type B enzyme.

Selective inhibitors and different substrates have been used to determine the relative proportions and the substrate specificity of the two forms of MAO. The relative proportions of the two forms vary widely among different tissues and species. Some tissues such as ox liver, pig brain, and human platelet have been shown to contain almost exclusively the B type, whereas human placenta contains only the A type. The livers and brains of rats and human contain both forms in different relative proportions, and human small intestine contains more than 80% type A. The human brain contains about 80% type B.

Substrate Specificity (see table X in Appendix)

The substrate specificities of the two forms are not absolute. Thus, the enzyme from a tissue known to contain exclusively type A or type B has been shown to deaminate the B-type or A-type substrates, respectively,(Fowler and Tipton, 1981) albeit relative poorly. The enzyme from tissues such as rat

liver, which contains both forms, oxidizes avidly a variety of amines now classified either as type-A or type-B substrates (Ekstedt, 1976).

In this experiment, the attempt had been made to develop the assay method for monoamine oxidase activity by using oxygen consumption technique. The developed assay, then, had been use to study the in vitro effects of piperine on the enzymatic activity of MAO in rat liver and brain mitochondrial preparations.