

**INFLUENCE OF TOBACCO SMOKE CONSTITUENTS ON MONOAMINE
OXIDASE ACTIVITY IN VITRO AND ON HUMAN PLACENTAL
MONOAMINE OXIDASE A ACTIVITY IN VIVO**

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SUMMARY

The aim of this study was to investigate the influence of tobacco smoke on monoamine oxidase activity, and of smoking during pregnancy on placental monoamine oxidase A (MAO-A) activity. The enzyme MAO exists in two isoforms, MAO-A and MAO-B, both of which are important in regulating monoamine status in the brain and periphery.

Some substrate selectivity for particular monoamines is a characteristic of these isoforms, and one form of the enzyme may predominate in particular organs. Recreational tobacco smoking influences the status of monoamine oxidases in the body. *In vivo* studies using PET imaging have shown that both MAO-A and MAO-B activity are inhibited in the brains of smokers compared to non-smokers. In support of these findings are data that suggest that MAO-B catalytic activity is attenuated in blood platelets of smokers.

Hitherto, no studies have assessed the influence of cigarette smoke on placental MAO activity. Mammalian placenta is rich in MAO-A and activity of this isoform of the enzyme is found virtually exclusively in this organ. The activity of MAO-A in the placenta is of interest since it is thought to play a major role in regulating serotonin (a monoamine neurotransmitter and highly selective MAO-A substrate) within the placenta. Serotonin plays a very important role in the development of the fetus and it is evident that any xenobiotically-induced influence on the status of placental serotonin, including alteration of the activity of its metabolizing enzyme (i.e. MAO-A), should be of concern in pregnancy.

A series of studies aimed at assessing the influence of tobacco smoke condensate and tobacco leaf extract on monoamine oxidase activity in general, has been performed. These included studies comparing the MAO inhibitory effects of tobacco leaf extracts compared to the (whole/total/crude) cigarette smoke condensate, and studies to determine whether this inhibition is reversible. Studies that focused on MAO-A included the determination of the activity of this enzyme isolated from healthy placentas and the study of the influence of cigarette smoke condensate on placental mitochondrial MAO-A activity *in vitro*. These studies showed that cigarette smoke condensate is much more potent in inhibiting MAO compared to tobacco leaf extracts. A very surprising finding was that smoke extracts of cigarettes that do not contain tobacco (Magic® brand cigarettes) showed similar potencies for inhibiting MAO than did smoke extracts of tobacco-containing cigarettes. It is clear that there is an irreversible component in the inhibition of MAO by cigarette smoke condensate (in the A as well as the B form). The series of *in vitro* and *ex vivo* settings confirmed that tobacco smoke has a profound influence on MAO activity by inhibiting the enzyme irreversibly.

In order to determine the effects of maternal smoking during pregnancy specifically on MAO-A activity in the placenta, term placentas from smoking and non-smoking mothers were collected and the MAO-A activity measured in each case. Furthermore, the influence of MAO-A activity on serotonin levels was investigated by measuring serotonin levels in the umbilical cord blood and maternal blood associated with each placenta. It was hypothesized that a decrease in placental MAO-A activity should result in an increase in umbilical cord and maternal blood serotonin levels in placentas obtained from mothers who smoked during pregnancy. Our results showed slightly lower MAO-A activity in term placentas of smokers compared to non smokers. However, this influence was not

significant. Platelet MAO-B activity was found to be significantly lower ($p < 0.1$) in maternal platelet rich plasma (PRP) of smokers compared to non smokers. Determinations in maternal PRP revealed that serotonin concentrations expressed per volume is not different between smokers and non smokers. However, when the maternal serotonin concentrations are expressed per platelet, non smokers have significantly more ($p < 0.05$) serotonin present per platelet than smokers. From the literature there seems to be an inverse relationship between mean platelet volume and serotonin content which would argue that smokers in our study had smaller mean platelet volumes than non-smokers. We speculate that there is a compensatory mechanism responsible for the subsequently higher maternal platelet count ($p < 0.05$) in the smokers compared to the non smokers. Determinations in cord blood PRP showed no significant differences between smokers and non smokers with regards to serotonin levels, platelet counts and MAO-B activity.

From these studies it can be concluded that extracts of tobacco leaves are less potent in inhibiting MAO than extracts of cigarette smoke, leading one to speculate that pyrolysis products are mostly implicated in MAO inhibition. Cigarette smoke extracts are more potent in inhibiting MAO-B than MAO-A *in vitro*. Despite the significant inhibition of MAO-A *in vitro* by extracts of cigarette smoke, placental MAO-A activity is not significantly inhibited by maternal smoking during pregnancy *in vivo* compared to women who do not smoke. However, platelet MAO-B activity was significantly inhibited in the smoker group compared to the non smoker group. These results leads one to speculate that the *in vivo* activity of placental MAO-A is somehow more “protected” against the effects of cigarette smoking than when this enzyme is tested in an *in vitro* environment.

OPSOMMING

Die doel van hierdie studie was om die invloed van sigaretrook op monoamienoksidase (MAO)-aktiwiteit, en van rook gedurende swangerskap op plasenta monoamienoksidase-A (MAO-A) aktiwiteit te ondersoek. Twee isovorme van MAO is reeds geïdentifiseer as MAO-A en MAO-B. Beide hierdie isovorme is belangrik ten opsigte van die regulering van monoamien in die brein sowel as in die perifere stelsel.

Tipies is MAO-A en MAO-B selektief teenoor verskillende spesifieke monoamien en oorheers een vorm van die ensiem in sekere organe. Tabakrook het 'n invloed op monoamienoksidases in die liggaam. Die inhibisie van MAO-A en MAO-B-aktiwiteit in die brein van rokers is reeds bewys deur middel van PET-skandering. Hierdie resultate word ondersteun deur data wat aantoon dat MAO-B aktiwiteit in bloedplaatjies van rokers geïnhibeer is.

Tot dusver is daar geen aanduiding dat die invloed van sigaretrook op plasenta-MAO-A aktiwiteit bestudeer is nie. Die menslike plasenta is ryk aan MAO-A en hierdie orgaan toon feitlik eksklusief MAO-A aktiwiteit. Die moontlike inhibisie van plasenta-MAO-A-aktiwiteit deur sigaretrook is van belang omdat MAO-A 'n belangrike rol speel in die regulering van serotonienvlakke (serotonien is 'n hoogs selektiewe MAO-A substraat) in die plasenta. Serotonien speel 'n belangrike rol in die ontwikkeling van die fetus. Enige xenobioties-geïnduseerde invloede op plasenta serotonien, onder andere 'n verandering in die ensiem verantwoordelik vir die metabolisme van serotonin (d.w.s. MAO-A), is daarom belangrik gedurende swangerskap.

Vir hierdie studie is 'n reeks toetse uitgevoer om die invloed van tabakrookekstrak en tabakblaarekstrak op MAO-aktiwiteit te bepaal. Die inhibisie van MAO deur tabakblaarekstrakte was onder andere vergelyk met ekstrakte van sigaretrook en die omkeerbaarheid van inhibisie van laasgenoemde ekstrak is ook getoets. Die invloed van sigaretrook op MAO-A is bepaal deur plasenta mitochondriale MAO-A aktiwiteit *in vitro* te meet in ensiem geïsoleer uit gesonde plasentas, in die teenwoordigheid van sigaretrookekstrak. Die resultate van hierdie studies dui onomwonde daarop dat sigaretrookekstrak MAO-aktiwiteit meer inhibeer as ekstrakte van die tabakblaar. 'n Verrassende bevinding was dat die rookekstrak van sigarette wat geen tabak bevat nie, maar wel 'n mengsel van kruie (sogenaamde "Magic" sigarette), volgens ons studies dieselfde vermoë het om MAO te inhibeer as die ekstrak van gewone tabakbevattende sigarette. Daar is heel duidelik 'n onomkeerbare komponent betrokke in die inhibisie van MAO deur sigaretrookkondensaat (MAO-A sowel as MAO-B). Die reeks *in vitro* toetse bevestig dat tabakrook 'n ernstige invloed het op MAO aktiwiteit deurdat dit hierdie ensiem onomkeerbaar inhibeer.

Die effek van maternale rook gedurende swangerskap op plasenta MAO-A aktiwiteit is bepaal deur die plasentas van rokende en nie-rokende moeders te versamel en die MAO-A aktiwiteit *ex vivo* te bepaal. Die invloed van plasenta MAO-A aktiwiteit op serotonienvlakke is ondersoek deur die serotonienvlakke in die ooreenstemmende maternale bloed en naelstringbloed te bepaal. Die hipotese is dat 'n afname in plasenta MAO-A aktiwiteit by rokende moeders sal lei to 'n toename in die serotonienvlakke in naelstringbloed en maternale bloed.

In ons studie het die moeders wat tydens swangerskap gerook het 'n effense afname in plasenta MAO-A aktiwiteit getoon in vergelyking met die nie-roker groep. Hierdie verskil in MAO-A aktiwiteit was egter nie statisties betekenisvol nie. Die MAO-B aktiwiteit in die bloedplaatjies was betekenisvol laer ($p < 0.1$) in die maternale plaatjieryk plasma (PRP) van die rokers in vergelyking met die nie-rokers. Daar was geen betekenisvolle verskille in die serotonienvlakke, uitgedruk as konsentrasie per volume, in die maternale PRP van rokers in vergelyking met nie-rokers nie. Wanneer die serotonienkonsentrasie egter per plaatjie bereken word, het die nie-rokers statisties betekenisvol meer ($p < 0.05$) serotonien per plaatjie as die rokers getoon. 'n Omgekeerde verhouding tussen gemiddelde plaatjievolume en serotonieninhoud word in die literatuur gerapporteer. Indien dit wel die geval is sal dit beteken dat die rokers in hierdie studie 'n kleiner gemiddelde plaatjievolume het as die nie-rokers. Ons spekuleer dat daar moontlik 'n kompensatoriese meganisme verantwoordelik is vir die hoër maternale plaatjietelling ($p < 0.05$) in die rokers in vergelyking met die nie-rokers. In die naelstringbloed-PRP was daar geen statisties-betekenisvolle verskille tussen rokers en nie-rokers ten opsigte van serotonien vlakke, plaatjie tellings, en MAO-B aktiwiteit nie.

Uit die studies wat gedoen is vir hierdie proefskrif kan ons die gevolgtrekking maak dat tabakblaarekstrakte MAO minder inhibeer as ekstrakte van sigaretrook. Hierdie resultaat lei tot die spekulasie dat dit hoofsaaklik piroliseprodukte sou wees wat verantwoordelik is vir die inhibisie van MAO. Sigaretrookekstrakte inhibeer MAO-B meer as MAO-A *in vitro*. Alhoewel die *in vitro* inhibisie van MAO-A deur sigaretrookekstrakte betekenisvol is, is die *in vivo* plasenta-MAO-A nie statisties betekenisvol geïnhibeer by moeders wat gedurende swangerskap gerook het, in vergelyking met moeders wat nie gerook het nie. Plaatjie-MAO-B-aktiwiteit was egter statisties betekenisvol geïnhibeer in die maternale

bloed van rokers in vergelyking met nie-rokers. Hierdie resultate lei tot 'n gevolgtrekking dat die *in vivo* aktiwiteit van plasenta MAO-A moontlik meer “beskerm” is teen die effekte van sigareetrook as in gevalle waar die ensiem in 'n *in vitro* omgewing getoets word.

INTRODUCTION

Cigarette smoking has been the most popular method of taking nicotine since the beginning of the 20th century. In 2002, 30 percent of the U.S. population, 12 years and older, (i.e. 71.5 million people) used tobacco at least once a month prior to being interviewed in one comprehensive study by the Substance Abuse and Mental Health Services Administration (SAMHSA, Substance Abuse and Mental Health Services Administration, 2003).

Tobacco use also is the single leading preventable cause of death in the United States (McGinnis and Foege, 1993) accounting for approximately 430,000 deaths each year (MMWR, 1996). The economic liability associated with tobacco use ranges from \$50 billion to \$73 billion per year in medical expenses alone (CDC, 1999).

In 2002, 17.3 percent of pregnant women in the USA aged 15 to 44 smoked cigarettes in the month prior to being interviewed in the SAMHSA study (Substance Abuse and Mental Health Services Administration, 2003). Maternal cigarette smoking during pregnancy has been shown to influence reproductive outcomes. The most well-known and documented of these effects is intrauterine growth retardation. Smoking during pregnancy reduces birthweight by an average of 200 g, and this effect is dose-related (Frazer *et al.*, 1961, Butler *et al.*, 1972, D'Souza *et al.*, 1981). Among maternal smokers the risk of a low birthweight baby is doubled and the risk increases with increasing number of cigarettes smoked (Frazer *et al.*, 1961, Meyer 1978; Cnattingius *et al.*, 1993). Women who

smoke during pregnancy have an estimated 30% increased risk of delivering before 37 weeks of gestation (Shiono, 1986). This risk also is dose-related (Shiono, 1986). Other health related effects associated with cigarette smoking include placenta previa (abnormal location of the placenta) (Kramer *et al.*, 1991; Handler *et al.*, 1994; Castles *et al.*, 1999) and abruption (early separation of the normally located placenta) (Misra and Ananth, 1999; Ananth *et al.*, 1999).

It is clear that studying the influence of cigarette smoking on humans is very relevant and information on how cigarette smoking influences the human body – also during pregnancy – is of utmost importance.

Interestingly cigarette smoking has been associated with a lower incidence of Parkinson's disease (Shahi *et al.*, 1991; Grandinetti *et al.*, 1994). This link between smoking and Parkinson's disease sparked interests to determine the mechanism by which cigarette smokers could be protected against this neurodegenerative disease. An important observation is that cigarette smokers have lower brain MAO-A and MAO-B activity (Fowler *et al.*, 1996a, 1996b) and platelet MAO-B activity (Oreland *et al.*, 1981; Yong and Perry, 1986; Norman *et al.*, 1987; Berlin *et al.*, 1995a) than do non-smokers. These observations, together with the knowledge that inhibitors of MAO-B – for example (R)-deprenyl – is neuroprotective (Ebadi *et al.*, 2002; Magyar *et al.*, 1998) lead to our in-vitro investigations on the influence of extracts of cigarette smoke on MAO-A and MAO-B.

The reported inhibition of MAO by cigarette smoke in the brains and platelets of smokers compared to non-smokers is of key interest during pregnancy. The placenta contains rich MAO-A expression (Riley *et al.*, 1989; Weyler and Salach, 1985) and this

enzyme is responsible for regulating the levels of serotonin in the placenta (Gujrati *et al.*, 1996). If smoking during pregnancy leads to placental MAO-A inhibition the levels of serotonin could increase to levels that could be detrimental to the health of the neonate. Of interest in this regard is that lower birth weight and shorter birth length has been reported in infants exposed to fluoxetine (a selective serotonin reuptake inhibitor and thus also responsible for increased extracellular serotonin levels) late in gestation (Chambers *et al.*, 1996) Studies assessing the effects of taking fluoxetine during pregnancy show a spontaneous abortion rate of 13.8% (Goldstein *et al.*, 1997). Furthermore, Fuchs and co-workers (1975) showed more than a 100% increase in serotonin levels during therapeutic abortion. It is therefore clear that serotonin plays an active role in the process of fetal expulsion. Hence it would be reasonable to speculate that if lower MAO-A activity in the placenta leads to higher levels of serotonin such events could lead to preterm births.

The main purpose of this study was focused on the investigation of placental events in smoking pregnant women to determine whether cigarette smoking during pregnancy leads to inhibition of placental MAO-A activity compared to the same activity in non-smokers, and to also measure the influence of such inhibition on serotonin levels.

LITERATURE REVIEW

2.1 The influence of tobacco smoke on monoamine oxidase (MAO) activity *in vitro*

2.1.1 Cigarette smoking and Parkinson's disease

It has been known for some time through strong epidemiological evidence that smokers have a lower incidence of Parkinson's disease than do non-smokers (Shahi *et al.*, 1991; Grandinetti *et al.*, 1994). The reason for this "inverse relationship" (Gorell *et al.*, 1999) between cigarette smoking and Parkinson's disease remains to be established.

One proposal is that such a "protective" action of cigarette smoking may be associated with inhibition of the monoamine oxidases (MAOs). Studies from the early 1980s until the mid 1990s have shown that cigarette smokers indeed have lower blood platelet MAO-B activities than do non-smokers (Oreland *et al.*, 1981; Yong and Perry, 1986; Norman *et al.*, 1987; Berlin *et al.*, 1995a). More importantly, Fowler and co-workers recently discovered that MAO-A and MAO-B activities are significantly lower in the brains of smokers than the same activities measured in the brains of non-smokers or former smokers (Fowler *et al.*, 1996a,b).

The link between MAO inhibition and neuroprotection in Parkinsonism has been investigated in laboratory animal models. It is known that inhibitors of MAO-B protect

against xenobiotic insult in animal models of Parkinsonism. For example, (R)-deprenyl, a well-known inhibitor of MAO-B, has been shown to be neuroprotective in models of neuronal insult, such as the MPTP/mouse parkinsonian model, where striatal dopamine (DA) depletion caused by treatment with MPTP is attenuated in animals pretreated with (R)-deprenyl (Ebadi *et al.*, 2002; Magyar *et al.*, 1998). The mechanism for this protection has been elucidated *in vivo*: MAO-B bioactivates the proneurotoxin MPTP (**1**) via the dihydropyridinium intermediate MPDP⁺ (**2**), leading to the formation of the neurotoxic pyridinium species, MPP⁺ (**3**) (Figure 2.1) (Chiba *et al.*, 1985). MPP⁺ acts as a mitochondrial toxin in dopaminergic neurons, resulting in an initial release and eventual depletion of dopamine in the striatum. Inhibitors of this MAO-B pathway block MPP⁺-induced neurodegeneration by protecting against the decrease in DA levels in the striatum after MPTP treatment (Chiba *et al.*, 1984; Fuller and Hemrick-Luecke 1985; Heikkila *et al.*, 1984).

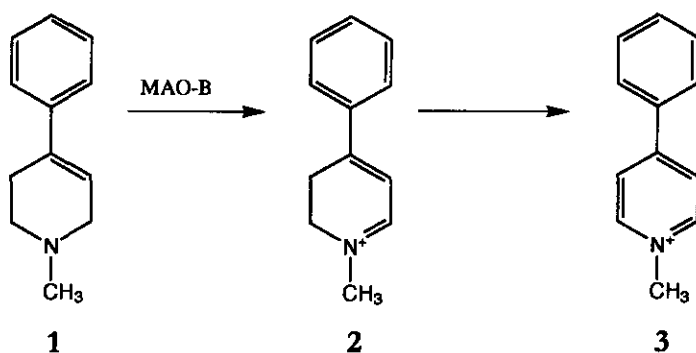


Figure 2.1. Metabolic bioactivation of MPTP (1) to the neurotoxic pyridinium species MPP⁺ (3).

The strong link between MAO-B inhibition and neuroprotection in parkinsonian laboratory animal models suggests that smoking also may be neuroprotective - among other reasons yet to be discovered - by inhibition of MAO [as in the case of (R)-deprenyl]. This MAO inhibition-associated neuroprotection may possibly be the result of enzyme inhibition by components found in cigarette smoke.

2.1.2. MAO

Monoamine oxidases (E.C. 1.4.3.4) are flavoenzymes that are responsible for catalyzing the oxidative deamination of biogenic amine neurotransmitters (including DA) and a variety of xenobiotic amines (Ramsay and Gravestock, 2003; Dostert *et al.*, 1989). This enzyme was first isolated in 1928 by Mary Hare (Hare, 1928) and was initially called tyramine oxidase because of the observation that this enzyme catalyses the oxidative deamination of tyramine. It was later shown that this enzyme also oxidizes catecholamines (Blaschko *et al.*, 1937) which led to the more generally accepted name of monoamine oxidase (MAO) (Zeller, 1938).

The MAO enzyme exists in two isoforms, MAO-A and MAO-B, which are different gene products (Berry *et al.*, 1994) and share 70% similarity in amino acid sequence (Bach *et al.*, 1988). These enzymes are localized in the outer mitochondrial membrane and occur in neuronal and non-neuronal cells in the brain and peripheral organs. Often only one form of the enzyme is present in a specific organ and/or within a specific cell type, the reasons for this being still unclear (Trendelenburg *et al.*, 1987; Yu *et al.*, 1992). MAO-A is found in catecholaminergic neurons (predominantly intraneuronally) while MAO-B is present in serotonergic neurons (Saura *et al.*, 1996).

MAO-A and MAO-B also have different inhibitor specificities and substrate preferences (Molodtsova, 2003; Severina, 1979; Deniker, 1984). MAO-A predominantly deaminates serotonin (5-HT) and noradrenaline (NA) and is inhibited by low concentrations of clorgyline (Fowler *et al.*, 1982). MAO-B predominantly deaminates benzylamine and β -phenylethylamine and is selectively inhibited by low concentrations of (R)-deprenyl (Yang and Neff, 1974; Fowler *et al.*, 1982). Dopamine has been shown to be an equally well preferred substrate for both forms of the enzyme (Yang and Neff, 1974; Garrick and Murphy, 1982; Strolin-Benedetti *et al.*, 1983; Molodtsova, 2003).

MAO-A inhibitors have mostly been associated with antidepressant activity (Ramsay and Gravestock, 2003; Kato *et al.*, 1998) while MAO-B inhibitors have strongly been linked to neuroprotection (Ramsay and Gravestock, 2003; Magyar *et al.*, 1998), although inhibitors of MAO-B have also been used in the treatment of depression (Mann *et al.*, 1984). The aforementioned facts suggest that these isoforms have different functions.

2.1.3. MAO and cigarette smoke

Essman (1977) was the first to report that cigarette smoke is implicated in MAO inhibition. Mice exposed to cigarette smoke showed significant inhibition of MAO as measured in the mouse skin, with serotonin as substrate (Essman, 1977). Since the work by Essman was first reported, it has been shown that cigarette smokers have lower blood platelet MAO-B activities than do non-smokers (Oreland *et al.*, 1981; Yong and Perry, 1986; Norman *et al.*, 1987; Berlin *et al.*, 1995a). This inhibition of platelet MAO-B activities in smokers were shown to be significant in both males and females (Norman *et al.*, 1987). In females (but not in males) there is a significant negative correlation between

the amount of cigarettes smoked and platelet MAO-B activity (Norman *et al.*, 1987). Importantly Fowler and co-workers documented that MAO-A and MAO-B activities are significantly lower in the brains of smokers than non-smokers or former smokers as measured by positron emission tomography (Fowler *et al.*, 1996a,b).

Bench and co-workers (1991) have studied platelet and brain MAO-B using dynamic positron emission tomography and probing with L-[¹¹C] deprenyl and Ro 19-6327 (a reversible MAO-B inhibitor), in eight normal individuals. The authors showed that inhibition of platelet MAO-B can be used as a marker for brain MAO-B inhibition.

Initially it was speculated that people with lower MAO activity might be more prone to smoking because of their personality traits e.g., high impulsiveness and sensation seeking behaviors (Coursey *et al.*, 1979; Orelund *et al.*, 1981). However, later studies were able to show an influence of cigarette smoke on MAO *in vitro* (Yu and Boulton, 1987). A cigarette smoke solution, collected by bubbling the smoke through a phosphate buffer solution (pH 7.5), showed inhibition of rat lung mitochondrial MAO. Human saliva collected after smoking also showed inhibition of rat lung MAO (Yu and Boulton, 1987). Tobacco smoke particulate matter extracted with dimethylsulfoxide (DMSO) and added to cerebral homogenates of mice, was capable of inhibiting MAO (Carr and Basham, 1991). This extract caused a concentration-dependent inhibition of MAO-A and MAO-B. The inhibitory effect observed *in vitro* showed characteristics of both competitive and noncompetitive inhibition (Carr and Basham, 1991).

2.1.4. Irreversible inhibition of MAO by cigarette smoke

There have been indications that the inhibition of MAO afforded by cigarette smoke is irreversible in nature. Of particular importance is the finding that platelet activity of MAO-B in ex-smokers was not different from that of non-smokers and significantly higher than that of smokers (Oreland *et al.*, 1981; Von Knorring and Oreland, 1985). This "normalization" of MAO-B activity in ex-smokers requires several weeks to occur after an individual quits smoking (Berlin *et al.*, 1995b). It may be argued, therefore, that the return of platelet MAO-B activity to normal levels may depend upon the turnover rate of thrombocytes and the rate of synthesis of the MAO-B enzyme itself. The life-span of human platelets is estimated to be about 10 days (Peerschke, 2002) and the rate of MAO-B synthesis is slow with a half life of approximately 40 days (Fowler *et al.*, 1994). These numbers suggest that the components of cigarette smoke putatively responsible for the inhibition of MAO may possibly be irreversible and non-competitive in nature. In early *in vitro* studies, Yu and Boulton (1987) and Carr and Basham (1991) reported that cigarette smoke has an irreversible inhibitory component towards MAO-B. These findings will have to be confirmed and studied in our own systems.

2.1.5. Components of cigarette smoke and tobacco leaf responsible for MAO inhibition

Lim and co-workers (1997) have reported MAO-B inhibitors to be present in cigarette smoke that was collected on Cambridge filter pads and extracted with methanol. After careful fractionation into acidic, basic, neutral and phenolic fractions, candidate components were isolated and tested for possible MAO-B inhibition in rat brain mitochondria. Among the isolated components farnesol, 3,4-dihydroxybenzylamine,

indole, eugenol, hydroquinone and methylcatechol showed the most MAO-B inhibition. 2-Naphthylamine, an aromatic primary amine is a well known carcinogen ($LD_{50}=727$ mg/kg) found in amounts of 1.0-20 ng/cigarette in cigarette smoke (Patrianakos and Hoffmann, 1979; Masuda and Hoffman, 1969). This compound has been shown to inhibit mouse brain MAO-A and MAO-B *in vitro* (Hauptmann and Shih, 2001).

More recent research lead to the isolation of a competitive inhibitor of MAO-B from extracts of tobacco leaf (Khalil *et al.*, 2000). This compound, 2,3,6-trimethyl-1,4-naphthoquinone (TMN), has been shown to be neuroprotective in the MPTP parkinsonian C57BL/6 mouse model when similar parameters of toxicity are considered to those used in studies with (R)-deprenyl. When mice were pretreated with TMN, the decrease in dopamine levels in the striatum was attenuated significantly compared with animals treated with MPTP only (Castagnoli *et al.*, 2001). These findings provide indirect evidence for *in vivo* MAO-B inhibition and subsequent neuroprotection by at least this one component (TMN) found in tobacco leaf and smoke (Fowler *et al.*, 2003; Chamberlain and Stedman, 1986).

Other than TMN, three other compounds in tobacco that show MAO-B inhibition have recently been isolated. These have been identified as farnesylacetone, an as yet unidentified compound that contains a chromone system, and a third compound containing a polyunsaturated macrocycle (Castagnoli *et al.*, 2002). These compounds all have been isolated from tobacco leaf only and their presence or absence in smoke still needs to be determined.

Studies in the early 1980s have shown that, although nicotine itself weakly inhibits MAO *in vitro*, it does so at concentrations considerably higher than the highest concentrations found in the blood of heavy smokers (Oreland *et al.*, 1981). Other researchers claimed that nicotine and cotinine did not show any inhibition of MAO-B. (Essman, 1977; Berlin *et al.*, 1995b; Hauptmann and Shih 2001; Castagnoli *et al.*, 2002).

2.1.6. Pyrolysis products and MAO inhibition

When a cigarette is lit, a burning zone forms at the end of the packed tobacco cylinder. Two types of burning take place with a cigarette: puffing, and smoldering between puffs. During puffing, air is drawn into the cigarette through the burning zone and mainstream smoke is formed. In the interval between puffs a natural convection flow of air around the burning zone in an upwards direction (because of buoyancy, Figure 2.2) sustains burning and forms the sidestream smoke (Baker, 1987).

Information on the conditions inside the burning zone of cigarettes has been obtained by inserting probes into burning cigarettes. Thermocouples and infrared probes measure the temperature and other probes connected to a mass spectrometer measure gas concentration. The approximate relationships of the major combustion processes involved in smoke generation are illustrated in Figure 2.2. The inside of the burning zone is oxygen-deficient and hydrogen-rich and can be divided into an exothermic combustion zone and an endothermic pyrolysis/distillation zone. As air is drawn into the cigarette during the puff, oxygen is consumed by combustion with carbonized tobacco and the simple combustion products carbon monoxide (CO), carbon dioxide (CO₂) and water (H₂O) are formed, together with the release of heat that sustains the whole burning

process. Temperatures in the region of 700 and 950 °C are generated and heating rates as high as 500 °C.s⁻¹ can be achieved. (Dube and Green, 1982).

Immediately downstream of the combustion region is the pyrolysis/distillation zone, where temperatures vary between approximately 200 and 600 °C and which is still low in oxygen levels. The majority of smoke products are generated in this region by a variety of mechanisms that are essentially endothermic (Baker, 1987). The composition of tobacco smoke depends on the smoking conditions as well as the physical and chemical properties of the leaf or the tobacco blend, the wrapper and the filter (Coggins, 2002). More than 3,800 components have been identified in cigarette smoke, the majority of which are formed in the pyrolysis-distillation zone (Baker, 1981).

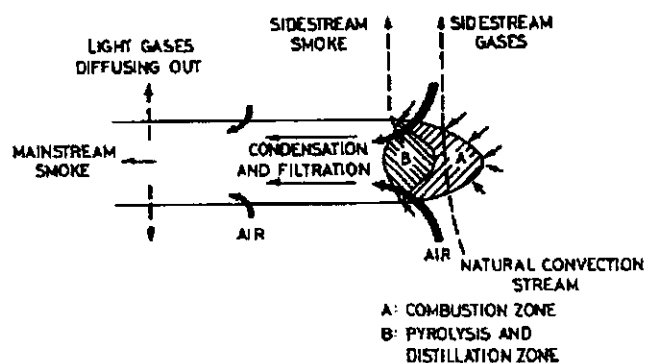


Figure 2.2. Illustration of the processes involved in the burning cigarette (Baker, 1987).

2.1.6.1. Tobacco leaf extracts vs. extracts of tobacco smoke

The overwhelming evidence that cigarette smoke inhibits MAO activity in smokers has already been discussed. This phenomenon has been shown in the brains and the platelets

of smokers. Subsequent in-vitro studies in order to determine the compounds responsible for this inhibition have been undertaken and some compounds have already been identified in cigarette smoke. Inhibitors of MAO have also been identified in extracts of tobacco leaf. However, only one study to date has directly compared the inhibitory potencies of leaf extracts to cigarette smoke extracts (Yu and Boulton, 1987). The inhibition of smoke extracts was compared to leaf extracts using sodium phosphate buffer as solvent. The study by Yu and Boulton indicated that leaf extracts are less potent in inhibiting MAO than extracts of tobacco smoke. Studies aimed at determining the influence of pyrolysis products on MAO inhibition are therefore of great importance in order to understand the influence of cigarette smoke on MAO and the nature of compounds implicated in the neuroprotection afforded by cigarette smoking.

It is clear that the process of burning tobacco results in many reactions occurring in parallel due to the severe pyrolytic conditions encountered in a burning cigarette. Based on these observations we speculate that extracts of cigarette *smoke* will have a higher potency in inhibiting MAO than extracts of the *tobacco leaf*.

2.1.6.2. Tobacco-less cigarettes

Is smoke from tobacco plant products the only source of compounds that may act as inhibitors of MAO? Cigarettes made from plant materials other than the tobacco plant are commercially available. These products often are advertised as "healthy" alternatives to tobacco cigarettes (Warner, 2002; Malson *et al.*, 2001). These cigarettes contain marshmallow, yerba santa, damiana, passion flower, jasmine and ginseng as well as natural and artificial flavoring. While not containing tobacco products (or nicotine), the phytochemical contents of such products will be subject to similar pyrolytic conditions as

those found during the smoking of regular cigarettes (White *et al.*, 2001). It may therefore be argued that the burning of a variety of plant materials (such as those also found in "tobacco-less" cigarettes (Pakhale *et al.*, 1990)) may lead to the formation of pyrolysis products capable of inhibiting MAO. As a first approach to investigate this possibility the smoke condensates of cigarettes containing tobacco alternatives were tested in the MAO assay in studies described later in this thesis. It is therefore also of much interest to investigate the MAO inhibitory activity of condensates obtained after smoking of such "alternative" cigarettes.

2.1.6.3. Cigarettes that primarily heat tobacco

White and co-workers (2001) showed that pyrolysis temperature influences the mutagenicity of cigarette smoke condensate (CSC). These authors showed increased levels of CO and CO₂ as well as an increase in mutagenicity with higher pyrolysis temperatures (above 400 °C). Studies (Bombick *et al.*, 1997; Bombick *et al.*, 1998) with a novel type of investigative cigarette ("Eclipse" or TOB-HT) that primarily heats tobacco, and burns only a small amount of the leaf, show that CSC from "Eclipse" and TOB-HT cigarettes exhibited *less* cytotoxicity and genotoxicity than CSC obtained from "regular" tobacco burning cigarettes. The authors attribute this phenomenon to fewer pyrolysis products being formed when the tobacco is primarily heated as opposed to being burned. These studies were expanded in later reports by the same group (Bowman *et al.*, 2002).

Philip Morris Inc. recently introduced a new type of cigarette (Accord®) that primarily heats tobacco as opposed to "regular" cigarettes that burn tobacco. The Accord® "system" consists of two parts, a cigarette-like roll of tobacco (which cannot be smoked

by conventional methods) and a battery-powered heating device (Figure 2.3). The special cigarette has to be inserted into a hole in the pager-sized heater and has to be smoked in conjunction with the heater. In order to smoke these cigarettes, a smoker sucks on a kazoolike box (Figure 2.4).

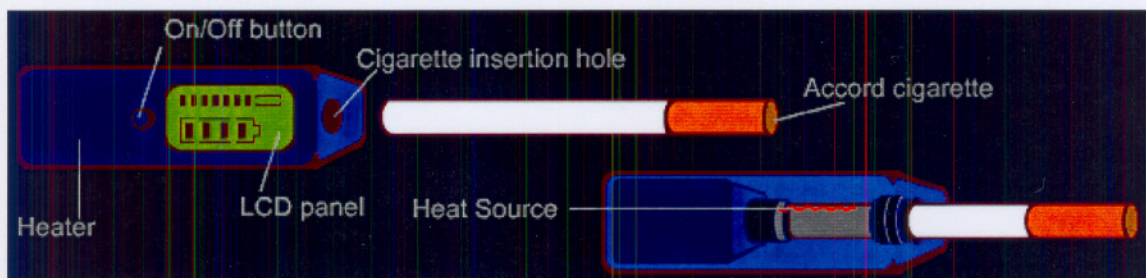


Figure 2.3. The Accord® “system” consists of the Accord® cigarette and a heater. This picture courtesy of http://www.pbs.org/wgbh/nova/cigarette/anat_text.html.



Figure 2.4. A picture of how to smoke an Accord® cigarette. This picture courtesy of <http://www.pbs.org/wgbh/nova/cigarette/history2.html>.

A microchip senses the puff and sends a burst of heat to the cigarette delivering a specific amount of heated tobacco vapor to the smoker each time he or she takes a drag. This process does not create ashes or smoke. Very little of the tobacco inside the Accord® cigarette actually burns and thus creates less pyrolysis products than conventional

cigarettes which primarily burns tobacco. (Info from: <http://www.pbs.org/wgbh/nova/cigarette/history2.html>). This type of cigarette creates a novel opportunity for studying the influence of pyrolysis products on the inhibition of MAO activity.

2.2. The influence of maternal cigarette smoking during pregnancy on placental MAO-A

2.2.1. Health related problems associated with cigarette smoking during pregnancy – a link with MAO-A inhibition?

The health related effects of cigarette smoking during pregnancy are well documented (Higgins, 2002). Fetal growth restriction resulting in low birth weight infants is the most well-known and documented reproductive outcome related to smoking (Jedrychowski *et al.*, 1998; Lam *et al.*, 1992; Geary *et al.*, 1997; Clausson *et al.*, 1998; Ogunyemi *et al.*, 1998 and Jacobson *et al.*, 1994). The extent of impaired growth is strongly related to serum cotinine concentrations (Haustein, 1999), a metabolic measure of nicotine exposure, and therefore also to the amount of cigarette smoke exposure (Haddow *et al.*, 1987). Maternal smoking also is strongly associated with abruption (early separation of the normally located placenta) (Misra and Ananth, 1999; Ananth *et al.*, 1999) and placenta previa (abnormal location of the placenta) (Kramer *et al.*, 1991; Handler *et al.*, 1994; Castles *et al.*, 1999). These conditions contribute to the increased prematurity and increased risk of perinatal mortality associated with cigarette smoking and possibly also to miscarriage rates (Naeye, 1978).

In the current study, we will not focus on potential links between lower MAO-A activity in the placenta and consequential health outcomes in the *later* life of the infant; but rather on whether some of the health related effects noted above could be linked to lower maternal activities of MAO-A and subsequent higher levels of serotonin (Mattson *et al.*, 2002) (the correlation between MAO-A activity and serotonin concentration in the placenta will be discussed later). Further, this study also will aim to determine the influence of smoking during pregnancy on the activity of placental MAO-A.

2.2.2. MAO-A in the human placenta

Auda *et al.* (1998) used specific primers for MAO-A and MAO-B mRNA in a reverse transcription-polymerase chain reaction to detect the predicted products for both enzymes on RNA from human placenta. The placenta, although believed to contain predominantly (or only) MAO-A protein, indicated the presence of both A and B gene transcripts (see Figure 2.5). The cellular distribution of MAO mRNA in placental tissue was analyzed by in situ hybridization of MAO-A and MAO-B mRNA-specific cRNA probes on paraffin sections. MAO-A mRNA was mainly evident in the syncytiotrophoblastic layer. None was detected in the vascular endothelium/smooth muscles (see Figure 2.5). Significantly, an MAO-B mRNA signal was also evident in the placental villi, notably in the syncytiotrophoblasts, intermediate trophoblasts, cytotrophoblasts, and the vascular endothelium (see Figure 2.6). Interestingly, the expression of MAO-B in placental tissue rather than in blood elements within placenta was unequivocally demonstrated by these authors.

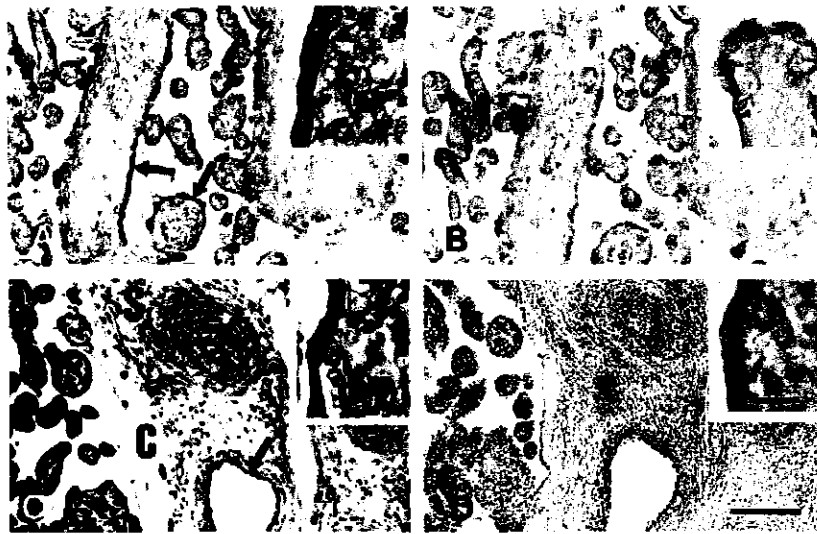


Figure 2.5. Placental sections hybridized with anti-sense (A,C) and sense (B,D) probes of MAO-A and MAO-B, respectively. (A) MAO-A mRNA is evident in the syncytiotrophoblastic layer of all villi (arrows). There is low signal in the smooth musculature and endothelial lining of blood vessels, and no signal in erythrocytes. Inset at higher magnification shows the presence of MAO-A mRNA in the syncytiotrophoblastic layer and in isolated cytotrophoblast cells. (C) MAO-B mRNA is strongly evident in the syncytiotrophoblastic layer of small villi and the endothelial lining of blood vessels (arrows). Signals are also evident in the vascular smooth musculature (s). There is no signal in the outer connective tissue layer (c) or in the erythrocytes. Inset at higher magnification shows the presence of MAO-B mRNA in the syncytiotrophoblastic layer and isolated cytotrophoblast cells. (B,D) Sense probes. No signal is evident in any part of these sections. Bars: A–D 5 1 mm; insets = 25 μ m. (Auda *et al*, 1998).

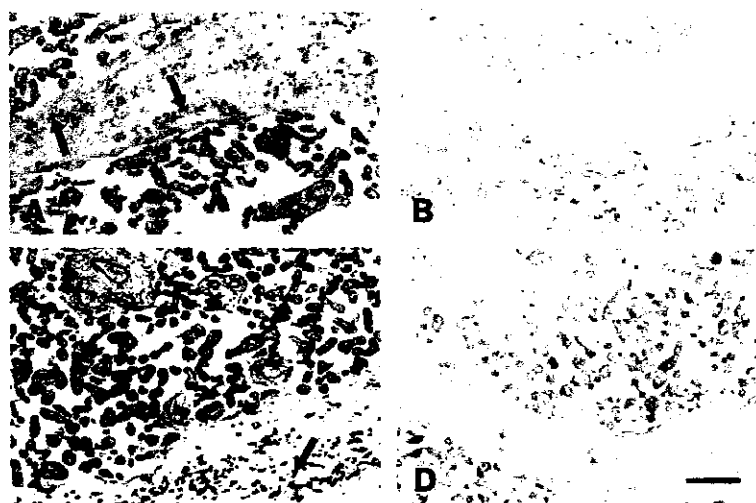


Figure 2.6. Placental sections (8 μ m) hybridized with MAO-A anti-sense (A), MAO-A sense (B), MAO-B anti-sense (C), and MAO-B sense (D) probes. (A) MAO-A mRNA signal is evident in all villi and villous buds and in the cytotrophoblast cell groups of the villous stem trunk (arrows). (C) MAO-B mRNA signal is strong in all villi and villous buds and in the cytotrophoblast cell groups of the villous stem trunk (arrows). Signal is also evident in the vessel walls (arrow). No signal is evident in sections hybridized with either the MAO-A sense or MAO-B sense probe. Bar = 0.2 mm. (Auda *et al.*, 1998).

Although gene transcripts for both MAO-A and MAO-B have been detected in the human placenta (Auda *et al.*, 1998), the levels of MAO-B activity in mitochondria obtained from term placentas (Riley *et al.*, 1989; Weyler and Salach, 1985) are extremely low. Thus, in humans, the placenta essentially contains exclusively MAO-A activity (Riley *et al.*, 1989; Weyler and Salach, 1985). In view of the evidence that cigarette smoking inhibits MAO-A activity in the brain of humans (Fowler *et al.*, 1996a) and compounds isolated from tobacco smoke inhibit MAO-A *in vitro* (Hauptmann and Shih, 2001; Carr and Basham, 1991), it is of interest to determine the effects of cigarette smoking on MAO-A activity in the human placenta.

2.2.3. The role of MAO and serotonin in the human placenta

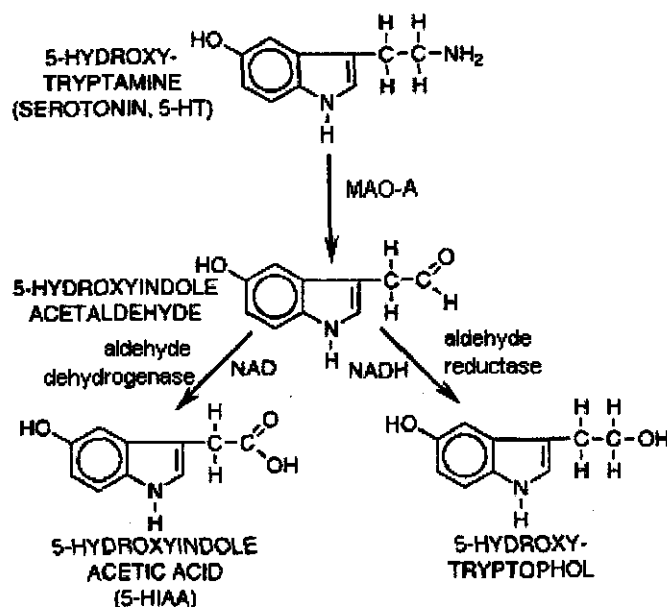


Figure 2.7. Metabolism of serotonin by MAO-A and resulting metabolites (Sanders-Bush and Mayer, 1996).

The importance of MAO-A in the placenta should be understood in the light of serotonin being a highly selective MAO-A substrate (Garrick and Murphy, 1982). There is evidence that placental MAO-A activity is a major factor in the regulation of serotonin levels within the placenta (Gujrati *et al.*, 1996, Figure 2.7). The placental brush border membrane serotonin transporter, responsible for transporting serotonin into the syncytiotrophoblast (the outer syncytial layer of the trophoblast) (Figure 2.8) from the maternal blood, also plays a major role in regulating fetal and maternal circulating serotonin levels (Sivasubramaniam *et al.*, 2002; Prasad *et al.*, 1996).

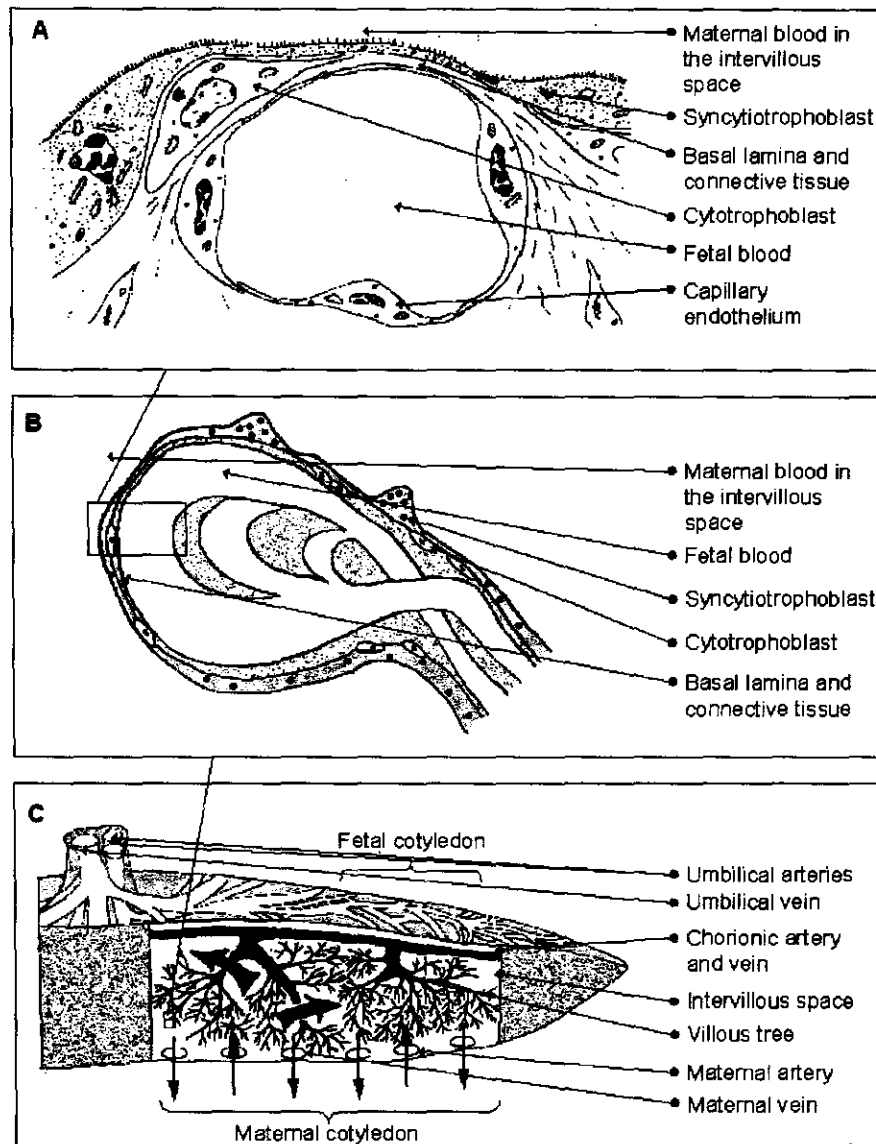


Figure 2.8. Human placental barrier between fetal and maternal blood circulation. (A) Schematic presentation of the cell layers separating the maternal and fetal circulations. **(B)** Structure of the terminal villus. **(C)** Schematic presentation of blood flow in a human placental cotyledon. The arrows indicate maternal blood flow (adapted from Ala-Koldko *et al.*, 2000).

The most widely studied cells that express the serotonin transporter are the serotonergic neurons and blood platelets (Rudnick and Clark, 1993). Serotonin, which is transported

into these cells via a transporter is either degraded by MAO-A or stored in vesicles. The vesicular monoamine transporter provides a mechanism to store the monoamines that enter the cytoplasm (Hayashi *et al.*, 1999). The uptake of serotonin from the cytoplasm into storage vesicles is catalyzed by a transporter present in the storage vesicular membrane (Njus *et al.*, 1986; Rudnick, 1986; Kanner and Schuldiner, 1987; Schuldiner, 1994).

The syncytiotrophoblast of the human placenta expresses a Na^+ - and Cl^- -dependent serotonin transporter in the maternally-facing brush border membrane (Balkovetz *et al.*, 1989). This transporter resembles the functional and pharmacological characteristics described for the serotonin transporter present in serotonergic neurons and blood platelets (Balkovetz *et al.*, 1989; Cool *et al.*, 1990a,b,c; Ramamoorthy *et al.*, 1992, 1993; Prasad *et al.*, 1994). The physiological role of this transporter in the human placenta remains highly speculative (Ganapathy *et al.*, 1993; Ganapathy and Leibach, 1994, 1995). The functional significance of the placental serotonin transporter (Martel and Keating, 2003) will depend primarily on what happens to serotonin inside the trophoblast once it is transported into the cell across the brush border membrane. Once inside the trophoblast, the high levels of MAO-A activity could participate in the degradation of serotonin (Salach and Detmer, 1979). The serotonin transporter also plays a role in the trans-placental transfer of this monoamine from mother to fetus as shown by studies with mouse placenta (Yavarone *et al.*, 1993).

Further evidence for a trans-placental transfer of serotonin is provided by findings that serotonin uptake sites appear in the plasma membrane of embryonic tissues much earlier than the development of serotonin synthesizing ability within the embryo itself (Lauder

and Zimmerman, 1988; Lauder *et al.*, 1988). It is therefore possible that maternal serotonin may be made available to the serotonin uptake sites in the embryo via transport across the placenta.

Serotonin has been shown to be a growth promoting factor for a number of cell types including neurons (Whitaker-Azmitia and Azmitia, 1986; Seurwen and Pouyssegur, 1990). During the early stages of life the blood-brain barrier is not fully developed and therefore serotonin in the circulation might be freely accessible to the developing neurons in the brain as was recently shown in rat studies (Manjarrez *et al.*, 2003). Normal embryonic development may therefore depend on trans-placental transfer of serotonin.

Prasad and co-workers (1996) indicated that the trophoblast cells from normal term placentas do not functionally express the vesicular monoamine transporter. This transporter is responsible for the transport of serotonin into storage vesicles. Since the normal placenta does not express such a transporter there is no mechanism for storage of the monoamines that enter the cytoplasm of the syncytiotrophoblast from the maternal blood (via the brush border membrane transporters of serotonin and norepinephrine). If the monoamines are not being stored by vesicular monoamine transporters, they must either be degraded inside the cell or transported into the fetal circulation or to adjacent uterine endometrial areas. In the absence of the vesicular monoamine transporter in the syncytiotrophoblast, the efficiency of the placenta to transfer serotonin from mother to fetus is determined primarily by the relative activities of the plasma membrane serotonin transporter and monoamine oxidases.

Another interesting observation is that the developmental state of the placenta may determine the ability of the placenta to transfer serotonin from the maternal side to the fetal side (Yavarone *et al.*, 1993). The trans-placental transfer of serotonin is very effective in the early stages of pregnancy and decreases markedly during later stages (Koren *et al.*, 1966). It appears that towards the end of pregnancy maternal serotonin enters the placenta but does not reach the fetal circulation. Speculation about the activities of monoamine oxidases and the plasma membrane serotonin transporter in the placenta has it that these two components may undergo marked changes during the development of pregnancy. In the early stages of pregnancy, where there is a need for effective transfer of maternal serotonin to the fetus for proper development, the activity of the plasma membrane serotonin transporter may be high while the activities of MAO-A may be low. This could enable serotonin to cross the placenta without being degraded. At later stages, however, the fetus develops the ability to synthesize serotonin on its own and thus is not dependent on maternal serotonin supply. During these stages, the activity of the plasma membrane serotonin transporter in the placenta may be low while the activities of monoamine oxidases may be high. Under these conditions, serotonin that is transported into the syncytiotrophoblast may undergo degradation and thus may not be available for transfer into the fetal circulation (Prasad *et al.*, 1996) (Figure 2.9).

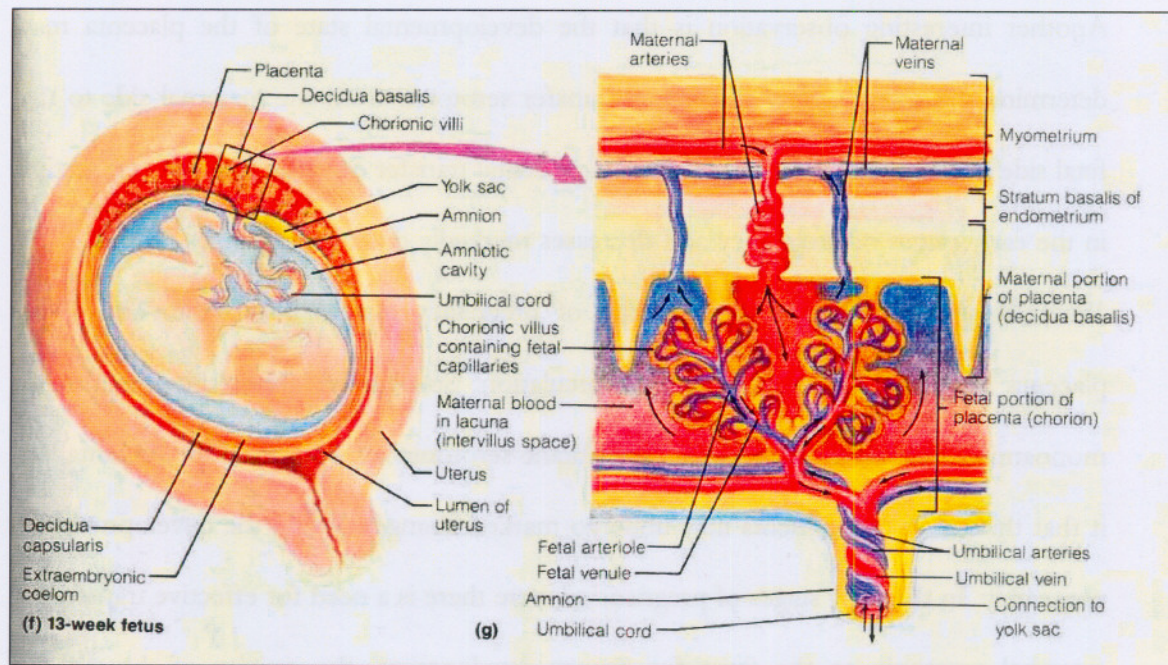


Figure 2.9. Illustration of the placenta. (f) 13 week fetus. (g) Detailed anatomy of the vascular relationships in the mature decidua basalis. This state of development has been accomplished by the end of the third month of development (Marieb, 1995).

Since serotonin inside the trophoblast that is not degraded by MAO-A may be transported to the adjacent endometrial areas (Carrasco *et al.*, 2000b), MAO-A is responsible also to keep a low cytosolic serotonin concentration (in the trophoblast cells) and thus enable the monoamine carriers to produce a net inward transport of serotonin. By this mechanism serotonin can be maintained at a very low level in the intervillous space (see Figure 2.9). If serotonin were not efficiently cleared from the intervillous space, it could cause vasoconstriction of uterine arteries and reduce the uteroplacental circulation (Ramamoorthy *et al.*, 1995). This could lead to alterations in the development of the fetus because exchange of nutrients and metabolic waste products between the maternal and fetal circulations would be compromised due to the decreased blood flow

through the intervillous space (Ramamoorthy *et al.*, 1995). Carrasco and co-workers (2000b) demonstrated that the impairment of placental MAO-A activity in pre-eclampsia (see below) appears to be an important factor leading to increased serotonin levels which induce intense vasoconstriction resulting in a compromise of the feto-placental circulation.

Inhibition of MAO-A (for example by cigarette smoke) may therefore lead to higher levels of serotonin available for transfer into the fetal circulation (intervillous space) and lead to the vasoconstrictive and subsequent effects described above.

2.2.4. The role of MAO-A and serotonin in pre-eclampsia

Pre-eclampsia has been described as a maternal syndrome characterized by hypertension and proteinuria. It is now believed that these clinical signs may only be secondary to placental ischaemia resulting from uteroplacental arterial insufficiency (Redman, 1991; Ness and Roberts, 1996). This syndrome is a major cause of maternal and perinatal mortality.

The phenomenon of lower levels of MAO-A associated with higher levels of serotonin is observed in pre-eclampsia. In pre-eclampsia, serotonin is significantly increased in the maternal blood and placenta (Laskowska *et al.*, 2001; Senior *et al.*, 1963; Fahim, 1964; Ramadan *et al.*, 1973; Gujrati *et al.*, 1985; Laskowska *et al.*, 2001) and the activity of MAO-A in the placenta has been reported to be decreased (Sagone and Arrotta, 1966; Carrasco *et al.*, 2000b). The levels of serotonin are closely related to the severity of this disease (Patel and Dass, 1962; Fahim and Botros, 1964). It has been shown that the metabolism

of serotonin is significantly higher in placental homogenates from normal pregnancies compared to placentas from severely pre-eclamptic pregnancies (Carrasco *et al.*, 2000b).

In this regard a very important study was done by Carrasco and co-workers (2000a) in pre-eclamptic pregnancies. These researchers determined the expression of MAO-A and of the serotonin transporter in maternally-facing brush border vesicles, and in placental homogenate by Western blotting. Their results showed no significant difference in serotonin transporter expression in placental maternally-facing brush border vesicles compared between normal and pre-eclamptic pregnancies. In addition, the uptake of serotonin in the maternally-facing brush border vesicles was similar between normal and pre-eclamptic pregnancies. Of exceptional importance was the discovery that the expression of MAO was almost undetectable in homogenates obtained from pre-eclamptic pregnancy placentas in comparison with homogenates obtained from placenta after normal pregnancy. In the latter case significantly higher activity and expression of this enzyme was found. These authors' data unequivocally suggest that the increased circulating serotonin associated with pre-eclampsia (Weiner, 1987) is mainly due to the diminished activity of MAO-A and not attributable to insignificant transport of serotonin into cells.

If smoking during pregnancy indeed inhibits MAO-A we might expect to see an increase in levels of serotonin in the placenta similar to cases of pre-eclampsia where lower levels of MAO-A are associated with increased levels of serotonin.

2.2.5 Cigarette smokers and pre-eclampsia

An apparent contradiction to the above reasoning is that smokers have been reported to have a lower incidence of pre-eclampsia compared to non-smokers (Klonoff *et al.*, 1993; Marcoux *et al.*, 1989; Cnattingius *et al.*, 1997), although this has been vigorously disputed in more recent epidemiological findings (Ioka *et al.*, 2003). A possible explanation for this apparent inconsistency and claim for a protective effect of smoking against pre-eclampsia may be that nicotine (the most abundant alkaloid found in tobacco smoke) induces relaxation of smooth muscle by inducing increased release of nitric oxide, a potent vasodilator (Toda *et al.*, 1994). There also have been reports of decreased plasma hemoglobin concentrations in smokers during the second trimester of pregnancy as opposed to the increased hemoglobin concentration in women with pre-eclampsia (Huisman and Aarnoudse, 1986). This finding may be explained by an increased plasma volume in the former subjects. Another possible mechanism for smoke protecting against pre-eclampsia is the increase in prostacyclin metabolites in smokers as the pregnancy advances versus a decrease among non-smokers. The increase in prostacyclin in the case of smokers may lead to an improved thromboxane A₂/prostacyclin ratio (Davis *et al.*, 1987). Still another possible mechanism for the protective effect of cigarette smoking is that chronic smoking may desensitize the endothelium, whereby it reduces the acute endothelial response characteristic of pre-eclampsia.

2.2.6. Cigarette smoking and placental MAO-A

The human placenta contains essentially exclusively MAO-A activity. Therefore, it has the enzymatic mechanism to degrade serotonin (Figure 2.7) which is transported via the

serotonin transporter. Thus the most likely physiological function of placental MAO appears to be regulation of the concentrations of MAO substrates (such as serotonin) at reasonably low levels in the maternal and fetal circulations.

Since serotonin is known to be a potent vasoactive agent, the ability of the placenta to transport and degrade this compound may be one mechanism to maintain optimal blood circulation in the uteroplacental vascular bed (Ganapathy and Leibach, 1995). This maintenance would have direct relevance to the growth and development of the fetus because optimal blood circulation in the placenta would ensure an adequate supply of oxygen and nutrients to, and efficient removal of metabolic waste products from the fetus (Ganapathy and Leibach, 1995).

It is therefore important to determine the extent to which maternal smoking may alter the efficiency with which placental MAO-A clears serotonin.

We hypothesize that the inhibitors of MAO present in tobacco smoke may impair placental MAO-A activity *in vivo* (Figure 2.10). Inhibited placental MAO-A activity implies that adequate turnover of serotonin in the placenta will be compromised and therefore lead to higher maternal and fetal serotonin concentrations. Consequently it will be important to determine the extent to which smoking may alter the efficiency with which placental MAO-A clears this biogenic amine.

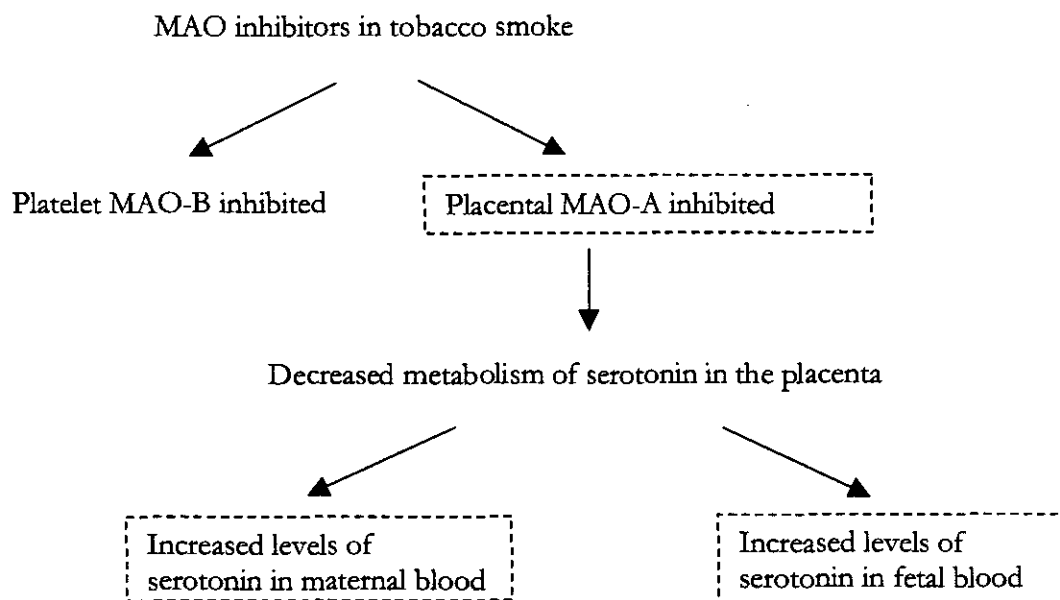


Figure 2.10. Hypothesis of the influence of cigarette smoke during pregnancy on placental MAO-A and the metabolism of serotonin. Dashed boxes indicate clinical signs of pre-eclampsia.

2.3. Background to methods

2.3.1. Methods for measuring MAO activity *in vitro*

Evaluation of the MAO inhibitory potency of extracts of cigarette smoke and tobacco leaf requires a robust assay for determining the activity of this enzyme. To date several methods have been documented in the literature. The most commonly used methods for the determination of MAO activity are based on spectrophotometric techniques (Houslay *et al.*, 1974; Kalgutkar *et al.*, 1994). Less frequently used assays use radiometric (Fuller *et al.*, 1970; Tipton, 1985; Da Prada *et al.*, 1989), fluorometric (Zhou *et al.*, 1996),

luminometric (O'Brien *et al.*, 1994) and ammonia detection (Meyerson *et al.*, 1978) methods.

Radiometric analyses are based on the detection of the labeled MAO-B catalyzed oxidation product after incubation with a radiolabeled substrate. For example Fuller *et al.* (1970) measured the rate of oxidation of ^3H -tyramine after selectively extracting the deaminated metabolite into an organic solvent. Radiometric assays, however, are limited to the availability of labeled compounds. Luminometric assays are based on measurement of light produced from the peroxidase-catalyzed chemiluminescent oxidation of luminol which is dependent on the amount of hydrogen peroxide produced in the MAO reaction (O'Brien *et al.*, 1994) (Figure 2.11). The advantage of this procedure is its sensitivity and that it is applicable to the oxidation of substrates that do not yield products that are readily detected. Methods dependent on hydrogen peroxide production however are very sensitive to the presence of even trace amounts of catalase.

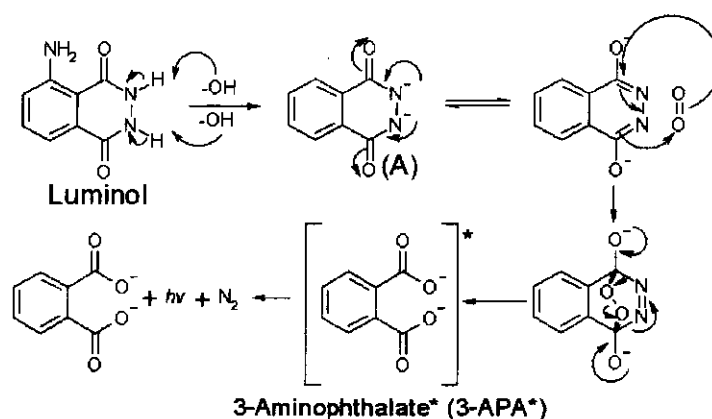


Figure 2.11. Luminol (3-Aminophthalic acid hydrazide) is oxidized in the presence of hydrogen peroxide and potassium hexacyanoferrate as catalyst. The reaction produces a bright yellow-green chemiluminescence.

Fluorometric detection is another very sensitive method for the determination of MAO activity. An extensively reported example of this procedure is the use of the non-selective MAO-A and MAO-B substrate kynuramine (4), which undergoes oxidative deamination, followed by the rapid intramolecular cyclization to form 4-hydroxyquinoline (5) (Figure 2.12). This compound is fluorescent and therefore readily measurable in the presence of the non-fluorescent substrate. Zhou *et al.* (1996) synthesized the fluorescent (*E*)-2,5-dimethoxycinnamylamine, an excellent MAO-B substrate, and found that the corresponding MAO-B catalyzed deaminated product is not fluorescent under assay conditions. This forms the basis for a direct, continuous assay for MAO-B used in their laboratory.

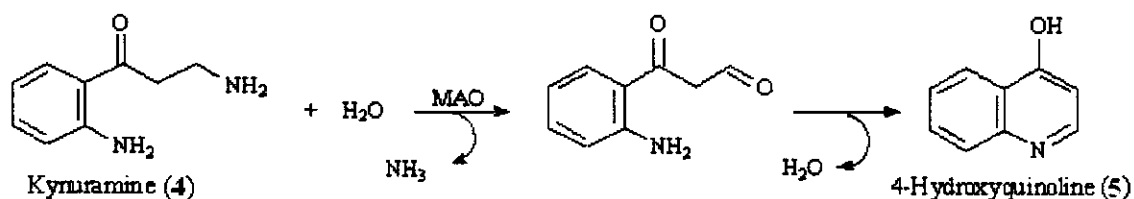


Figure 2.12. The MAO-catalyzed oxidation of kynuramine (4) followed by intramolecular cyclization to form 4-hydroxyquinoline (5).

Finally, assays based on the detection of ammonia are only rarely reported in the literature and are only applicable for detecting the oxidative deamination of primary amines (Meyerson *et al.*, 1978).

Because of their ease and speed of operation, many laboratories make use of spectrophotometric assays to measure MAO-B catalytic activity. Spectrophotometric detection of 4-hydroxyquinoline (5), the MAO-catalyzed oxidation product of

kynuramine (4), is a frequently reported example of this procedure (Figure 2.12.). 4-Hydroxyquinoline has a high extinction coefficient at 314 nm, a wavelength at which light absorption from the parent compound is negligible. This makes it possible to estimate the rate of MAO-catalyzed kynuramine oxidation spectrophotometrically (Gnerre *et al.*, 2000).

The discovery that MAO catalyzes the allylic ring α -carbon oxidation of tetrahydropyridinyl substrates forms the basis of the assay routinely used in our laboratory (Figure 2.13). The two electron oxidation products are dihydropyridinium species with excellent chromophoric properties that can be reliably measured by spectrophotometric techniques (Inoue *et al.*, 1999; Nimkar *et al.*, 1996). The first documented tetrahydropyridinyl substrate is the pro-neurotoxin MPTP (1) (Figure 2.13), which is an MAO-B selective substrate (Chiba *et al.*, 1984). As discussed earlier in this chapter, the neurotoxic properties of MPTP are dependent on its bioactivation by MAO-B. MAO-B catalyzes the oxidation of MPTP to the corresponding dihydropyridinium intermediate [MPDP⁺ (2); λ_{max} = 345 nm] which undergoes a second two-electron oxidation to generate the pyridinium species [MPP⁺ (3); λ_{max} = 295 nm]. To measure MAO-B catalytic activity quantitative estimations of both MPDP⁺ and MPP⁺ are required making it necessary to employ chromatographic techniques for accurate kinetic measurements (Castagnoli *et al.*, 1997). Because of its ease and speed of operation, most laboratories prefer spectrophotometry above chromatography, making MPTP a less suitable substrate for exploratory studies of MAO-B inhibition.

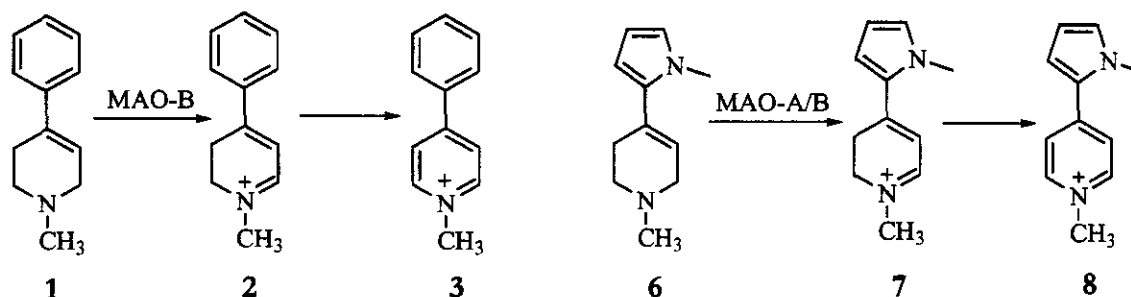


Figure 2.13. The MAO-catalyzed oxidation of the 1-methyl-4-aryl-1,2,3,6-tetrahydropyridinyl substrates MPTP (1) and MMTP (6).

It has been found that several other tetrahydropyridinyl species can serve as substrates for MAO. Of particular interest is 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine [MMTP (6)] which is both an MAO-A and -B substrate. MAO catalyzes the oxidation of MMTP to the corresponding dihydropyridinium species [MMDP⁺ (7); $\lambda_{\text{max}} = 420 \text{ nm}$] which, unlike MPDP⁺, is stable to further oxidation to the pyridinium species 8 (Figure 2.13). MMTP is, therefore, a superb substrate for screening both MAO-A and -B activity and is frequently used in our laboratory (Inoue *et al.*, 1999; Nimkar *et al.*, 1996).

2.3.2. Determination of substrate concentration to be used in inhibition studies

In order to know which concentration of substrate to use in the MAO assay it is important to determine the specific K_m value of the enzyme to be used with the substrate of choice. If the concentration of an enzyme substrate [S] is increased while all other conditions are kept constant, the initial velocity (V_i , the velocity when very little substrate has been consumed) of an enzymatic reaction increases to a maximum value, V_{max} . At this

point the enzyme is saturated with substrate and V_i is unaffected by further increases in substrate concentration. $V_{\max}$ is therefore the limiting maximum velocity of the reaction when all the enzyme is bound to substrate. The substrate concentration $[S]$ that produces half-maximal velocity ($V_{\max}/2$), termed the K_m value or Michaelis constant, is determined experimentally by graphing V_i vs. $[S]$ (Figure 2.14). The K_m value may estimate, with certain assumptions, a binding constant (K_d) for the enzyme-substrate complex. The affinity of an enzyme for its substrate is equal to the inverse of the K_d value. Therefore, a numerically small K_m indicates that the substrate has a high affinity for the enzyme (Stryer, 1988). The behavior of many enzymes under the influence of varied substrate concentrations is described by the Michaelis-Menten equation (Equation 2.1).

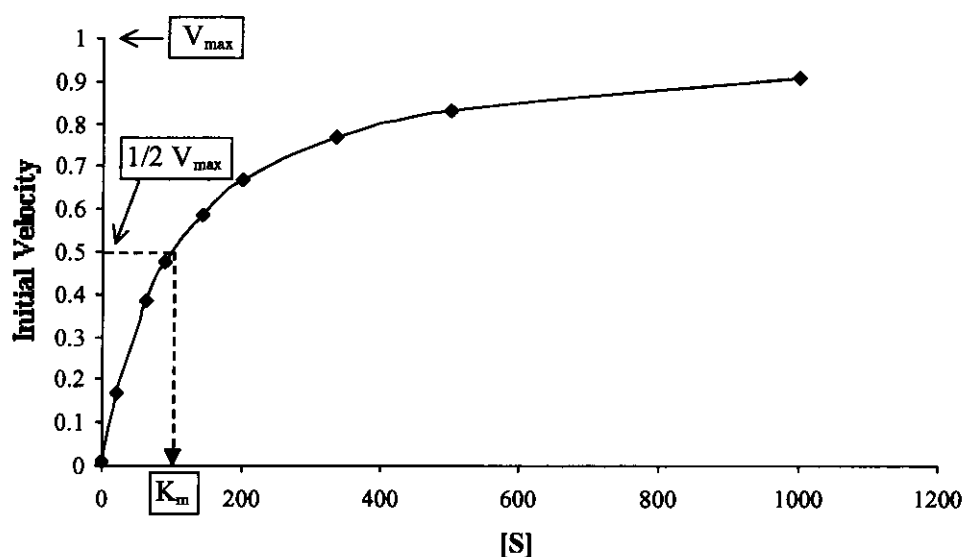


Figure 2.14. Graphical representation of the Michaelis-Menten equation (V_i versus $[S]$).

$$V_i = \frac{V_{\max} \times [S]}{K_m + [S]}$$

Equation 2.1.

Many enzymes give saturation curves that do not readily permit accurate measurement of $V_{\max}$ (and therefore K_m) when V_i is plotted vs $[S]$. By inversion of the Michaelis-Menten equation (Equation 2.2) and graphing the inverse of the initial velocity ($1/V_i$) as a function of the inverse of substrate concentration ($1/[S]$), a straight line is obtained. This plot is called a double reciprocal plot or Lineweaver-Burke plot (Figure 2.15). The K_m and $V_{\max}$ values can be easily obtained from this plot since the y-axis intercept is equal to $1/V_{\max}$ and the slope is $K_m/V_{\max}$. The x-axis intercept is equal to $-1/K_m$ (Stryer, 1988).

$$\frac{1}{V} = \left(\frac{K_m}{V_{\max}} \right) \times \left(\frac{1}{[S]} \right) + \frac{1}{V_{\max}}$$

Equation 2.2.

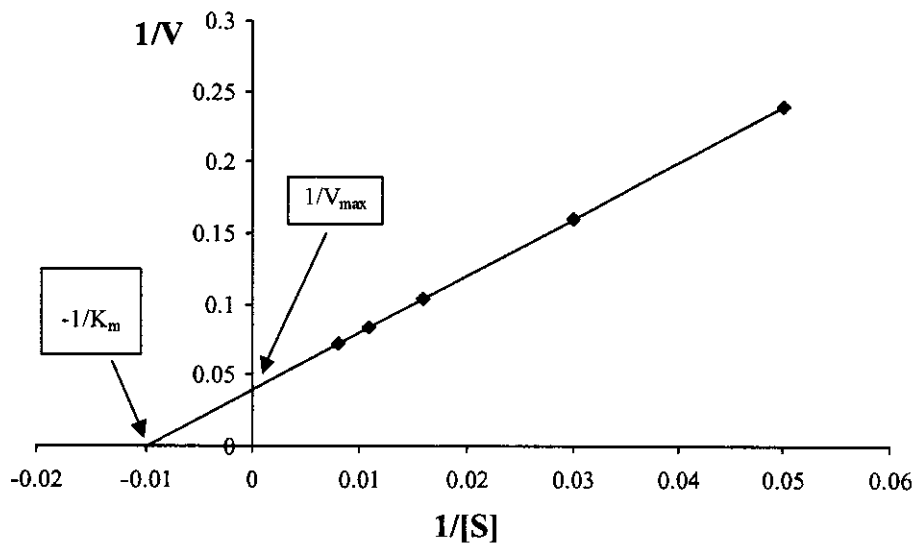


Figure 2.15. An example of the Lineweaver-Burke plot ($1/V_i$ versus $1/[S]$).

When $[S]$ is approximately equal to K_m , V_i is very responsive to changes in substrate concentrations and the presence of inhibitors. Consequently the determination of the K_m value under certain conditions (i.e. substrate used and source of enzyme used) is of

importance since K_m is the constant that relates the velocity of an enzyme catalyzed reaction to the substrate concentration. It also establishes an approximate value for the intracellular level of the substrate. The K_m value is constant for a given enzyme. Enzymes from different organisms or from different tissues in the same organism or from the same tissue at different stages of development will have different K_m values. Determination of the K_m therefore makes it possible to compare these enzymes (Stryer, 1988). Hence, when examining the effects of possible inhibitors in cigarette smoke extracts on MAO-B and MAO-A activity, substrate concentrations that bracket the reported apparent K_m value are used. The K_m and V_{max} values for baboon liver mitochondria with MMTP as substrate has previously been determined to be 60.9 μ M and 24.8 nmol product formed/mg prot/min respectively (Inoue *et al.*, 1999). However, the K_m and V_{max} values for human placental mitochondria in the presence of MMTP as substrate have not been published. This necessitated the determination of K_m and V_{max} values for human placental mitochondria with MMTP as substrate.

2.3.3. Platelet counting

Platelet counting is part of routine procedures used in hospitals and other clinical laboratories. Currently automated electronic instruments are available for platelet counting. For example the instrument used in this study, the Beckman Coulter® model Z2, uses the electrical sensing zone technique. This method of sizing and counting particles is based on measurable changes in electrical conductance produced by nonconductive particles suspended in an electrolyte. This produces a current pulse of short duration that has a magnitude proportional to size. A small opening (aperture) between electrodes is the sensing zone through which suspended particles pass (see

Figure 2.16). In the sensing zone each particle displaces its own volume of electrolyte. Volume displaced is measured as a voltage pulse; the height of each pulse being proportional to the volume of the particle. The quantity of suspension drawn through the aperture is precisely controlled to allow the system to count and size particles for an exact reproducible volume. This method is independent of particle shape, color and density.

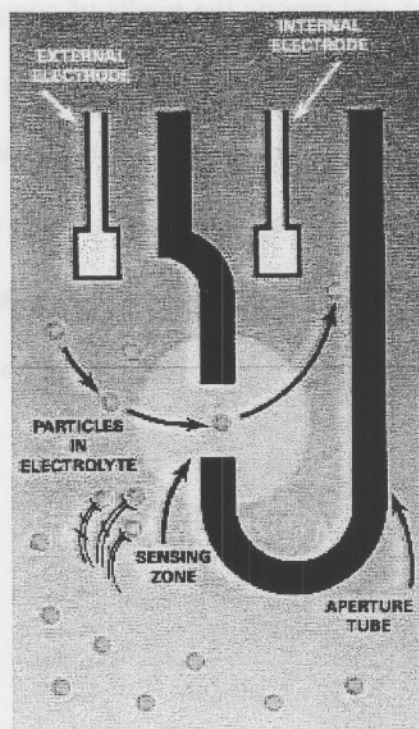


Figure 2.16. An illustration of the principle used for platelet counting in the electrical sensing zone technique (http://scooter.cyto.purdue.edu/pucl_cd/flow/vol3/7/coulter/ss000103.htm.)

2.4. Conclusion

This chapter provided a review of the literature regarding the influence of cigarette smoking on MAO. Furthermore, the impact of smoking on maternal biochemical

parameters related to MAO inhibition was discussed in view of the current state of knowledge. Certain preliminary hypotheses derived from these earlier findings will form the bases of specific aims for the remainder of the study. These will be outlined in 2.5. Chapter 3 reports on the influence of tobacco smoke and tobacco smoke constituents on MAO activity in *in vitro* studies. The results of these studies and the impact of these findings are discussed in detail. Chapter 4 proceeds to further expand on these *in vitro* findings and their significance, and reports the results of comprehensive studies in two patient cohorts that represent non-smoking, and smoking pregnant women. The influence of cigarette smoking during pregnancy on placental MAO-A activity and other biochemical parameters in maternal and umbilical cord blood is detailed and discussed. In Chapter 5, concluding remarks on the significance of the findings reported in Chapters 3 and 4, attempt to outline the significance of the studies presented in this thesis.

2.5. Aims of the study

2.5.1. Research goals (part I)

In view of the reported literature on the influence of tobacco smoke constituents on MAO activity *in vitro* we propose to further investigate what influence pyrolysis may have on MAO inhibition. Our hypothesis to address this investigation makes a threefold statement:

- A. the inhibition of MAO by extracts of tobacco leaves (i.e. not subjected to pyrolysis through burning) will be less than the inhibition observed with cigarette smoke extracts.

- B. smoke extracts from non-tobacco cigarettes will be subject to the same pyrolysis conditions as regular cigarettes when smoked and may therefore also inhibit MAO;
- C. smoke extracts from cigarettes with reportedly lower yields of pyrolysis products ("heating" versus "burning" of tobacco at lower temperatures) may yield smoke extracts that are less active as MAO inhibitors than extracts obtained from "regular" cigarettes;

2.5.2. Research goals (part II)

A study of the literature concerning the influence of cigarette smoking during pregnancy on placental MAO-A, has lead us to compile the following hypothesis:

- A. since cigarette smoking inhibits platelet MAO-B activity in males and non-pregnant females, it will also inhibit maternal platelet MAO-B activity in pregnant females;
- B. cigarette smoking during pregnancy should then also inhibit placental MAO-A activity;
- C. inhibition of placental MAO-A activity which is responsible for the metabolism of serotonin, will lead to higher serotonin levels in maternal and fetal blood as seen in the case of pre-eclampsia;

Chapter 3

THE INFLUENCE OF TOBACCO SMOKE CONSTITUENTS ON MAO ACTIVITY IN VITRO

3.1. The inhibition of MAO by tobacco leaf extracts versus cigarette smoke extracts

3.1.1. Introduction

Inhibitors of MAO have already been found in hexane extracts of tobacco leaves (Kahlil *et al.*, 2000; Castagnoli *et al.*, 2002). It has also been shown that tobacco smoke contains inhibitors of MAO (Essman, 1977; Orelan *et al.*, 1981, Norman *et al.*, 1982; Yu and Boulton, 1987; Berlin *et al.*, 1995b; Fowler *et al.*, 1996a;b; Hauptmann and Shih, 2001). Yu & Boulton (1987) have done preliminary experiments comparing the inhibition of smoke extracts to leaf extracts using 0.1 M sodium phosphate buffer (pH 7.4) as solvent.

We speculate (see hypothesis, chapter 1, p. 9) that cigarette smoke extracts will have a higher potency in inhibiting MAO than tobacco leaf extracts since the process of burning tobacco likely results in many reactions that occur due to the severe pyrolytic conditions encountered in a burning cigarette. These conditions are amenable to the formation of numerous pyrolysis products that would not be present in leaf extract (Baker, 1987). In order to investigate this hypothesis we propose to determine the inhibitory potency of tobacco leaf extracted in acetone and compare that with acetone extracts of cigarette

smoke. Using acetone as solvent, non-polar compounds will also be extracted from the cigarette smoke while an aqueous buffer will mostly extract polar compounds.

Cigarette smoke consists of mainstream and sidestream smoke (Seeman *et al.*, 2003). Mainstream smoke is generated during puff-drawing in the burning cone and hot zones of a burning cigarette or cigar. Sidestream smoke is that fraction of smoke emitted freely from the smoldering tobacco product into the air (Nelson, 2001). Although both sidestream and mainstream smoke have been shown to possess compounds that may be involved in human health after passive or active inhalation (Nelson, 2001), our studies will focus on the effects of mainstream smoke.

3.1.2. Experimental procedure

3.1.2.1. Materials and instrumentation

Chemicals and reagents, unless otherwise stated, were purchased from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade. Water was obtained from a Water Milli-Q UV Plus system (Millipore, Bedford, MA). Acetone (HPLC grade), the solvent for smoke and leaf extracts, was purchased from Honeywell International Inc. Burdick and Jackson, (Muskegon, MI). The substrate for the MAO activity assay, MMTP•HCl, was synthesized according to the method of Nimkar and co-workers (1996). NaH_2PO_4 , Na_2HPO_4 and glycerol were purchased from Fisher Scientific Products (Fair Lawn, NJ). Marlboro® filter cigarettes were kindly provided by Philip Morris Inc. Non-tobacco Magic® cigarettes were purchased from a local tobacco store. UV data were obtained using a Beckman DU-7400 spectrophotometer.

3.1.2.2. Preparation of leaf extracts

In order to compare the MAO inhibitory potency of tobacco leaf extracts (TLE) and cigarette smoke extract (CSE), packed dry tobacco leaves and cigarette smoke, both from Marlboro® cigarettes, were extracted. The tobacco leaves of 2 Marlboro® cigarettes - each containing 15 mg tar and 1.2 mg nicotine (www.philipmorrisusa.com) - were crushed and then stirred for 40 min at room temperature in 40 mL acetone. The mixture was filtered through glass wool, the filtrate transferred to a round bottom flask and evaporated to dryness using a rotary evaporator (manufactured by Büchi LaborTechnik AG, Switzerland). The residue was subsequently dissolved in a small volume of HPLC grade acetone and the resulting solution was filtered through glass wool and then transferred to a pre-weighed glass vial. The sample was again evaporated to dryness and weighed. The extract was stored at 4 °C until tested in the MAO assay which was usually performed on the next day.

3.1.2.3. Collection of cigarette smoke with an in-house designed smoking device

Cigarette smoke was extracted according to the protocol described by Yu and Boulton (1987) on an in-house designed smoking device. The smoke was bubble dispersed in acetone through a fine frit as illustrated in Figure 3.1. Individual Marlboro® cigarettes were puffed every thirty seconds for a duration of 3 seconds. The smoke was bubble dispersed through 35 mL acetone, using a 50 mL syringe to develop a slight vacuum. The acetone was then transferred to a round bottom flask and was evaporated to dryness using a rotary evaporator. The residue was subsequently dissolved in a small volume of acetone and the resulting solution was filtered through glass wool and then transferred to

a pre-weighed glass vial. The sample was again evaporated to dryness. After weighing the sample, the extract was stored at 4 °C until tested in the MAO assay, which was usually performed on the next day.

Cigarette smoke was also collected in 0.1 M sodium phosphate buffer (pH 7.4) and hexane as solvent according to the same protocol. In the case of the sodium phosphate buffer the solvent was not evaporated, instead the entire solution was used in the MAO assay as described below.

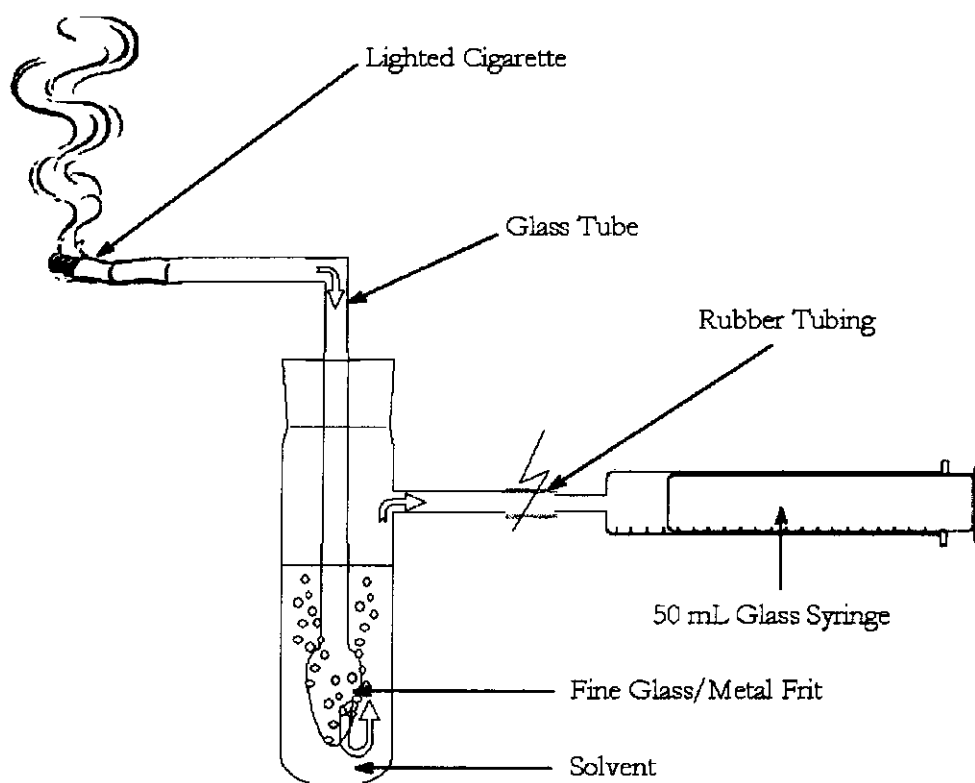


Figure 3.1. In house designed smoking device for the collection of cigarette smoke.

Smoke is bubble dispersed through the solvent by using a fine frit.

3.1.2.4. Control samples for smoke collected on the in-house designed smoking device

The control samples were prepared as described for the samples above with the exception that the cigarette was not lighted. Thus a cigarette would be “puffed” according to the above protocol without lighting the cigarette. The acetone was then evaporated and filtered as described above and the final “residue” dissolved in DMSO and tested in the MAO assay. The control sample did not show inhibition of MAO.

3.1.2.5. Preparation of mitochondrial fractions

Protocols for obtaining human placenta were approved by the Internal Review Boards of Montgomery County Hospital and Virginia Tech. The collection of baboon liver (*Papio ursinus*) was approved by the Animal Care Committees of Virginia Tech, Blacksburg, VA, USA and Potchefstroom University for CHE, Potchefstroom, South Africa. The preparation of the mitochondrial fractions followed literature procedures (Salach and Weyler, 1987). The homogenates were prepared from individual human placenta and baboon livers. These preparations were stored in aliquots of 100 – 200 μ L at -70°C. A previous study showed that these preparations are stable for 12 months when stored under these conditions (Inoue *et al.*, 1999). The aliquoted samples were thawed as needed and mixed with glycerol-containing buffer [50 mM sodium phosphate buffer, pH 7.4, containing 50% (w/v) glycerol] for the initial protein concentration determination. All protein concentrations were determined according to the Coomassie Brilliant Blue dye binding method of Bradford (1976) with bovine serum albumin used as standard. For the assays, aliquoted samples as described above were further diluted using 50 mM sodium phosphate buffer, pH 7.4, to a concentration appropriate for the experiment.

3.1.2.6. K_m and V_{max} determinations of human placental mitochondria with MMTP as substrate

In order to determine the K_m and V_{max} of human placental mitochondria with MMTP as substrate, MMTP was dissolved in 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate buffer was prepared with Na_2HPO_4 and NaH_2PO_4 . 50 μL of the MMTP solution was added to 350 μL of a 0.1 M sodium phosphate buffer, pH 7.4 to give final concentrations of 20, 40, 80 and 160 μM . These four concentration samples were prepared in duplicate. The samples were preincubated in a shaking water bath at 37 °C for 15 min. Following the preincubation, 25 μL human placental mitochondrial homogenate (final protein concentration = 0.15 mg/mL) was added to the vial. Preparation of the mitochondrial homogenate is described above. The samples were incubated for 15 min at 37 °C after which the reactions were terminated by the addition of 20 μL 70% HClO_4 . The samples were vortexed and the denatured protein removed by centrifugation at $16,000 \times g$ for 5 minutes. The absorption of the resulting supernatants containing the product, MMDP^+ , was read at 420 nm on the spectrophotometer.

The rate of this reaction was determined by equation 3.1. Thus under our experimental conditions: $V = \text{Absorption}/24000 \times 0.5/0.025 \times 1/3 \times 10^6 \times 1/15\text{min}$.

$$V = \frac{\text{Abs}}{\epsilon} \times \frac{\text{total assay volume}}{\text{enzyme volume}} \times \frac{1}{\text{initial enzyme conc.}} \times 10^6 \times \frac{1}{\text{incubation time}}$$

Equation 3.1.

The reciprocal of the V (rate) values was then plotted against the reciprocal of the substrate values to create a Lineweaver-Burke plot. The resulting line was then extrapolated so that it intercepts the x-axis, thus: x-intercept = $-1/K_m$ and the y-intercept = $1/V_{max}$ (see Figure 2.14).

3.1.2.7. Assays to determine the IC_{50} values for the inhibition of MAO-A and MAO-B by cigarette smoke

The activity of MAO-A and MAO-B in the presence of CSEs and TLEs were determined. The TLE and CSE were dissolved in DMSO to yield concentrations ranging from 25 $\mu\text{g/mL}$ to 25 mg/mL . Baboon liver mitochondrial homogenates which contain only MAO-B activity (Inoue *et al.*, 1999) were used as the enzyme source when determining MAO-B activity. Human placental mitochondrial homogenates which contain exclusively MAO-A activity (Riley *et al.*, 1989) were used as the enzyme source when determining MAO-A activity. See paragraph 4.1.2.5. for the preparation of these mitochondrial homogenates. MMTP was used as substrate (Nimkar *et al.*, 1996; Inoue *et al.*, 1999). The activity of either MAO-A or MAO-B enzyme was measured in the presence of tobacco leaf or smoke condensates as follows: mixtures (500 μL , final volume) consisting of 355 μL 0.1 M sodium phosphate buffer pH 7.4, 100 μL 0.25 mM MMTP [50 μM (K_m) final concentration] in 0.1 M sodium phosphate buffer pH 7.4, and 20 μL of Marlboro®, Magic®, Accord® or Parliament® smoke or leaf extracts were prepared. The Marlboro®, Magic®, Accord® or Parliament® smoke or leaf extracts were prepared in DMSO in varying concentrations ranging from 25 $\mu\text{g/mL}$ to 25 mg/mL . When added to the buffer and substrate mixtures this protocol resulted in final

concentrations of 1, 3, 10, 30, 100, 300, 600 and 1000 $\mu\text{g/mL}$ smoke or leaf extracts and no more than 4% (v/v) DMSO in the assay mixture. This mixture of buffer, substrate and smoke/leaf extract was preincubated with gentle agitation in a water bath at 37 °C for 15 min. For every experiment a control was included which contained 4% (v/v) DMSO but no inhibitor. DMSO concentrations higher than 4% were found to negatively affect MAO-B activity (Gnerre *et al.*, 2000). Following the preincubation, 25 μL baboon liver mitochondrial homogenate (for the MAO-B determinations) or human placenta mitochondrial homogenate (for MAO-A determinations) (final protein concentration = 0.15 mg/mL) was added to the vial and the sample was incubated for 15 min at 37 °C. The increase in the concentration of the dihydropyridinium metabolite (MMDP^+) with time is reported to be linear for at least 15 minutes after initiation of the reaction (Inoue *et al.*, 1999). The reactions were quenched by the addition of 20 μL of 70% perchloric acid. The samples were vortexed and the denatured protein removed by centrifugation at $16,000 \times g$ for 8 minutes. The resulting supernatants contained the MAO-B and MAO-A catalyzed product [MMDP^+ (7)] of MMTP (6). The absorbance of the tetrahydropyridine metabolite [MMDP^+ (7)] was measured on the spectrophotometer at a wavelength of 420 nm [$\epsilon = 24,000 \text{ M}^{-1} \text{ cm}^{-1}$, (Nimkar *et al.*, 1996)]. Inhibition of the MAO-A/B catalytic activity would consequently lead to less absorbance of the tetrahydropyridine metabolite.

3.1.2.8. Determination of IC_{50} values

The absorbance of MMDP⁺ (reflecting MAO-A/B catalytic activity), measured in the presence of different concentrations of TLE and CSE (as determined above), was expressed as a percentage of the control absorbance, i.e. the absorbance of MMDP⁺ without the presence of an inhibitor. This percentage of MAO-A or MAO-B activity was plotted against the log of the inhibitor concentrations and fitted as sigmoidal dose response curves with GraphPad Prism™ software (see Figures 3.2 and 3.3). Curves were compared for inhibitory potency as IC_{50} values. The 95% confidence intervals of the IC_{50} values were then fitted to a standard sigmoidal four parameter logistic equation. The IC_{50} value is defined as the concentration at which the extract-solution will cause 50% inhibition of the enzyme activity.

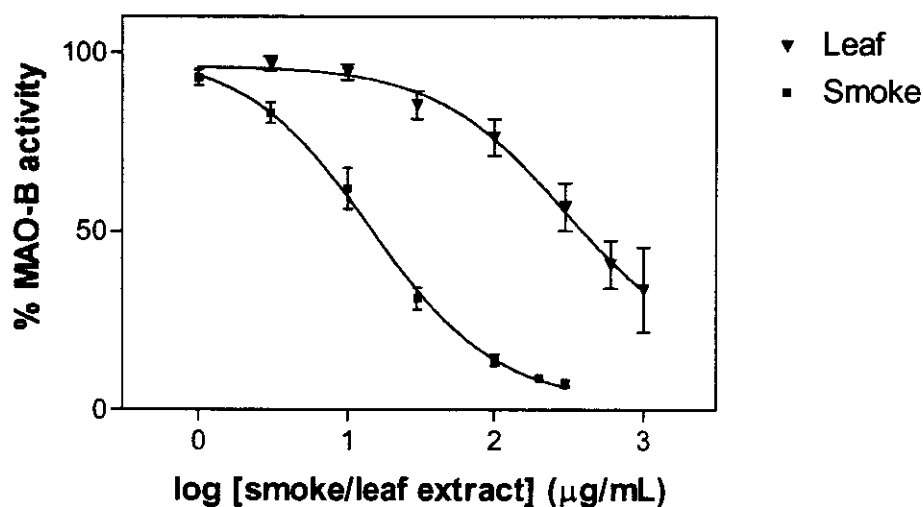


Figure 3.2. MAO-B inhibition by Marlboro® cigarette smoke and tobacco leaf extracts.

Each data point represents the mean ($\pm$ S.E.M.) of triplicate replications with three determinations in each replicate. Lack of error bars indicates that the S.E.M. resides within the size of the symbol.

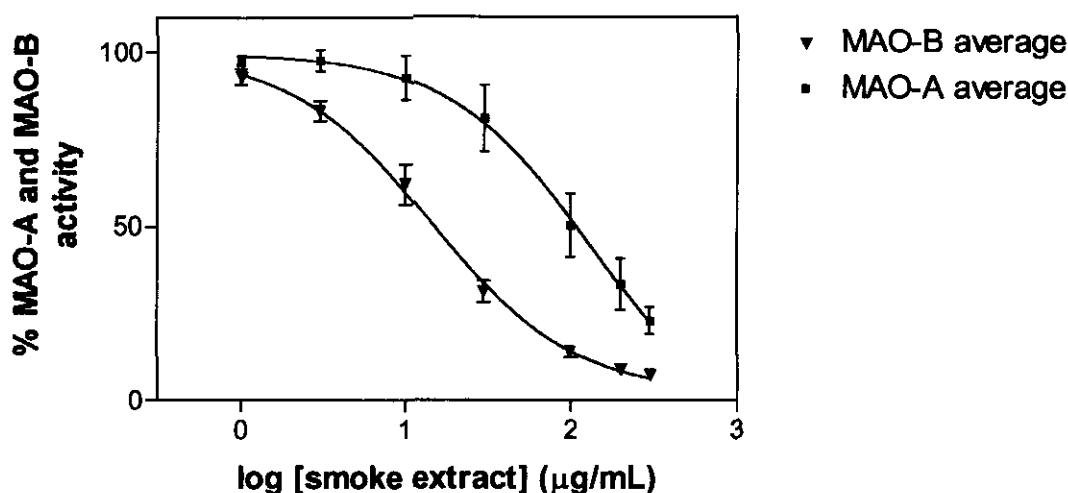


Figure 3.3. MAO-A and MAO-B activity of three replicate experiments after incubation with Marlboro® cigarette smoke extract. Each data point represents the mean ($\pm$ S.E.M.) of triplicate replications with three determinations in each replicate. Lack of error bars indicates that the S.E.M. resides within the size of the symbol.

3.1.2.9. Measurement of MAO-B activity in the presence of cigarette smoke extracted with different solvents

CSEs collected in 0.1 M sodium phosphate buffer, hexane and acetone were tested for their inhibitory potency on MAO-B. The hexane and acetone extracts were dissolved in DMSO to yield concentrations of 1000 $\mu\text{g/mL}$ and 750 $\mu\text{g/mL}$ respectively. For the extractions into 0.1 M sodium phosphate buffer (pH 7.4) the smoke of eleven cigarettes was trapped in 33 mL of the buffer which was stored at $-20\text{ }^{\circ}\text{C}$ prior to analysis. The buffer was not evaporated and this mixture was used directly in the MAO-B assay. The samples were tested in the MAO-B inhibition assay as follows: for testing the hexane and acetone extracts, mixtures (500 μL) containing the following were prepared: 355 μL 0.1 M sodium phosphate buffer pH 7.4; 100 μL MMTP (50 μM final concentration)

dissolved in 0.1 M sodium phosphate buffer pH 7.4 and 20 μ L of either hexane or acetone smoke extract (respectively 40 and 30 μ g/mL final concentration) in DMSO (4% v/v final concentration). In the case of the smoke extract in buffer, the mixture contained 375 μ L of smoke extract and 100 μ L MMTP (50 μ M final concentration) dissolved in 0.1 M sodium phosphate buffer pH 7.4. The mixtures were preincubated with gentle agitation in a water bath at 37 °C for 15 min. Following the preincubation, 25 μ L baboon liver mitochondrial homogenate (final protein concentration = 0.15 mg/mL) was added to the mixtures and the samples incubated for 15 min at 37 °C after which the reaction was terminated by the addition of 20 μ L perchloric acid. The samples were vortexed and the denatured protein removed by centrifugation at $16,000 \times g$ for 5 min. The absorption of the resulting supernatants containing the product MMDP⁺ was read at 420 nm on the spectrophotometer.

3.1.3. Results and discussion

3.1.3.1. Determination of an appropriate solvent for smoke collection

Previous investigators determining the inhibitory potency of cigarette smoke and leaf extracts on MAO used either aqueous buffer (Yu and Boulton, 1987) or hexane (Khalil *et al.*, 2000) as solvent for extraction. After collecting Marlboro® cigarette smoke in different solvents such as 0.1 M sodium phosphate buffer (pH 7.4), hexane and acetone and testing these extracts for MAO inhibition, it was evident that the acetone extracts showed considerably more inhibition of MAO-B than the aqueous buffer extract and slightly more inhibition than hexane extract (Figure 3.4, investigative data, no statistics

performed) of cigarette smoke. It was therefore decided to do al subsequent extractions with acetone.

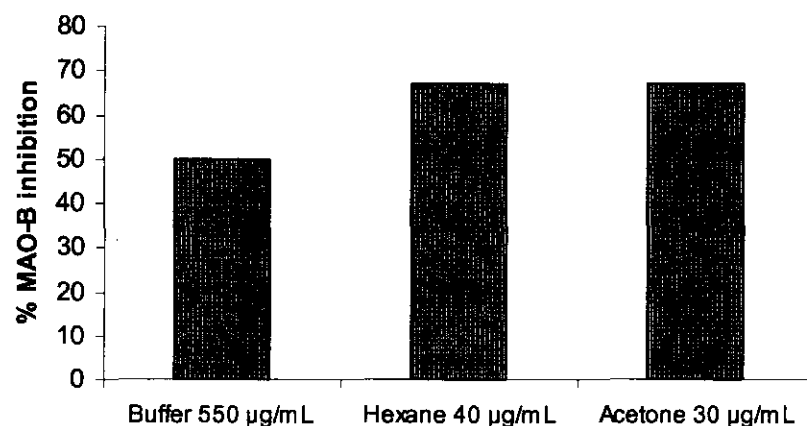


Figure 3.4. Comparing the MAO-B inhibition obtained with different solvents for smoke extraction.

3.1.3.2. Determination of K_m and V_{max} for human placental mitochondria with MMTP as substrate

The importance of working at K_m concentrations of the substrate for inhibition studies have been discussed earlier (paragraph [2.13.2.](#)) Figure 3.5 shows the Lineweaver-Burke plot obtained from human placental mitochondria with MMTP as substrate. From this figure the K_m value (x-intercept = $-1/K_m$) is calculated as $51.72 \pm 2.72 \mu\text{M}$ (CV = 5.3) and the V_{max} value (y-intercept = $1/V_{max}$) is calculated to be $18.35 \pm 0.75 \text{ nmol/mg protein/min}$ (CV = 4). The K_m and V_{max} values determined for the human placental mitochondria with MMTP as substrate is close to the reported values of baboon liver mitochondria with the same substrate ($K_m = 60.9 \mu\text{M}$, $V_{max} = 24.8 \text{ nmol product formed/mg protein/min}$). Subsequently, MAO-A assays with human placental

mitochondria determining the inhibitory activity of cigarette smoke and leaf extracts, were conducted with 50 μM of protein. This is the same protein concentration used when baboon liver mitochondria are used as protein source.

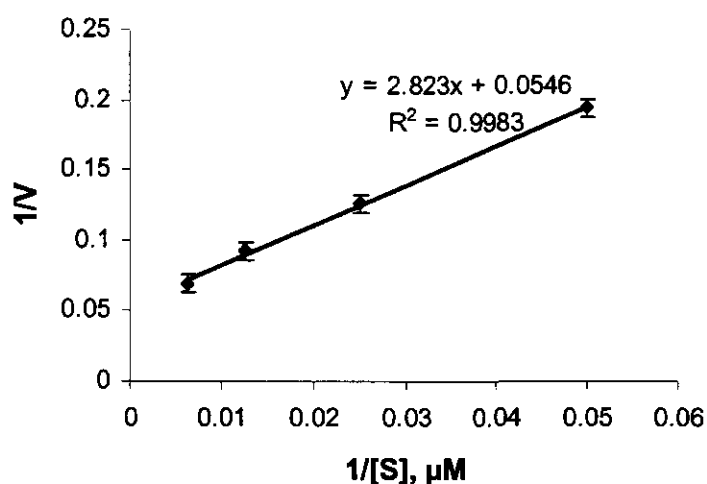


Figure 3.5. Lineweaver-Burke plot for the determination of the K_m and V_{max} of human placental mitochondria with MMTP (6) as substrate. Each data point represents the mean [$\pm$ standard deviation (SD)] of duplicate replications.

3.1.3.3. The inhibition of MAO by tobacco leaf extracts versus cigarette smoke extracts

The IC₅₀ values determined for TLE and CSE samples are given in Table 3.1.

Table 3.1. The IC₅₀ values for MAO-A and -B inhibition determined for Marlboro® tobacco smoke and leaf extracts on MAO-A and MAO-B enzyme preparations.

Sample	MAO-A		MAO-B	
	IC ₅₀ , µg/mL	95% C.L. ^a	IC ₅₀ , µg/mL	95% C.L. ^a
Marlboro®				
Smoke ^b	133	70-252	14	12-18
Leaf ^c	n.d.		301	162-560

Smoke and leaf samples were prepared in triplicate. The results are the average of three assays.

^a 95% C.L. = 95% confidence limits.

^b An average of 9 puffs per cigarette generated the smoke and the total particulate matter (TPM) collected was 14.7 ± 3.5 mg.

^c The leaf extract had a TPM of 11.7 ± 3.3 mg.

n.d. = not determined due to color interference caused by sample color at high concentrations.

It is clear that the burning process of tobacco leads to an increase in the inhibitory potency of the tobacco extract. The inhibitory potency of the smoke extract on MAO-B is more than 21 times higher than that of the leaf extract (Table 3.1). The higher inhibition seen with the CSE compared to the TLE may be due to pyrolysis products formed during the process of burning tobacco.

The smoke extract showed significantly more inhibition of MAO-B (89%) compared to MAO-A (Table 3.1). In the case of the leaf extracts it was not possible to prepare a high enough concentration in DMSO to achieve 50% inhibition of MAO-A since high concentrations of the DMSO solution interfered with the spectrophotometric

determinations due to the dark color of the solution. The highest concentration of Marlboro® leaf extract tested was 1 mg/mL and it gave approximately 10% inhibition of MAO-A (thus 90% of residual enzyme activity remains in the presence of the tobacco leaf extract). The leaf extracts also showed less inhibition of MAO-A compared to MAO-B (301 µg/mL gave 50% inhibition). These results are consistent with the findings of Fowler and co-workers (1996a,b) that reported 40% MAO-B inhibition in the brains of smokers compared to only 28% inhibition of MAO-A. It is however contradictory to the results of Yu and Boulton (1987) and Pavlin and Sket (1993) who reported that MAO-A activity was slightly more affected by cigarette smoke condensate (CSC) than MAO-B activity *in vitro*. The reason for this difference in MAO-A and MAO-B inhibition is not clear. However, the different solvents used by different authors to collect the cigarette smoke might have had an influence. Yu and Boulton used an aqueous buffer for the collection of cigarette smoke while we used acetone. An aqueous buffer will collect more polar compounds whereas acetone will collect polar and non-polar compounds. A study done by Khalil and co-workers (2000) revealed that the inhibitory components of tobacco leaf extracts are found in the non-polar solvents and that these inhibitors could not be extracted into water, thus indicating that the inhibitors are lipophilic in nature.

3.2. Comparing the inhibition of smoke and leaf extracts of non-tobacco cigarettes with “regular” cigarettes

3.2.1. Introduction

In order to further investigate the influence of possible pyrolysis products the "leaf" ("leaf" in the context used here denotes the unburnt dry contents of the cigarette) and

smoke extracts of one brand of non-tobacco cigarettes (Magic®) were tested for their inhibition of MAO-A and MAO-B. Magic® cigarettes contain a blend of herbs that can be burnt and smoked. These were listed on the packaging as: marshmallow, yerba santa, damiana, passion flower, jasmine and ginseng as well as flavoring agents. Importantly, they do not contain any tobacco. When smoking these cigarettes, the herbs and flavoring agents will be subject to similar processes as burning tobacco. We hypothesize that the burning of most plant material may lead to pyrolysis products capable of inhibiting MAO and therefore that the smoke from cigarettes that do not contain any tobacco may also inhibit MAO. The purpose of this experiment was to compare the inhibitory properties of smoke extracts from non-tobacco cigarettes with the smoke of Marlboro® cigarettes.

3.2.2. Experimental procedure

3.2.2.1. Preparation of cigarette smoke and tobacco leaf extracts

Smoke and leaf extracts of the Magic® (non-tobacco) cigarettes were prepared in the same way as described for the Marlboro® cigarettes [described in the previous section, 3.1.2.2. (leaf extracts); 3.1.2.3. (smoke extracts)].

3.2.2.2. Assay for MAO inhibition

The extracts of Magic® cigarette leaves and smoke were assayed for MAO inhibition as described previously (see section 3.1.2.6.). The curves were drawn with GraphPad Prism™ software and the algorithms available in that software package were used to determine the IC₅₀ values are shown in Figures 3.6 and 3.7.

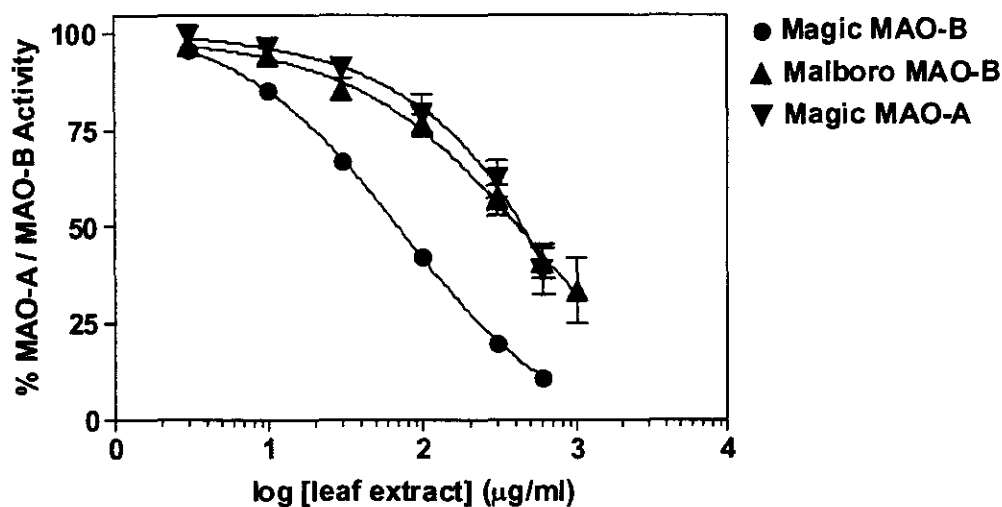


Figure 3.6. MAO-B and MAO-A activity of three replicate experiments after incubation with Magic® and Marlboro® leaf extracts. Each data point represents the mean ($\pm$ S.E.M.) of triplicate replications with three determinations in each replicate. Lack of error bars indicates that the S.E.M. resides within the size of the symbol.

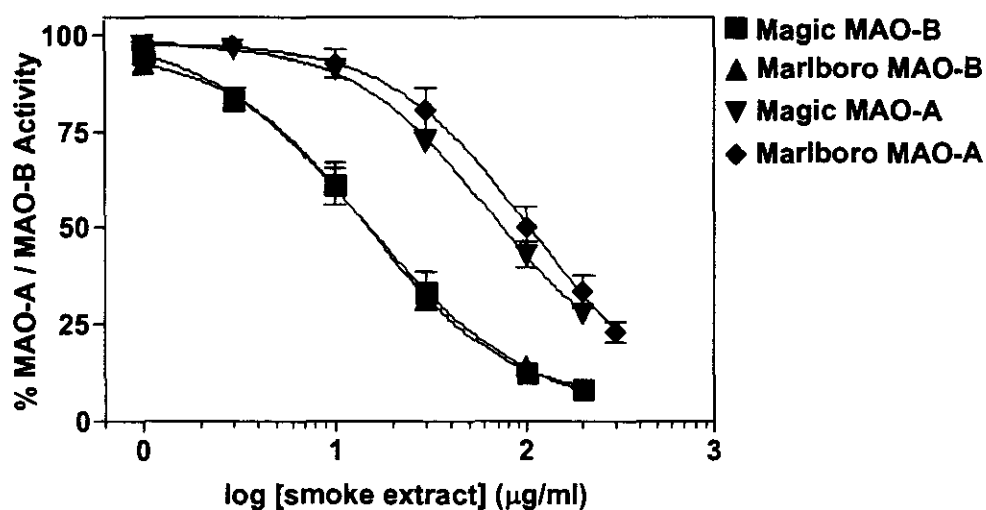


Figure 3.7. MAO-B and MAO-A activity of three replicate experiments after incubation with Magic® and Marlboro® smoke extracts. Each data point represents the mean ($\pm$ S.E.M.) of triplicate replications with three determinations in each replicate. Lack of error bars indicates that the S.E.M. resides within the size of the symbol.

3.2.3. Results and discussion

The IC₅₀ values determined for leaf and smoke extracts of Magic® and Marlboro® cigarettes are given in Table 3.2.

Table 3.2. The inhibition of MAO-A and MAO-B by acetone extracts obtained from of the smoke and "leaf" of Marlboro® and Magic® cigarettes.

Sample	MAO-A		MAO-B	
	IC ₅₀ , µg/mL	95% C.L.*	IC ₅₀ , µg/mL	95% C.L.*
Magic				
Smoke	83	59-115	14	10-21
Leaf	1214	667-2208	64	56-73
Marlboro				
Smoke	133	70-252	14	12-18
Leaf	n.d.		301	162-560

Preparation of the samples and subsequent assays were replicated three times with three determinations in each replicate. The Magic® and Marlboro® cigarettes were puffed an average of 9 times per cigarette.

* 95% C.L. = 95% confidence limits.

n.d. = not determined due to color interference caused by sample color at high concentrations.

Table 3.3. Weight of the residues extracted from the leaves and smoke of Marlboro® and Magic® cigarettes.

Sample	Smoke mg TPM ^a /cigarette	Leaf mg TPM ^a /cigarette
Magic®	16.0 ±2.6	8.7 ±1.0
Marlboro®	14.7 ±3.5	11.7 ±3.3

^aTotal particulate matter.

Despite the fact that Marlboro® and Magic® cigarettes differ widely in their contents, our results clearly show that extracts of Magic cigarette smoke are as potent as Marlboro

smoke extracts in inhibiting MAO (see Table 3.2), also, the two types of cigarettes create similar amounts of total particulate matter (see Table 3.3). Marlboro® cigarettes contain only tobacco whereas Magic® cigarettes contain no tobacco but a mixture of herbs and flavoring agents. Both of these types of cigarettes however, are smoked by the same method i.e. lighting a cigarette and taking a drag of the resulting smoke. Therefore the two types of cigarettes will be subject to the same combustion processes. Our results clearly show that the pyrolysis products thus formed have the same potency for inhibiting MAO whether it is tobacco or other plant materials being smoked.

The possibility of volatile compounds being responsible for the inhibition of MAO is ruled out by our method of obtaining the smoke extract, i.e. the smoke is bubbled through acetone which is then evaporated on a rotary evaporator. Any volatiles present would be evaporated by this process and would therefore not have an influence on MAO inhibition.

Interestingly, even though the smoke extracts of the Marlboro® and Magic® cigarettes showed a similar potency for inhibiting MAO, the leaf extracts of these cigarettes differ significantly in their inhibition of MAO-B. The Magic® leaf extracts have IC_{50} values four times smaller than that of the Marlboro® leaf extracts (Table 3.2) for MAO-B. This suggests that the compounds found in Magic® leaf extracts are more potent MAO inhibitors than the compounds found in tobacco leaf extracts. However, this result also supports the fact that formation of pyrolysis products plays an important role in the inhibition of MAO in these assays. Despite the significant differences found in the MAO-B inhibition assay between the leaf extracts of Marlboro® and Magic® cigarettes, the smoke extracts (containing the pyrolysis products) do not show a comparable significant

difference in inhibiting MAO-B, but are substantially more potent inhibitors in both cases, compared with the respective leaf extracts. This also suggests that the MAO inhibiting effect elicited by the pyrolysis products overshadows that elicited by the natural "unpyrolyzed" products extracted from the leaves of Magic® cigarettes.

The significant difference in MAO-B inhibition exerted by the leaf extracts of these two cigarette types also indicates that the herbs or flavoring agents used in the Magic® cigarette has a higher potency for inhibiting MAO-B. A study done by Bhattacharya and co-workers (2000) indicates that ginseng lowers stress induced MAO activity. No research has thus far been reported on the MAO inhibition properties of the other herbs and flavoring agents contained in the Magic® cigarettes. It is therefore unclear whether these herbs or perhaps the flavoring agents could be responsible for the higher inhibition of MAO-B seen with the extracts of Magic® leaves.

Control samples were prepared using the same method for preparation as the smoke samples, except that the cigarette was not lighted. These showed no inhibition of MAO. This finding confirms that the inhibition seen with the different samples is exclusively due to components extracted from the tobacco leaf or smoke, and not to due to the effect of solvent.

3.3. Comparing the MAO inhibition of cigarettes that primarily heat tobacco to cigarettes that primarily burn tobacco

3.3.1. Introduction

The first cigarette that primarily heats tobacco, Premier®, was introduced in 1988. Later another cigarette aimed at heating tobacco was introduced. The experimental TOB-HT cigarette was designed to contain combustion only within a small portion of the cigarette. Studies on this type of cigarette showed that the concentrations of most target compounds are significantly lower with the TOB-HT cigarette when compared to other light tar reference cigarettes (Borgerding *et al.*, 1997).

More recently Philip Morris Inc. introduced a new type of cigarette (Accord®) that primarily heats tobacco as opposed to “regular” cigarettes that burn tobacco. No ashes or smoke are created in the process of smoking an Accord® cigarette. This system has been discussed in detail in chapter 1. Whereas smoking regular cigarettes lead to the formation of a complex chemical mixture through the processes of combustion, pyrolysis, distillation, pyrosynthesis, condensation and elution (Dube and Green, 1982), very little if any of the tobacco inside the Accord® cigarette actually burns. It could therefore be expected that Accord® cigarettes will create less pyrolysis products than conventional cigarettes.

To further test our hypothesis that the presence of pyrolysis products increases the inhibition of MAO we proposed to compare the smoke extracts of Accord®, Marlboro® (representing a high tar cigarette) and Parliament® (a low tar cigarette) cigarettes. We

anticipated that the smoke extract from the Accord® cigarettes will show comparatively less MAO inhibition because of less pyrolysis products being formed in the process of "smoking" the cigarette.

3.3.2. Experimental procedure

3.3.2.1. Collection of Accord, Marlboro and Parliament cigarette smoke on filter pads

Of each of the Accord®, Marlboro® and Parliament® cigarette brands the smoke of 5 cigarettes was collected on 44 mm filter pads (Whatman) with a Filtrona SM400 (Cerulean Corporation) linear smoking machine. The above cigarette samples were prepared in triplicate by Philip Morris, USA. These samples were mailed to us wrapped in foil and placed in plastic sealed bags.

3.3.2.2. Extraction of Accord, Marlboro and Parliament cigarette smoke from filter pads

The filter pads, containing the total particulate matter (TPM) collected from Accord®, Marlboro® and Parliament® cigarette smoke, were cut into small pieces (1 cm by 0.5 cm) and placed in 250 mL Erlenmeyer flasks. Freshly distilled acetone (150 mL) was added to each flask and the resulting suspensions stirred for 40 minutes at room temperature. The resulting suspensions contained intact pieces of the pad together with some fibrous material resulting from cutting the pads. The resulting suspensions (after stirring for 40 minutes) were filtered through glass micro-fiber filters (25 mm diameter) under reduced pressure. The solution appeared clear after this filtration step. The acetone was removed under reduced pressure (water pump with rotary evaporator) after transferring the

solution to a 500 mL round bottom flask. Four mL of acetone was added to the round bottom flask in order to remove the remaining residue. This solution of residue in acetone consisted of small particles that would not dissolve in the acetone and was therefore filtered through a plug of glass wool held in a Pasteur pipette. This filtrate was transferred to a pre-weighed glass vial and the acetone was again evaporated under reduced pressure. After evaporation the weight of the residue was determined. The samples were stored at 4 °C until analyses which were generally performed on the next day.

3.3.2.3. Control samples for smoke collected on filter pads

In order to determine, on MAO-B inhibition, the possible influences of other components (besides those contained in smoke) in the process of sample preparation, the following control sample was prepared:

One control filter pad (not used for smoke collection) was treated exactly the same as the filter pads containing TPM from the Accord® cigarettes. The residue, extracted from the filter pad with acetone, was diluted with DMSO similarly to the dilution of Accord® smoke and was also tested in the same MAO assay. This dilution was prepared in the same way as for a typical 2.5 mg/mL Accord® sample. This solution did not show any inhibition of MAO-B.

3.3.2.3. Assay for MAO inhibition

The extracts of Accord®, Marlboro® and Parliament® smoke were assayed for MAO inhibition as described previously (see section 3.1.2.7). The curves were drawn with GraphPad Prism™ software in order to determine the IC_{50} values and are presented in Figures 3.8 and 3.9.

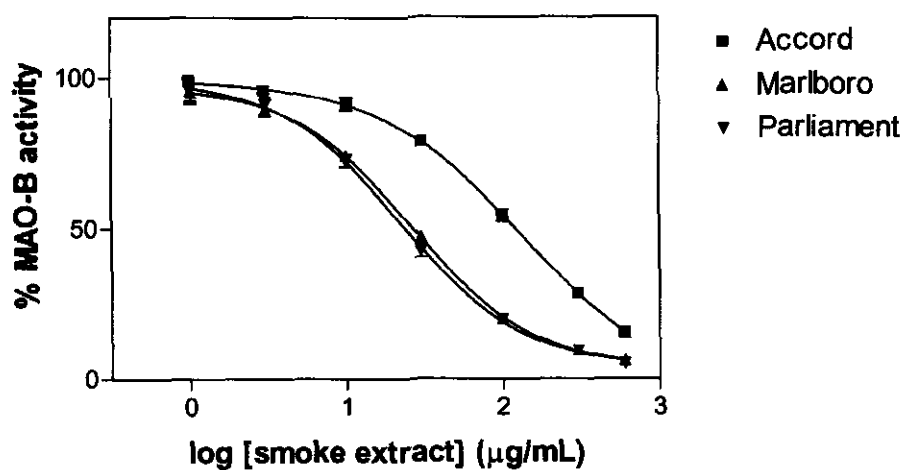


Figure 3.8. MAO-B activity of three replicate experiments after incubation with Accord®, Marlboro® and Parliament® smoke extracts. Each data point represents the mean ($\pm$ S.E.M.) of triplicate replications with three determinations in each replicate. Lack of error bars indicates that the S.E.M. resides within the size of the symbol.

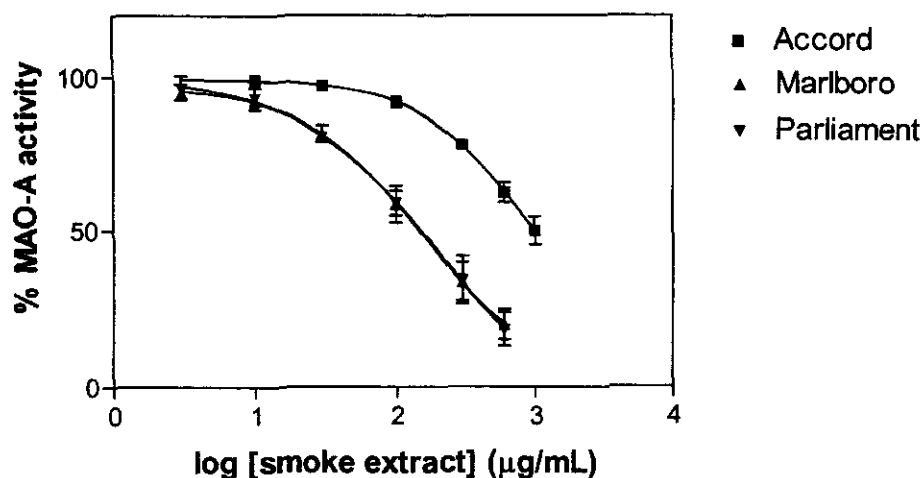


Figure 3.9. MAO-A activity of three replicate experiments after incubation with Accord®, Marlboro® and Parliament® smoke extracts. Each data point represents the mean ($\pm$ S.E.M.) of triplicate replications with three determinations in each replicate. Lack of error bars indicates that the S.E.M. resides within the size of the symbol.

3.3.3. Results and discussion

Cigarette smoke extracts obtained from Accord®, Marlboro® and Parliament® cigarettes were tested for MAO-A and MAO-B inhibition. The IC_{50} values determined for these samples are presented in Table 3.4.

The cigarette smoke collected on the filter paper was extracted with acetone. After evaporation of the acetone the smoke residues were weighed. Table 3.5 gives the average weight determined for the Accord®, Marlboro® and Parliament® samples.

Table 3.4. The average IC₅₀ values for MAO-A and -B inhibition determined for Accord®, Marlboro® and Parliament® cigarette smoke.

Sample	MAO-A		MAO-B	
	IC ₅₀ μg/mL	95% C.L.*	IC ₅₀ μg/mL	95% C.L.
Accord®	1258	729-217	126	112-143
Marlboro®	152	105-223	26	24-30
Parliament®	155	103-231	22	19-26

Three samples each of Accord®, Marlboro® and Parliament® cigarette smoke collected on filter paper were analyzed for MAO inhibition.

* 95% C.L. = 95% confidence limits.

Table 3.5. Average weight of the cigarette smoke residues extracted from filter paper.

Sample	Average Weight of the Residue (mg)
Accord®	33.33±1.53
Marlboro®	87.67±6.66
Parliament®	102.33±8.37

The extracted cigarette smoke was dissolved in DMSO in order to be tested in the MAO assay. The stability of this solution, kept at 4 °C was assessed over a period of 2 months by determining the inhibition of MAO-B at different time intervals. The activity of MAO-B in the presence of the smoke samples was determined on the first day after preparation of the samples, after one month and after two months of preparation (Figure 3.10).

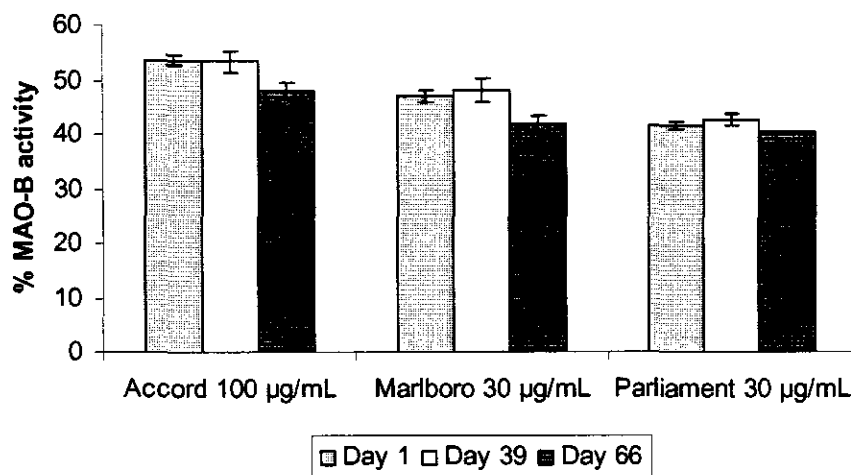


Figure 3.10. Stability (as a factor of MAO-B inhibitory activity over time stored) of Accord®, Marlboro® and Parliament® cigarette smoke samples dissolved in DMSO stored at 4 °C for 2 months. The error bars indicate the standard deviation (SD).

The inhibition of MAO-B and MAO-A by the smoke of Accord® cigarettes were significantly less than the inhibition of the Marlboro® and Parliament® smoke extracts. Accord® cigarettes were approximately 8 times less potent in inhibiting MAO-A and 5 times less potent in inhibiting MAO-B (see Table 3.4). We speculate that this difference in inhibition is due to a smaller amount of pyrolysis products being formed in the process of smoking the Accord® cigarettes and that the higher amount of pyrolysis products present in the smoke of “regular” cigarettes are partly responsible for MAO inhibition – regardless of the tar content of the cigarette.

The high tar cigarette (Marlboro®) and the low tar cigarette (Parliament®) showed similar inhibition of MAO-A and MAO-B. Marlboro® smoke had an IC_{50} of 26 µg/mL and Parliament® an IC_{50} of 22 µg/mL when tested on MAO-B. When tested on MAO-A Marlboro® had an IC_{50} of 152 µg/mL and Parliament® an IC_{50} of 155 µg/mL (Results

are given in Table 3.4, Figure 3.8 and Figure 3.9). In each case there was no significant difference between Marlboro® and Parliament® smoke when assayed for MAO inhibition. From this it may be concluded that the smoke of a lower tar cigarette (for example Parliament®) inhibits MAO to a similar extent as smoke from a cigarette with higher tar content (for example Marlboro®).

As expected the amount of total particulate matter (TPM) of the Accord® sample was significantly less than the TPM of the Marlboro® and Parliament® samples (see Table 3.5). Surprisingly the high tar cigarette and low tar cigarette showed similar amounts of TPM suggesting that it is the burning process of a cigarette and not the tar content that determines the amount of TPM formed.

The control experiment for these data was done by cutting clean 44 mm filter pads (Whatman) and stirring them in acetone in the same way as for the sample preparations. These samples were then also dissolved in DMSO and tested in the MAO-assay. The control experiments showed that the process of extracting the cigarette smoke from the filter pads had no influence on the inhibition of MAO and that the inhibition being reported is solely due to the cigarette smoke extracts contained in the pads.

3.4. Studies on the Irreversible (Slowly Reversible) Inhibition of Baboon Liver Mitochondrial MAO-B by Marlboro®, Parliament® and Accord® Cigarette Smoke as Determined by Gel-Filtration

3.4.1. Introduction

Fowler and co-workers (2000) compared the MAO-B binding activity in a group of smokers who were scanned after an overnight smoke-free interval and once at 10 min. after smoking to determine if MAO-B binding activity increased immediately following smoking. MAO-B binding activity in this group was similar to that found in their earlier study (Fowler *et al.*, 1996b) and averaged 42% lower binding activity than in a control group. The brain MAO-B activities were not significantly different 10 min. or 11 h after the cigarette. This lack of binding recovery is consistent with preclinical studies showing that MAO-B inhibitory substances cannot be removed by gel filtration or dialysis (Yu and Boulton, 1987) and by recent studies of platelet MAO in human smokers showing no recovery of MAO during the first week of abstinence but full recovery by 4 weeks (Rose *et al.*, 2001). These findings suggest that the components of cigarette smoke putatively responsible for the inhibition of MAO may possibly be irreversible in nature.

Since irreversible inhibitors will have a long-term effect on the enzyme it is important to investigate the reversibility of the inhibition exerted by tobacco smoke. We decided for two important reasons to focus on cigarette smoke extracts in this study to investigate irreversible inhibition. Firstly smokers are primarily exposed to smoke, rather than the tobacco leaf (as opposed to chewers and "dippers"). Secondly we have shown earlier that

tobacco leaf extracts inhibit MAO-A and MAO-B much less than extracts of tobacco smoke (section 3.1).

Previously, *in vitro* studies done by Yu and Boulton (1987) showed that aqueous extracts of tobacco smoke irreversibly inhibit MAO. We hypothesize that smoke collected in acetone will also show irreversible inhibition of this enzyme. Acetone extracts of Accord®, Marlboro® and Parliament® cigarettes were therefore tested for irreversible MAO inhibition.

3.4.2. Experimental procedure

3.4.2.1. Preparation of cigarette smoke samples

The Accord®, Marlboro® and Parliament® samples collected for IC₅₀ determinations were used for the irreversible assay as well. The preparation of these samples is described in section 3.3.2.

3.4.2.2. Assays to measure irreversible inhibition of MAO-B by extracts of Marlboro®, Parliament® and Accord® smoke extracts

To determine whether the smoke samples irreversibly inhibit MAO-B activity we utilized gel filtration procedures similar to that used by Yu and Boulton (1987). In addition to the crude smoke extracts, we included TMN, a *reversible* inhibitor of MAO (Khalil *et al.*, 2000; Castagnoli *et al.*, 2001). TMN served as a control for recovery of MAO-B activity after gel filtration.

Samples (dissolved in DMSO) were prepared in 0.1 M sodium phosphate buffer, pH 7.4, by adding a known inhibitor or extracts of interest to yield in a total of 1000 μ L solution, the following concentrations: 60 μ g/mL TMN; 1000 μ g/mL Accord® smoke extract; 200 μ g/mL Marlboro® smoke extract or 200 μ g/mL Parliament® smoke extract. All samples contained a final concentration of no more than 4% DMSO. After 15 min preincubation with gentle agitation in a water bath at 37 °C for 15 min, 30 μ L of baboon liver mitochondria (1.5 mg-protein/mL final concentration) was added to the vial and the mixture immediately vortexed. At $t = 0$, a 50 μ L aliquot was taken from each of the individual mixtures and immediately added to a preheated solution (37 °C) containing MMTP 50 μ M in phosphate buffer; (final volume 500 μ L, final protein concentration 0.15 mg/mL). This step diluted each of the samples 10 times. The samples were incubated for 15 min at 37 °C after which the reaction was terminated by adding 20 μ L of perchloric acid. The samples were then vortexed and centrifuged at $16,000 \times g$ for 8 minutes. The absorption of the supernatants at 420 nm was read on the spectrophotometer. The remainder of the initial mixtures (containing 1.5 mg/mL enzyme and inhibitor) were kept at 10 °C until gel filtration using PD-10 columns (Amersham Pharmacia, Sephadex™ G-25 M). After column equilibration with buffer, the columns were loaded with the remainder of each initial mixture diluted to a volume of 2.5 mL with phosphate buffer. The column was eluted with 3.5 mL of 0.1 M sodium phosphate buffer, pH 7.4, and fractions 2 and 3 were collected (1000 μ L each) based on results from preliminary activity experiments. These two fractions were combined, and the protein concentration was determined (Bradford, 1976). The activity of the resulting samples was assayed as described above using the same final concentrations of substrate and protein which were achieved by adjusting volumes as necessary (total volume 500

μL). Irreversible inhibitor-enzyme complexes will elute intact and show no enzyme activity, whereas loosely bound inhibitors will dissociate and the free enzyme (as a relatively large molecule) will elute early while the relatively small inhibitor molecules will be retarded by the gel beads (see Figure 3.11). When both types of inhibitors are present, the regained activity following gel filtration can be attributed to reversible inhibition.

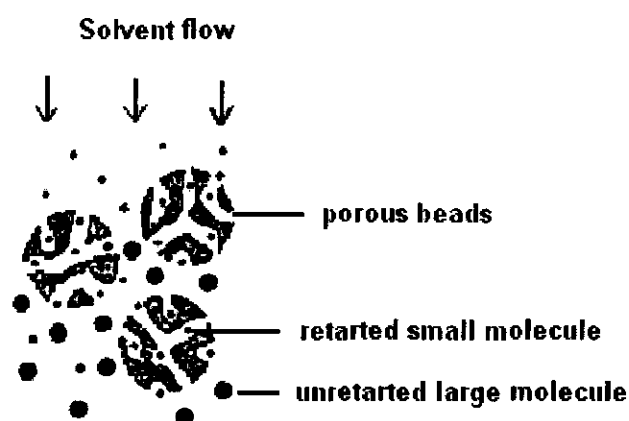


Figure 3.11. The principle of gel-filtration chromatography.

3.4.3. Results and discussion

Our results clearly show irreversible inhibition of MAO-B with acetone extracts of Accord®, Marlboro® and Parliament® cigarette smoke (see Figure 3.12). The partial recovery of MAO-B activity after gel filtration also suggests the presence of reversible inhibitors in the smoke extracts. Our results therefore support the findings of Fowler and co-workers (1996a, 1996b) that components of tobacco smoke may be responsible for the lower activity of brain and platelet MAO-B seen in human smokers, and that these effects can subsist for some time due to irreversible inhibition. Our data also support the

earlier findings of irreversible inhibition in tobacco smoke concentrates by Yu and Boulton (1987).

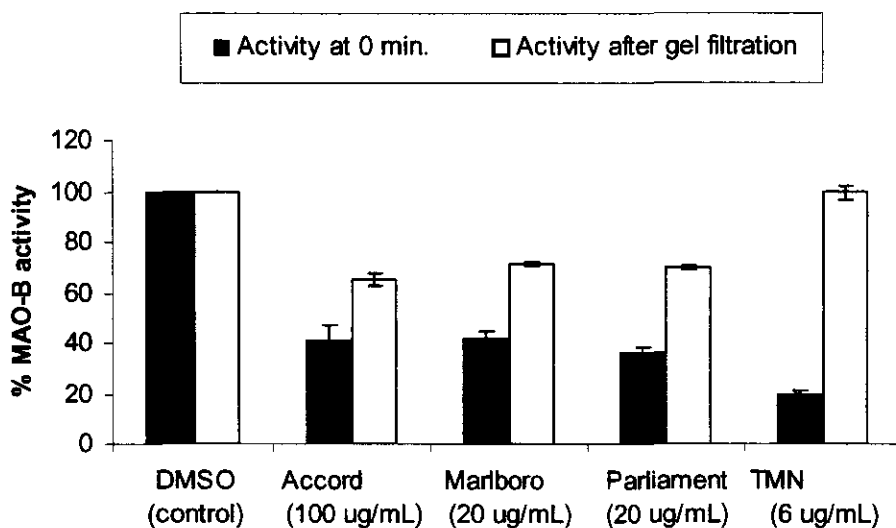


Figure 3.12. MAO-B inhibition by Accord®, Marlboro® and Parliament® smoke samples, and TMN. Baboon liver mitochondrial MAO-B (final protein concentration 1.5 mg/mL) was incubated with TMN and smoke samples. Aliquots were taken at 0 minutes, and after gel filtration. The activity of the enzyme (final protein concentration 0.15 mg/mL) was determined using MMTP (50 μ M final concentration) as substrate. The results are the average % MAO-B activity of three replicates of each of the smoke samples. The error bars indicate the SD.

Some advances have been made in finding the compounds in cigarette smoke and leaf extracts responsible for MAO inhibition. TMN, a competitive inhibitor of MAO-B was recently isolated from flue cured tobacco leaves (Khalil *et al.*, 2000). 2-naphtylamine, an MAO inhibitor was later identified in tobacco smoke (Hauptmann and Shih, 2001). This compound inhibits both MAO-A and MAO-B but is 10-fold less potent than TMN.

These studies should be continued in order to also find the compounds responsible for irreversible inhibition.

The irreversible inhibition of Accord® cigarette smoke extracts on MAO-B has not been reported in the literature. The significance of this result is that Accord®, a cigarette that primarily heats tobacco and therefore creates less pyrolysis products, also shows irreversible inhibition of MAO-B. However, this inhibition requires a 5 times higher concentration compared with cigarettes that primarily burn tobacco. It seems as though the different smoke samples contain proportionally the same amount of irreversible inhibitors since the activity of the enzyme that was regained seem to be parallel in all three smoke sample experiments. This gives rise to the question of whether the same compounds in Accord® smoke are responsible for the irreversible inhibition than those found in Marlboro® or Parliament® smoke, and if so, whether these compounds are only formed in smaller quantities. If the answer is “yes” to the last statement, it would mean that primarily heating the tobacco leads to the formation of the same or at the least, similar irreversible compounds in the smoke as opposed to the smoke resulting from burning tobacco. Our results support the findings of Fowler and co-workers (1996a, 1996b) that components of tobacco smoke may be responsible for the lower activity of brain and platelet MAO-B seen in human smokers and that at least some of these lead to irreversible enzyme inhibition.

The sample containing the known reversible inhibitor TMN showed $19.8 \pm 1\%$ activity at 0 min, but regained full activity ($99.4 \pm 2.7\%$) following column filtration. This indicates

that the enzyme, in the presence of a competitive inhibitor, will regain full activity after column separation.

In previous experiments we also included (R)-deprenyl a known irreversible, mechanism-based MAO-B inhibitor, (Knoll, 1986) as control to verify that the activity of the enzyme in the presence of this inhibitor will not return after column separation. Our results (not shown here) showed complete inhibition of the enzyme [with 2 $\mu\text{g/mL}$ of (R)-deprenyl] at $t = 0$ that did not recover following filtration. In previous experiments we also determined the activity of the enzyme after 60 minutes of incubation with the inhibitor and smoke samples to determine whether there would be time dependent inhibition in the course of 60 minutes. The inhibition observed at 60 minutes was the same as the inhibition observed at 0 minutes of incubation thus indicating that there was no further inhibition that occurred during that time.

3.4.3.1. Recovery of enzyme after a second gel filtration

To evaluate if complete recovery of free enzyme is achieved by single column filtration, we subjected a hexane extract of cigarette smoke and enzyme mixture to a second column separation and observed that there was a slight increase (10%) in activity after separation on the second column but that such a step still did not lead to complete recovery of the enzyme (see Figure 3.13).

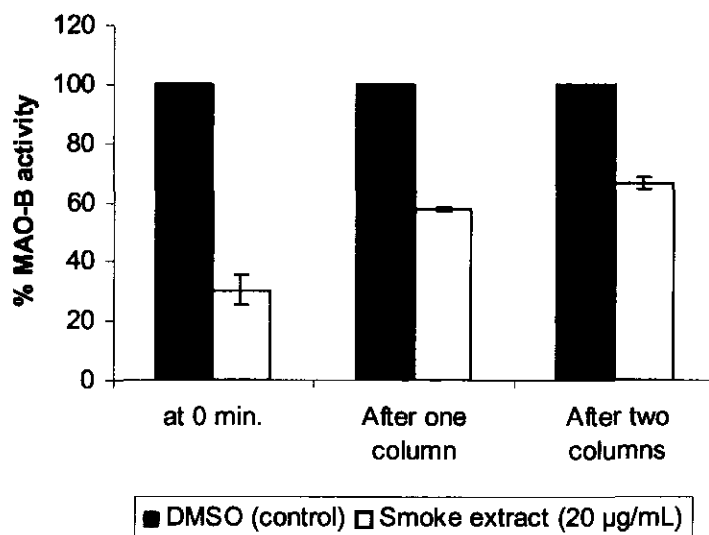


Figure 3.13. Recovery of enzyme after a second gel filtration. Percentage MAO-B activity when incubated with a 20 µg/mL hexane extract of cigarette smoke. "At 0 min." indicates that the enzyme-smoke extract mixture was immediately added to the substrate and not incubated before addition to the substrate. One enzyme-smoke extract mixture and control was assayed after gel filtration on one sephadex column and another enzyme-smoke extract mixture and control was assayed after being passed through a second sephadex column. This figure is the result of two replicate assays and the error bars indicate the SD.

3.4.3.2. Irreversible inhibition of placental mitochondrial MAO-A by Marlboro® cigarette smoke

Preliminary studies to determine the irreversibility of MAO-A inhibition were performed with smoke extracts from Marlboro® cigarettes (collected on our in-house designed smoking device). The irreversibility of MAO-A inhibition was determined by using gel filtration. First a smoke concentration of 20 µg/mL was used but this did not give satisfactory inhibition of MAO-A, therefore, a concentration of 160 µg/mL (8 times

more than that used for the MAO-B assay reported in section 3.4.2.2.) was used in order to achieve sufficient inhibition of the enzyme. After gel filtration the activity returned to 80% of the control (see Figure 3.15). These studies were not repeated to enable statistical analyses of the data. However, even from these preliminary studies it can be concluded that *in-vitro* cigarette smoke extracts have less potency for irreversibly inhibiting MAO-A compared to MAO-B.

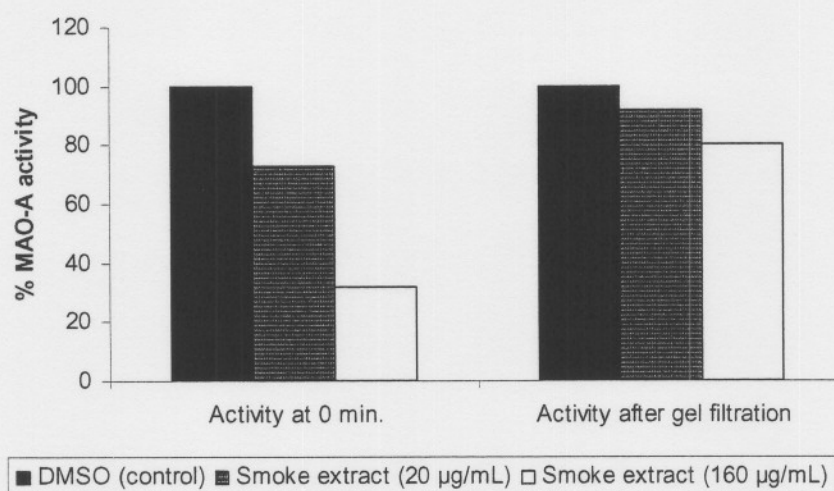


Figure 3.14. Activity of MAO-A in the presence of a Marlboro® smoke sample collected with hexane. Human placental mitochondrial MAO-A (final protein concentration 1.5 mg/mL) was incubated with the smoke sample. Aliquots were taken at 0 minutes and following gel filtration and the activity of the enzyme (final protein concentration 0.15 mg/mL) determined using MMTP (50 µM final concentration) as substrate.

**THE INFLUENCE OF CIGARETTE SMOKING DURING PREGNANCY
ON PLACENTAL MAO-A**

4.1. The influence of cigarette smoking on serotonin and placental MAO-A in vivo

4.1.1. Introduction

Several researchers have documented health related problems associated with smoking during pregnancy. Fetal growth restriction (Jedrychowski *et al.*, 1998), abruption (Misra and Ananth, 1999; Ananth *et al.*, 1999) and placenta previa are among the most documented conditions associated with cigarette smoking during pregnancy. Cigarette smoking during pregnancy is further associated with increased prematurity and increased risk of perinatal mortality and possibly also to increased miscarriage rates (Naeye, 1978).

Research findings showing MAO-A and MAO-B inhibition in the brains (Fowler *et al.*, 1996a,b) of smokers and MAO-B inhibition in platelets (Oreland *et al.*, 1981; Yong and Perry, 1986; Norman *et al.*, 1987; Berlin *et al.*, 1995a) of smokers also are of particular interest in considering possible biological effects of cigarette smoking on pregnancy health outcomes. The observation that long term abstinence from smoking results in a return to normal brain MAO activity levels (Fowler *et al.*, 1996a) argues that this effect is not genetically determined. It is likely that similar processes may be operative in smoking

pregnant women and a salient question to ask would be whether cigarette smoking during pregnancy will also inhibit placental MAO.

Even though the human placenta expresses both MAO-A and MAO-B mRNA (Auda *et al.*, 1998), the MAO activity present in the placenta has been shown to be predominantly due to MAO-A (Nagy and Salach, 1981; Hsu and Shih, 1988). This is important since serotonin is a highly selective MAO-A substrate (Garrick and Murphy, 1982). In Chapter 2 it was speculated that the most likely physiological role of MAO-A in the placenta is the regulation of serotonin concentrations at reasonably low levels in the maternal and fetal circulations. If serotonin were not efficiently cleared from the placenta, it could cause vasoconstriction of uterine arteries and reduce the uteroplacental circulation (Ramamoorthy *et al.*, 1995).

The influence of inhibited MAO-A activity on serotonin levels can be observed in pre-eclampsia. In this maternal syndrome, lower MAO-A activity (de Maria, 1964; Sandler and Coveney, 1962) is associated with increased levels of placental (Senior *et al.*, 1963; Marley *et al.*, 1967), maternal (Ramadan *et al.*, 1973; Patel and Dass, 1962) and fetal (Gujrati *et al.*, 1985) serotonin. Carrasco and co-workers (2000a) have been able to show that the expression of MAO was almost undetectable in placental homogenates obtained from pre-eclamptic pregnancies in comparison with homogenates obtained from placentas after normal pregnancy. These authors gave convincing proof that the increased circulating serotonin associated with pre-eclampsia (Weiner, 1987) is mainly due to the diminished activity of MAO-A and not attributable to insignificant or diminished transport of serotonin into cells.

It can therefore be speculated that cigarette smoking during pregnancy will inhibit placental MAO-A and that the same effects regarding serotonin observed in pre-eclampsia should be expected in pregnant smokers (see also Figure 2.10). In order to test the hypothesis that cigarette smoking will inhibit placental MAO-A, this activity will be measured in women who smoked during pregnancy and in women who did not smoke. The consequent influence of placental MAO-A inhibition on maternal and fetal serotonin levels will be studied by measuring serotonin levels in blood collected from the mothers and the umbilical cord. Platelet MAO-B activity will also be determined in maternal blood samples as a control to verify that cigarette smoking by these pregnant women inhibited platelet MAO-B activity as reported elsewhere in the literature.

4.1.2. Experimental procedure

4.1.2.1. Materials and instrumentation

Chemicals and reagents, unless otherwise stated, were purchased from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade. Water was obtained from a Milli-Q UV Plus system (Millipore, Bedford, MA). The substrate for the MAO activity assay, MMTP·HCl was synthesized according to the method of Nimkar and co-workers (1996). NaH_2PO_4 , Na_2HPO_4 and glycerol were purchased from Fisher Scientific Products (Fair Lawn, NJ). Three mL Vacutainer® tubes containing 5.1 mg (K_3) EDTA were used for blood collection and were manufactured by Becton Dickinson Vacutainer Systems (Franklin Lakes NJ). UV data were obtained using a Beckman DU-7400 spectrophotometer. Platelets were counted with a Beckman Coulter® model Z2 manufactured by Beckman Coulter Corporation (Miami, FL). Isoton® II electrolyte

solution and Accuvettes® sample vials for platelet counting were purchased from the same company.

4.1.2.2. Patient pool and recruitment

Cases for the present study were recruited from the Montgomery Obstetrics and Gynecology and Women's Health Center of Virginia, with an office in Blacksburg, Virginia. All collections of human placenta were approved by the Internal Review Boards of Montgomery County Hospital and Virginia Tech. Inclusion of subjects in the study was based on clinical data and the discretion of their obstetricians. Only healthy subjects were recruited. An informed consent document, stating the willingness to donate a placenta, was signed by each patient before inclusion in the study. The subjects comprised of two groups:

Group 1: non-smoking pregnant women. Included were 9 pregnant women (mean age 32.3 ± 5.4 years old) who did not smoke during pregnancy. Six of these women never smoked. Three of the women were former smokers, all of whom quit at least 2 years ago.

Group 2: smoking pregnant women. Included were 10 pregnant women (mean age 21.7 ± 7.1 years old) who smoked 5-15 cigarettes per day during pregnancy. One of these women had been smoking for two years while the remaining 9 women in this group smoked for at least 5 years prior to the study.

Information on the patients' age, history of smoking and current smoking status was obtained by questionnaire. The questionnaire is given in appendix A.1. Information on

length of gestation and weight of the infant at birth was provided by medical personnel involved in the birthing process (see Table 4.1).

Table 4.1. Clinical information of the women who participated in the study.

	Smoking during pregnancy (n=10)	Not smoking during pregnancy (n=9)
Maternal age, years	22.8 ± 1.8	32.3 ± 1.8
Gestational age, weeks	39.7 ± 0.3	39.8 ± 0.5
Gestational weight, g	3427 ± 140	3474 ± 162
Cigarettes smoked/day	10.7 ± 0.7	-
Period of smoking, years	8.2 ± 1.6	-
Age started smoking	14.6 ± 0.6	-
<u>Deliveries:</u>		
Uncomplicated	1	5
Complicated ¹	9	4

¹Complicated deliveries include deliveries by Caesarean section, augmented and induced deliveries as well as a combination of induction and Caesarean section.

The results are expressed as mean ± S.E.M.

4.1.2.3. Preparation of human placental mitochondrial fractions

Human placental mitochondria were prepared according to the method of Salach and Weyler (1987). The following solutions were used in the preparation of the human placental mitochondrial fraction:

Solution #1: 10 mM potassium phosphate buffer containing 0.5 mM EDTA and 0.25 mM sucrose (pH 7.0-7.2). This solution was mixed with 50 mM solution of phenylmethylsulfonyl fluoride (PMSF) in absolute ethanol just before use to yield a concentration of 1% (v/v).

Solution #2: 10 mM tris-phosphate buffer containing 0.5 mM EDTA and 0.15 M potassium chloride.

Processing of the placentas started within one hour post partum. All the procedures were performed in a cold room (6 °C). The first step was to cut off and discard the umbilical cord as well as removing as much tissue surrounding the placenta (amniotic sac) as possible. The placenta was then cut into small pieces (approximately 1" square) and rinsed with solution #1 containing 1% PMSF to get rid of as much blood and blood clots as possible.

Blood was further removed by putting the placenta pieces in cheese cloth and squeezing the mass to remove as much blood as possible. The placental pieces were placed in 1.5 L of solution #1 with 1% PMSF and homogenized using a blender. The resulting homogenate was centrifuged at $1,250 \times g$ for 15 min at 4 °C.

After centrifugation the supernatant was poured through a few layers of cheese cloth. The supernatant was then centrifuged at $26,000 \times g$ for 15 min at 4 °C. Following centrifugation the supernatant was discarded and the pellets (mitochondria) stirred into a total of 500 mL solution #1 containing 1% PMSF and transferred to a glass homogenizer sitting in ice. The pellets were homogenized and centrifuged at $26,000 \times g$ for 15 min. at 4 °C. The supernatant was discarded and the pellets stirred into 70 mL of solution #2.

The pellets (in solution #2) were homogenized and centrifuged at $40,000 \times g$ for 20 min at 4 °C. The supernatant was discarded. The pellets were left in the centrifuge tubes and

stored in a -70 °C freezer until used in the MAO assay. Mitochondrial homogenates from other tissues prepared in the same way has been shown to be stable for 12 months when stored at -70 °C (Inoue *et al.*, 1999).

A day before determining placental MAO-A activity the frozen mitochondrial homogenates were removed from the -70 °C freezer, initially placed in a -20 °C freezer and later at room temperature to thaw. The mitochondrial pellet was then transferred to a 10 mL glass homogenizer together with 2 mL of a 0.1 M sodium phosphate buffer (pH 7.4), containing 50% weight/volume glycerol. The pellet was then homogenized and the resulting homogenate aliquoted into Eppendorf tubes. One aliquot of the homogenate was stored at -20 °C for MAO-A activity determination on the next day. Other aliquots were stored in a -70 °C freezer. This step was necessary in order to have a homogenous mitochondrial mixture for analysis.

4.1.2.4. MAO-A activity in placental tissue

In order to compare the MAO-A activity for the different samples of mitochondrial fractions collected, the substrate concentration should be well above the K_m of the substrate so that the enzyme will be saturated with substrate (see Chapter 2 paragraph 2.1.3.2). For this reason the final substrate concentration in our assay was 2 mM, a value well above the K_m of 50 μ M.

The protein concentrations of the placental homogenates were determined according to the Coomassie Brilliant Blue dye binding method of Bradford (1976) with bovine serum

albumin used as standard. The activity of the MAO-A enzyme was measured as follows. A mixture (500 μL , final volume) consisting of 225 μL 0.1 M sodium phosphate buffer (pH 7.4) and 250 μL 4 mM MMTP [2 mM final concentration] in 0.1 M sodium phosphate buffer (pH 7.4), was preincubated with gentle agitation in a water bath at 37 °C for 15 min. Following the preincubation, 25 μL human placental mitochondrial homogenate (final protein concentration = 0.15 mg/mL) was added to the vial and the sample was incubated for 15 min at 37 °C after which the reaction was terminated by the addition of 20 μL 70% perchloric acid. The samples were vortexed and the denatured protein removed by centrifugation at $16,000 \times g$ for 8 minutes. The resulting supernatants contained the MAO-A catalyzed product (MMDP⁺) of MMTP. The absorbance of the tetrahydropyridine metabolite (MMDP⁺) was measured on the spectrophotometer at a wavelength of 420 nm [$\epsilon = 24,000 \text{ M}^{-1} \text{ cm}^{-1}$, (Nimkar *et al.*, 1996)].

4.1.2.5. Preparation of platelet rich plasma (PRP) from blood samples

Maternal blood samples were collected at the onset of labor and venous umbilical cord blood samples were collected shortly after birth. Blood samples were drawn into 3 mL Vacutainer® tubes containing EDTA. Blood sampling was done by personnel at Montgomery hospital. As soon as possible (generally within 20 minutes) after the blood was collected samples were centrifuged at $200 \times g$ for 10 minutes in order to obtain the PRP. A small volume (20 μL) of PRP was immediately used for platelet counting and the remainder of PRP was stored at -70 °C until further analysis

4.1.2.6. Platelet counting

After collection, the mean platelet volume in EDTA-blood will increase over time. This increase is caused by a change in the shape of the platelets after removal from the body. The changing volume is relatively stable for a period of one to three hours after collection. This period is the best time to count the sample when using electronic instruments, because the platelets will be within a standard size range (Rowan, 1986). Platelets in the PRP were counted on a Beckman Coulter® model Z2 within one and a half hour after blood collection. A small volume (20 μL) of PRP was diluted with Isoton® II (balanced electrolyte solution) in two steps to give a final dilution (as defined by the instrumentation used) of 5050. Particles with volumes ranging from 1.767 fl to 33.510 fl were counted as platelets. The platelet counts were performed in duplicate and three readings were done for each replicate.

4.1.2.7. Platelet MAO-B activity

Platelet MAO-B activity was determined according to a similar spectrophotometric assay used to determine MAO-A activity. The MAO-B activity was determined in PRP after thawing the samples stored at $-70\text{ }^{\circ}\text{C}$. A mixture (500 μL , final volume) consisting of 150 μL 0.1 M sodium phosphate buffer (pH 7.4), and 250 μL 4 mM MMTP [2 mM (V_{max}) final concentration] in 0.1 M sodium phosphate buffer (pH 7.4), was preincubated with gentle agitation in a water bath at $37\text{ }^{\circ}\text{C}$ for 15 min. Following the preincubation, 100 μL PRP was added to the vial and the sample was incubated for 60 min at $37\text{ }^{\circ}\text{C}$ after which the reaction was terminated by the addition of 30 μL 70% perchloric acid. The samples

were vortexed and the denatured protein removed by centrifugation at $20,500 \times g$ for 10 min at 10 °C. The resulting supernatants contained the MAO-A catalyzed product MMDP⁺. The absorbance of MMDP⁺ was measured on the spectrophotometer at a wavelength of 420 nm [$\epsilon = 24,000 \text{ M}^{-1} \text{ cm}^{-1}$, (Nimkar *et al.*, 1996)].

4.1.2.8. Serotonin content of maternal and umbilical cord PRP

Serotonin measurements were performed as previously described by Kumar *et al.* (1990) by high performance liquid chromatography coupled with electrochemical detection (HPLC-EC).

The maternal and cord blood PRP samples stored at -70 °C were thawed at room temperature. Serotonin was extracted from these samples with perchloric acid. To 100 μL PRP, 100 μL NMET (N-methyl-5-hydroxytryptamine, internal standard, 0.84 $\mu\text{g}/\text{mL}$) was added. Perchloric acid, 10 μL , was then added and the mixture vortexed vigorously and left on ice for 30 minutes for complete precipitation of proteins. Samples were then centrifuged at $20,800 \times g$ for 15 minutes at 4 °C. The supernatant was carefully removed and diluted 5 fold with mobile phase (see below) before HPLC analysis (this procedure yielded 80 ng/mL of internal standard).

The standard solutions were prepared as follows: Standard solutions of serotonin and its major metabolite 5-hydroxyindole acetic acid (5-HIAA) were prepared to give final concentrations of 1, 10, 30, 80 and 150 ng/mL. The internal standard had a concentration of 80 ng/mL. These solutions were prepared in a buffer containing 0.1 M

NaH_2PO_4 , 0.5 mM EDTA and 0.15 g/L sodium octyl sulfate with the pH adjusted to pH 5. Since serotonin and 5-HIAA are unstable at a low pH (Huang and Kissinger, 1996) it was decided to prepare the standards in this buffer. Figure 4.1 shows a chromatogram of the standard solutions. The separation of serotonin (5-HT) and the internal standard (NMET) in a sample of maternal PRP can be seen in Figure 4.2. The metabolite (5-HIAA) was not detected in any of the samples. Analyses were performed within 12 hours of sample preparation. According to Kumar *et al.* (1990) samples were found to be stable for 24 hours when kept at 4 °C.

The mobile phase for HPLC contained the following: 0.1 M sodium acetate, 0.1 M citric acid, 0.5 mM 1-octanesulfonic acid, 0.15 mM EDTA, 1.0 mM di-ethylamine and 10% methanol. The solution was prepared fresh in deionized water and filtered under vacuum using a 0.45 μm filter. The solution was not recirculated during analysis.

The HPLC-EC system (Hewlett Packard 1049A) had a three-electrode detection system consisting of a carbon working electrode, an auxiliary electrode and a silver-silver chloride reference electrode. The potential of the working electrode versus the reference electrode was set at +0.65 V (detection potential). The chromatography column, reverse phase (100 mm $\times$ 3.2 mm) packed with 3 μM spherical C_{18} bonded silica gel particles was obtained from BAS.

ChemStation software was used to analyze the data. An internal standard calibration method was used to integrate individual peaks and to calculate concentrations using ratios of the peak areas with respect to the internal standard. The data module was also

programmed to apply the correction for dilution factors and to express the final values as ng/mL of sample.

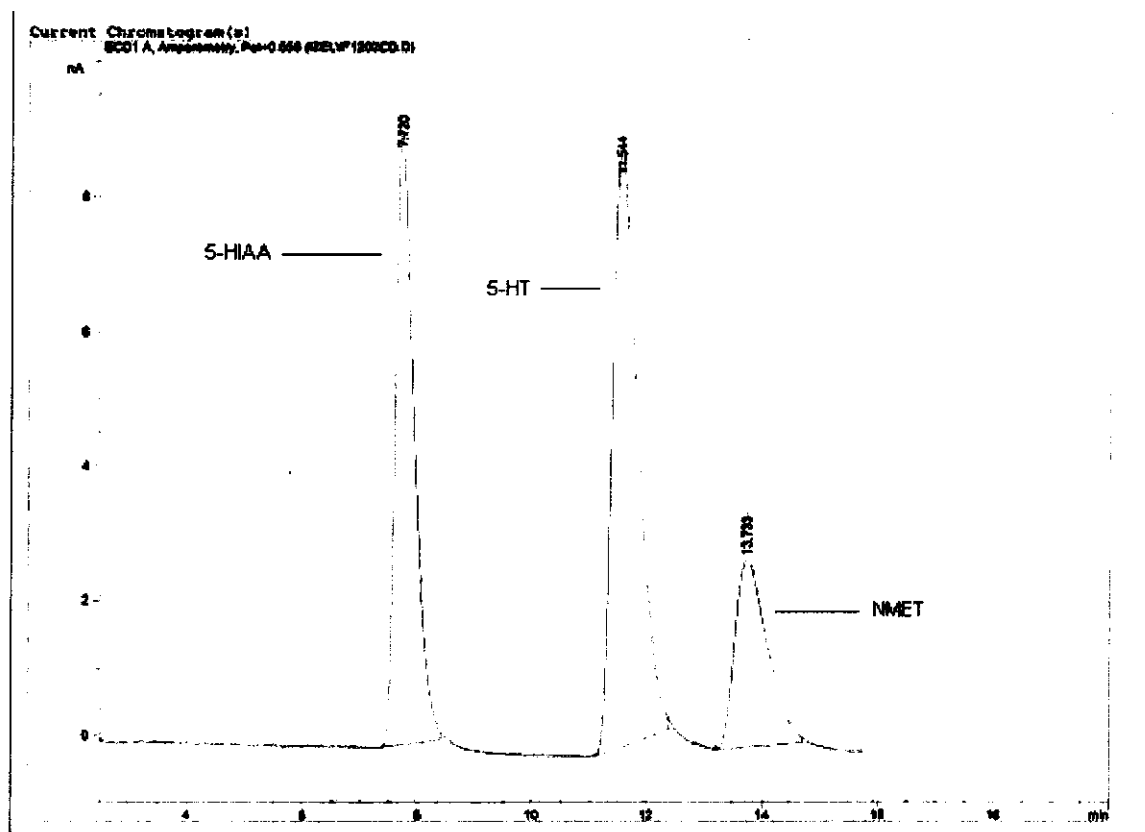


Figure 4.1. Separation of 5-HIAA, serotonin (5-HT) and NMET in a standard solution.

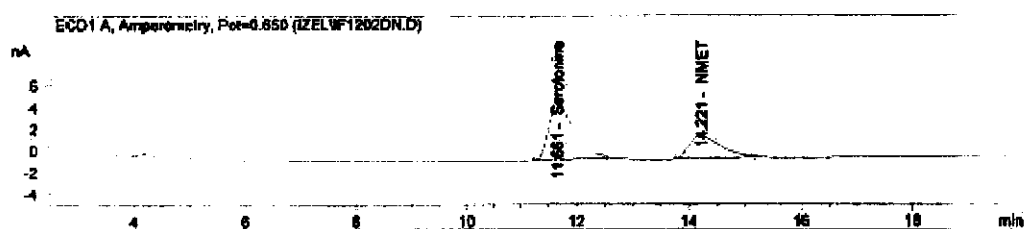


Figure 4.2. Separation of serotonin and the internal standard (NMET) in a sample of maternal PRP. The metabolite (5-HIAA) was not detected in any of the samples.

4.1.3. Results and discussion

4.1.3.1. Placental MAO-A activity

In women who smoked during pregnancy, a trend was seen wherein placental MAO-A activity was slightly inhibited (by ~12%) compared to women who did not smoke during pregnancy (see Figure 4.3; Table 4.2), but this finding did not reach statistical significance. We hypothesized that cigarette smoking should inhibit placental MAO-A. Although statistical significance was not reached in assessing the difference between inhibition of placental MAO-A activity in smokers versus non-smokers, our data do follow the trend stipulated in our hypothesis. To date no studies determining the influence of smoking during pregnancy on MAO-A activity have been reported and we are therefore unable to directly compare these data with the literature.

In addition to placental MAO-A inhibition we found that platelet MAO-B activity was decreased by 43% in the smoking group compared with the non-smoking group ($p < 0.1$) (see Figure 4.4). The more moderate inhibition of placental MAO-A (~12%) compared to platelet MAO-B inhibition in the smokers reflects a thesis that the A-enzyme is less susceptible to inhibition by smoke components. This is shown in our *in-vitro* experiments in Chapter 3. In Chapter 3 we also showed that irreversible inhibition of MAO-A activity is less severe than inhibition of MAO-B activity even in the presence of much higher concentrations of cigarette smoke extract (160 $\mu\text{g/mL}$ vs. 20 $\mu\text{g/mL}$). This already gave an indication that cigarette smoking might have a lesser long-term effect on placental MAO-A (compared with MAO-B measured in platelets, for instance) *in vivo*. *In vivo* experiments by Fowler and co-workers (1996a,b) support this observation by showing

that MAO-A activity in the brains of smokers is less susceptible to inhibition by cigarette smoking than MAO-B activity.

Table 4.2. A summary of the placental MAO-A activity, and platelet MAO-B activity in maternal and umbilical cord blood and serotonin concentration in PRP in maternal and umbilical cord blood (expressed as concentration per volume and concentration per platelet) of smoking and non-smoking pregnant women.

Sample tissue	Smoker	Non Smoker
Placenta		
MAO-A activity (nmol/mg protein/min)	26.0 ± 1.1	29.4 ± 2.3
Platelet Rich Plasma (PRP)		
<i>Maternal</i>		
Serotonin (ng/10 ⁹ platelets)	213 ± 20	416 ± 101**
Serotonin (ng/mL)	80 ± 8.0	82 ± 6.5
Platelets (× 10 ⁸ /mL)	3.9 ± 0.4	2.5 ± 0.4**
MAO-B activity (nmol/10 ⁹ platelet/min)	1.2 ± 0.2	2.0 ± 0.4*
<i>Umbilical Cord Vein</i>		
Serotonin (ng/10 ⁹ platelets)	1272 ± 506	617 ± 376
Serotonin (ng/mL)	140 ± 15	149 ± 27
Platelets (× 10 ⁸ /mL)	4.1 ± 1.1	6.8 ± 1.9
MAO-B activity (nmol/10 ⁹ platelet/min)	1.1 ± 0.2	1.4 ± 0.2

The results are expressed as means ± standard error of the mean (S.E.M.). The symbol * indicates p<0.1 and ** indicates p<0.05 for statistically significant differences between women who smoked during pregnancy (n = 10) and women who do not smoke during pregnancy (n = 9).

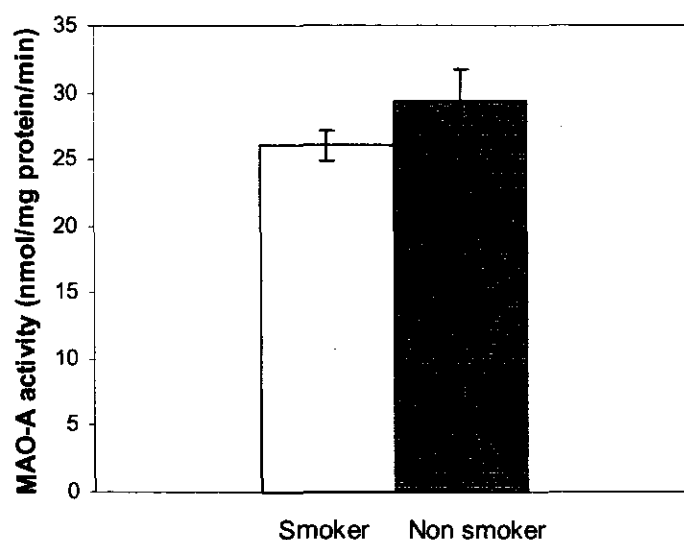


Figure 4.3. Placental MAO-A activity of women who smoked and women who did not smoke during pregnancy. There is not a statistically significant difference between smokers and non-smokers. The error bars indicate standard error of the mean (S.E.M).

The reasons for not finding statistically significant placental MAO-A inhibition in women who smoke during pregnancy is not clear. However, it is possible that a larger study population than the cohort available to us in the current study could shift the trend towards a statistically significant realm relative to lower placental MAO-A activity in smokers compared to non-smokers. It is also possible that the inhibition of placental MAO-A, *in-vivo*, may be restricted due to an accelerated clearance of the compounds in cigarette smoke that are responsible for MAO-A inhibition. It has been reported that pregnancy is a state known to cause more rapid pharmacodynamic clearance of xenobiotics compared with the nonpregnant state (Loebstein *et al.*, 1997). A very important study by Dempsey and co-workers (2002) showed that the metabolic clearance of nicotine and cotinine (the major metabolite of nicotine) is markedly accelerated during

pregnancy. The placenta, as one of the extrahepatic sites of drug metabolism, may be involved in the increased clearance not only of nicotine and cotinine but also of other components – some of which may contribute to MAO inhibition.

It is also possible that the placenta would try to compensate for lower MAO-A activity by expressing more of the enzyme. Such a possibility could be investigated by quantifying (by Western blot experiments) the amount of MAO-A enzyme present in the protein samples used for determining MAO-A activity (Carrasco *et al.*, 2000a). It may be possible that, although we measured similar *activities* of MAO-A per weight of protein for the two groups, the protein of the smoking subjects may have expressed larger amounts of MAO-A enzyme than the non-smoking subjects. If indeed the smoking group expressed larger amounts of MAO-A enzyme per weight of protein it would imply that lower activity of the enzyme would show the same relative activity as found in the non-smoking group.

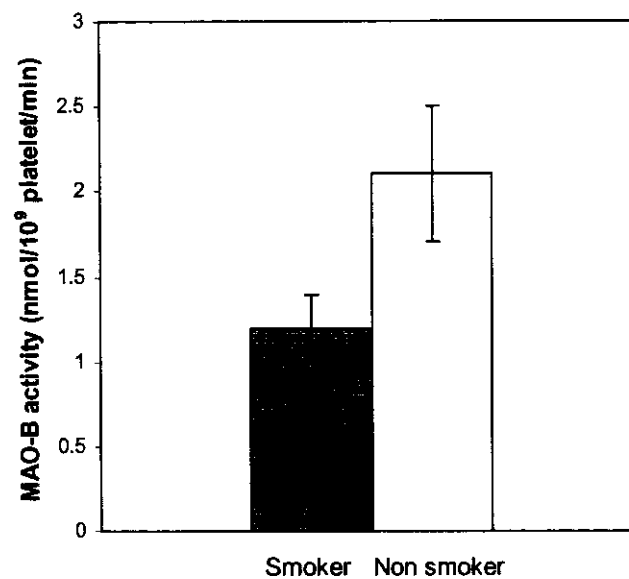


Figure 4.4. Maternal platelet MAO-B activity of women who smoked during pregnancy and women who did not smoke during pregnancy. Smokers showed significantly lower ($p<0.1$) platelet MAO-B activity than non-smokers. Error bars indicate S.E.M.

4.1.3.2. Maternal platelet MAO-B activity

As previously mentioned, cigarette smoking during pregnancy significantly inhibited ($p<0.1$) platelet MAO-B activity (see Figure 4.4). This finding is consistent with all other research data to date that report lower platelet MAO-B activity in smokers compared to non-smokers (Oreland *et al.*, 1981; Yong and Perry, 1986; Norman *et al.*, 1987; Berlin *et al.*, 1995a). The lowered platelet MAO-B activity in the smoking group can be viewed as confirmatory data for our study, showing that the smoking women enrolled in the study, did smoke sufficient amounts to decrease platelet MAO-B activity significantly.

4.1.3.3. Maternal serotonin concentrations

If indeed placental MAO-A activity was inhibited by cigarette smoke a significant *increase* of maternal serotonin concentrations would be expected (see Figure 2.10). In our study however, there was only slight inhibition of placental MAO-A activity, not sufficient in magnitude to result in such an unequivocal outcome. According to our hypothesis our result should consequently show similar or perhaps a slight increase in serotonin concentrations for the smoking compared to the non-smoking group. Within the circulation, more than 99% of serotonin is stored in platelets (reviewed by Kema *et al.*, 2000). Therefore, platelet serotonin measurements are key in determining serotonin fluctuations *in vivo*. Interestingly, in our laboratory, when the serotonin concentration is expressed *per platelet rich plasma volume*, the smoking and non-smoking group appear to have similar serotonin concentrations (see Figure 4.5).

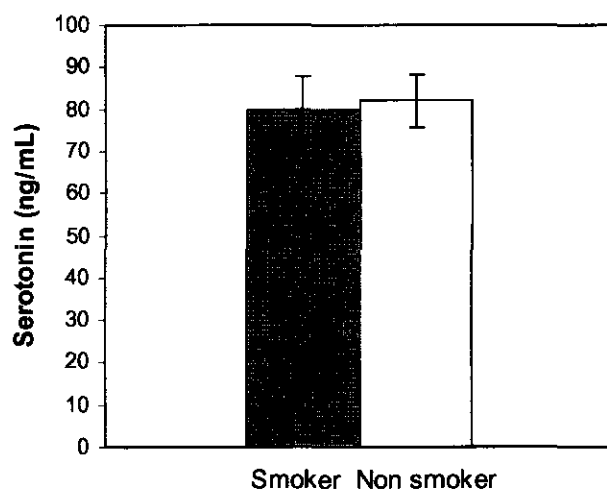


Figure 4.5. Maternal serotonin concentrations (ng/mL) as measured in PRP of women who smoked during pregnancy and women who did not smoke during pregnancy (i.e. equivalent to expressing serotonin concentration against platelet rich plasma volume in mL: for explanation, see text). The error bars indicate S.E.M.

However, when the serotonin concentration is expressed *per platelet number*, the smoking and non-smoking group appear to have different serotonin concentrations (see Figure 4.6). In the maternal blood the serotonin concentration expressed per platelet was significantly lower ($p < 0.05$) for the smokers compared to the non-smokers (see Figure 4.6). The smokers had 33% less serotonin per platelet compared to the non-smokers. This result is influenced by both the platelet count as well as the amount of serotonin present in the platelet rich plasma (where the serotonin is measured) since this result is expressed as serotonin concentration per platelet. Thus, expressed per volume the smokers and non-smokers have the same amount of serotonin but the smoking group has significantly less serotonin per platelet. In this regard it is important to note the number of platelets determined for each group (see Figure 4.7). The maternal platelet counts for the smoking group are significantly higher ($p < 0.05$) than for the non-smoking group. Our results show a 56% higher platelet count for women who smoke during pregnancy compared to their non-smoking peers.

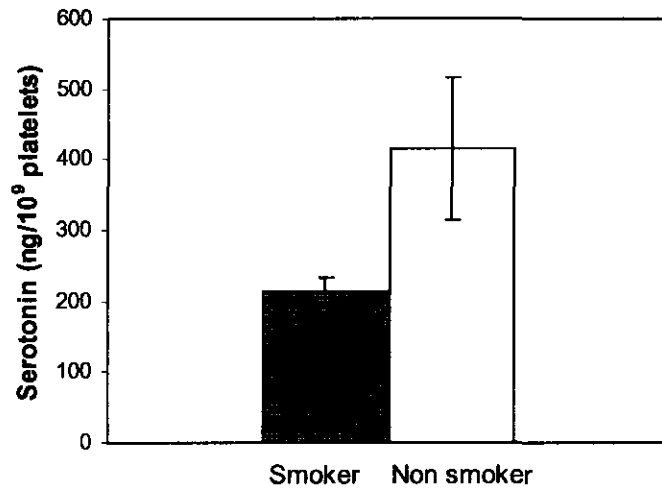


Figure 4.6. Maternal serotonin concentration as measured in PRP of women who smoked and women who did not smoke during pregnancy. The concentration is expressed as ng serotonin/10⁹ platelets (i.e. expressed against platelet number: for explanation, see text). Smokers showed significantly lower ($p < 0.05$) serotonin concentrations when expressed per platelet number. Error bars indicate S.E.M.

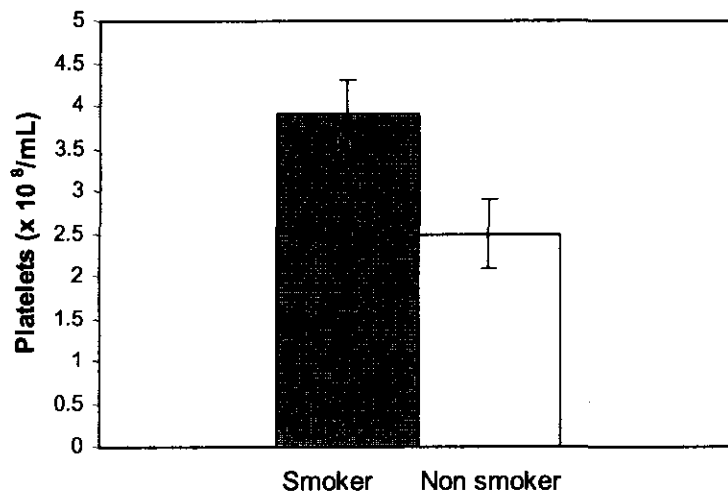


Figure 4.7. Platelet count as measured in maternal PRP of smoking vs. non-smoking pregnant women. Smokers had a significantly higher (by ~60%; $p < 0.05$) platelet count than non-smokers. Error bars indicate S.E.M.

The maternal platelet count determined in our laboratory ($2.5 \pm 0.4 \times 10^8$ platelets/mL) for the non-smokers compares well with platelet counts reported by Mercelina-Roumans and co-workers (1995) in 247 women during their 31st-40th week of pregnancy (reported $2.58 \pm 0.36 \times 10^8$ platelets/mL). A large study by Boehlen and co-workers (2000) consisting of 6,770 women also showed platelet counts (2.13×10^8 platelets/mL) that are consistent with our result for the non-smoking group.

Interestingly a study conducted in rats exposed to cigarette smoke for twelve weeks showed a significant increase in their platelet counts (Nagvekar and Abraham, 2002). However, literature about the influence of cigarette smoke on platelet counts in women is not consistent and most studies have been done in non-pregnant women. Previous studies determining the platelet MAO-B activity in smoking and non-smoking non-pregnant women showed no difference in platelet counts between the two groups (Oreland *et al.*, 1981). Green and co-workers (1992) reported lower platelet counts for female smokers (non-pregnant) compared to non-smokers. Van Tiel and co-workers (2002) reported a 4% increase in platelet count in female smokers (non-pregnant). Mercelina-Roumans and co-workers (1995) determined the platelet counts of smokers and non-smokers at different stages of pregnancy and showed a significant decrease in the platelet count (from 2.87×10^8 to 2.58×10^8) with gestational age for non-smokers, whereas women who smoked during pregnancy *did not show this decrease*. This implies a higher platelet count for the smokers compared to the non-smokers.

Of importance to our observations are reports indicating that there is a correlation between platelet count and mean platelet volume. During the last 8 weeks of gestation in 2,066 pregnant women there was a significant fall in platelet count together with a

significant increase in mean platelet volume (Fay *et al.*, 1983). During pregnancy pre-eclamptic women showed significantly lower platelet counts compared to control groups and correspondingly significantly higher platelet volumes (Järemo *et al.*, 2000). Interestingly, a significantly *lower* mean platelet volume has been reported for pregnant smokers compared to non-smokers (Merzelina-Roumans *et al.*, 1995).

Considering the reported lower mean platelet volume for smokers and the correlation between platelet volume and platelet count, our results showing a significantly higher platelet count for the smoking women might suggest a smaller platelet volume in these women. The smaller platelet volume in women who smoke during pregnancy would explain the observed lower serotonin content *per platelet*. This observation is confirmed by an earlier study showing that serotonin uptake is decreased in small platelets (Thompson *et al.*, 1982).

We speculate that a smaller platelet volume will decrease the ability of platelets to store serotonin which could lead to the production of more platelets in order to maintain the required amount of serotonin in the blood. Platelets have been reported to deteriorate in functional ability with decreasing size (Karpachin, 1964).

4.1.3.4. Cord blood serotonin concentrations

The serotonin concentration (expressed per volume) in the cord blood of the smokers (140 ± 15 ng/mL) and non-smokers (149 ± 27 ng/mL) are similar. Importantly the serotonin concentrations determined in the cord blood of both smoking and non-

smoking subjects were significantly higher than the serotonin concentrations measured in maternal blood samples ($p < 0.01$ for smokers and $p < 0.05$ for non-smokers; see Figure 4.8 and Table 4.2). This result is consistent with data reported by Jones and Rowsell (1973) showing that the mean concentration of circulating serotonin is higher in umbilical cord blood than in maternal blood immediately post partum. These researchers showed that the fetoplacental circulation contains high levels of serotonin in late pregnancy until delivery.

Jones and Rowsell (1973) determined serotonin concentrations in arterial and venous cord blood and demonstrated similar levels in both. They hypothesized that the increase in placental serotonin concentrations can be accounted for by an increasing production of serotonin in the developing fetus. Earlier, Loose and Paterson (1966) reported that 5-HIAA, a metabolite and major marker for the *in vivo* presence of serotonin (see Figure 2.9), only appeared in the amniotic fluid of women in the second half of their pregnancy. During the second half of pregnancy the concentration of 5-HIAA in the amniotic fluid shows a tendency to increase with the maturity of the fetus. This same group of researchers showed serotonin-producing argentaffin cell granules in the appendix of fetuses (Loose and Paterson, 1966).

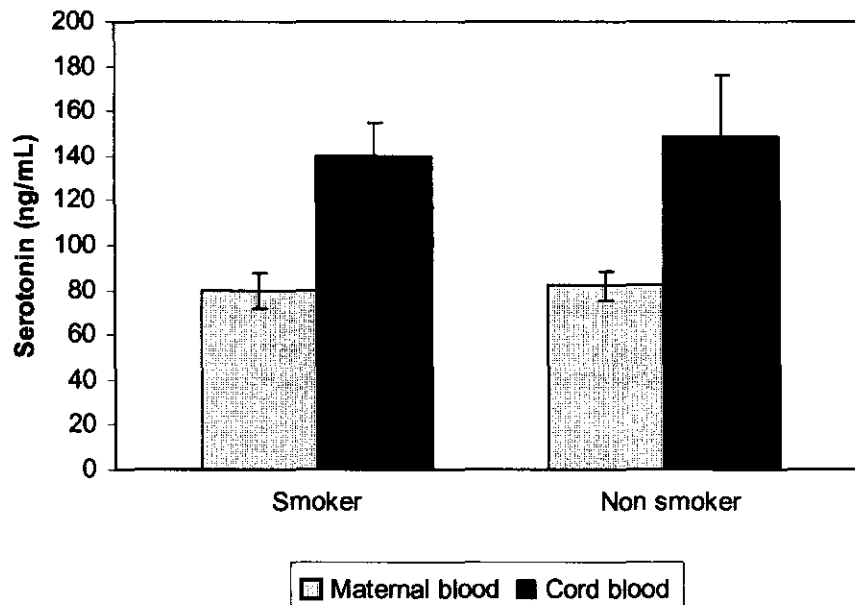


Figure 4.8. The maternal and cord blood serotonin concentrations measured in the smoking and non-smoking group. In both the smoking and the non-smoking group the serotonin concentration contained in cord blood is significantly higher ($p < 0.01$ for smokers; $p < 0.05$ for non-smokers) than contained in maternal blood.

The serotonin concentration per platelet in the cord blood of the smoking pregnant women was 106% higher than in the cord blood of non-smokers; however this difference was not statistically significant due to a large variance (see Figure 4.9). It is once again important to remember that this result (serotonin concentration per platelet) is a function of the platelet count. Also, in the cord blood the platelet count for the non-smoking group was 72% higher than the platelet count for the smoking group, even though this difference did not reach statistical significance (see Figure 4.10). It has previously been reported that the platelet count in the cord blood of smokers and non-smokers do not differ significantly (Mercelina-Roumans *et al.*, 1995; Anthony, 1989) or that smokers show a slight decrease in platelet count (Ahlsten *et al.*, 1985). In our study – in the non-smoking

cohort – the platelet count in the cord blood and the maternal blood did not correspond (Figure 4.11/Table 4.2.). The cord blood platelet count was found to be significantly higher (tested at $p > 0.01$) than the maternal blood platelet count. However, some studies contend that there is no difference in cord and maternal blood platelet counts (Tsao *et al.*, 2002; Andrew *et al.*, 1990). The platelet counts determined in the maternal and cord blood of the *smoking* group were similar and thus consistent with the literature reports. Our data could be explained by arguing that the large value of the platelet count determined in the cord blood of the non-smoking group might be an artifact. Cord blood samples were not available for all the women enrolled in the study ($n = 5$ vs. $n = 9$ for maternal blood determinations) and there was variation in the sample volume of the cord blood samples that were analyzed. Differences in sample volume will result in differences in the platelet count (Day *et al.*, 1975). The small sample size and differences in sample volume were confounding factors in this study and may be the cause for the high platelet count measured in the cord blood of our non-smoking group.

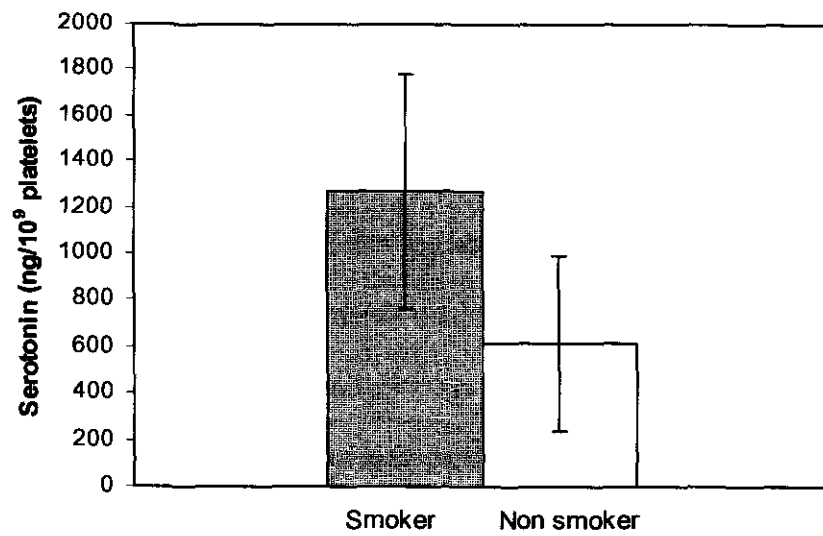


Figure 4.9. Cord blood serotonin concentration as measured in PRP of women who smoked and women who did not smoke during pregnancy. The concentration is expressed as ng serotonin/10⁹ platelets. Smokers showed a trend towards higher serotonin concentrations when expressed per number of platelets although this trend did not reach significance. Error bars indicate S.E.M.

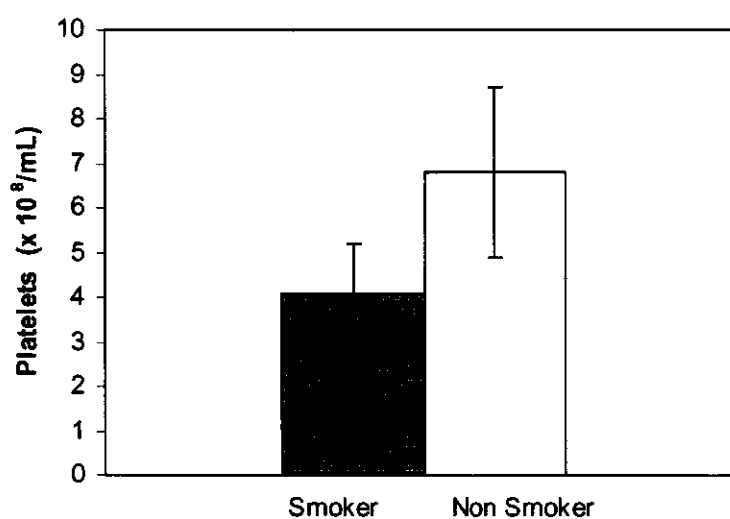


Figure 4.10. Platelet concentration as measured in the cord blood PRP. Smokers had a lower platelet count than non-smokers but this trend did not reach significance. Error bars indicate S.E.M.

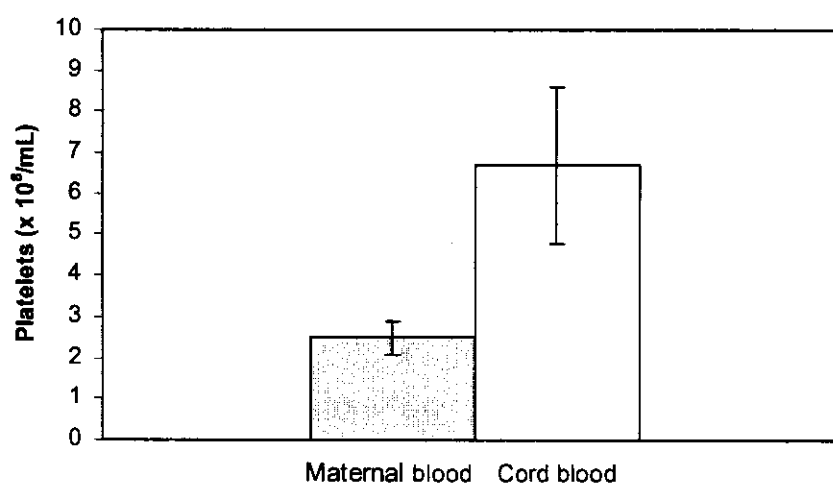


Figure 4.11. Platelet counts determined in the maternal blood (n = 9) and cord blood (n = 6) of non-smokers. The cord blood platelet count is significantly higher ($p < 0.01$) than the maternal platelet count. Error bars indicate S.E.M.

4.1.3.5. Cord blood results as calculated with a hypothetical platelet count

The remainder of the cord blood results will be discussed, with the assumption that the platelet count obtained in the cord blood of the non-smokers is an artifact. It was decided to use a hypothetical platelet count for the cord blood of the non-smoking group in order to see what the results would have looked like when a smaller, more realistic platelet count was measured. Thus, a hypothetical serotonin concentration per platelet and MAO-B activity per platelet will be calculated by assuming that the non-smoking cord blood platelet count is equal to the cord blood platelet count for the smoking group. Thus, we assumed that the platelet count for the cord blood in the non-smoking group is closer to $4 \times 10^8/\text{mL}$.

When assuming that the non-smoking cord blood platelet count should be equal to the cord blood platelet count of the smoking group, the serotonin concentration per platelet in the cord blood of the non-smokers will be closer to the serotonin concentration per platelet in the cord blood of the smokers (compare Figure 4.12 and Figure 4.9). Both the smoking and non-smoking cord blood serotonin concentrations will be higher than for the maternal blood serotonin concentrations. These results are expected in the light of researchers reporting higher serotonin concentrations in cord blood compared to maternal blood (Jones and Rowsell, 1973).

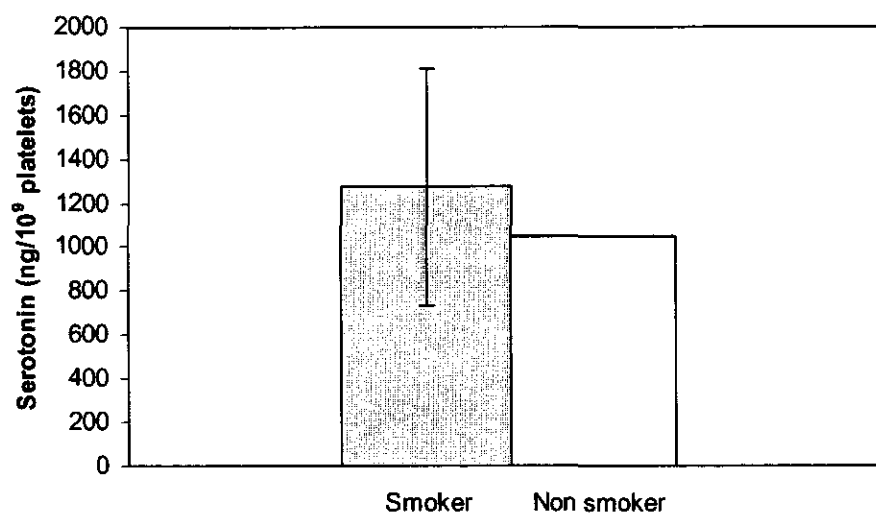


Figure 4.12. Cord blood serotonin concentration as measured in the smoking group compared to the hypothetical non-smoking serotonin concentration, calculated by using the maternal platelet value as described in the text. Error bar indicates S.E.M.

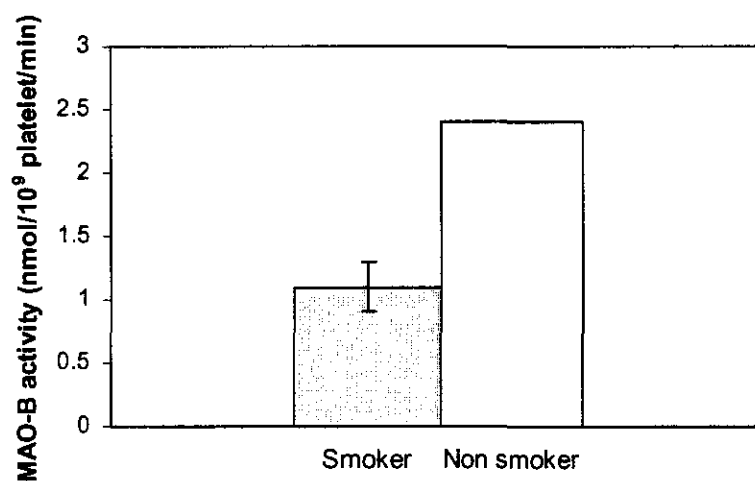


Figure 4.13. Cord blood MAO-B activity in smokers compared with hypothetical MAO-B activity determined for the non-smokers (by using the maternal platelet count in the calculation; see text for details). Error bar indicates S.E.M.

Interestingly, platelet MAO-B activity determined in the cord blood did not show a significant difference between smokers and non-smokers (1.1 ± 0.2 vs. 1.4 ± 0.2 nmol/ 10^9 platelets/min). When the cord blood platelet count of the non-smokers is adjusted to the hypothetical value (i.e. equal to the cord blood platelet count in smokers), the cord blood MAO-B activity of non-smokers is considerably higher than that found for the smokers (see Figure 4.13).

Also, in the smoking group, the maternal and cord blood platelet MAO-B activities were similar (1.2 ± 0.2 vs. 1.1 ± 0.2 nmol/ 10^9 platelets/min) (Table 4.2). This result is consistent with reports by Jones and Southgate (1976) stating that platelet MAO activity in the human fetus of 40 weeks gestation is equivalent to that found in the adult. In the non-smoking group the maternal MAO-B activity was 43% higher than the cord blood MAO-B activity even though this difference was not statistically significant. The hypothetical MAO-B activity for the non-smoking cord blood samples will be 2.38 nmol/ 10^9 platelets/min. Thus the maternal MAO-B activity in the non-smoking group will be 15% lower than the cord blood MAO-B activity in the same group (see Figure 4.14). The adjusted value is therefore closer to the expected value.

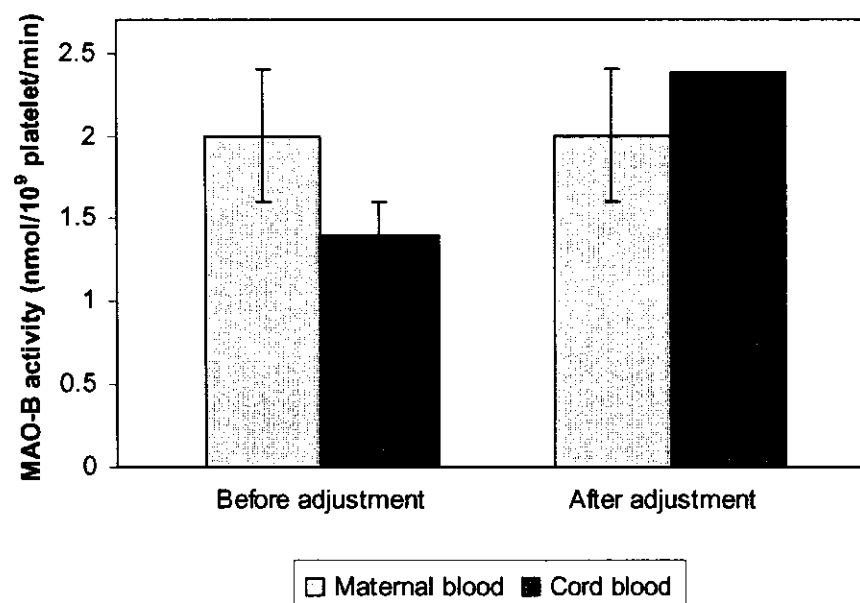


Figure 4.14. Platelet MAO-B activity in the non smoking group as determined with the measured platelet count in maternal blood and cord blood vs. determined with the hypothetical platelet count in the cord blood.

CONCLUSIONS

5.1. The influence of tobacco smoke constituents on MAO activity *in vitro*

Prompted by literature reports on the influence of tobacco smoke constituents on MAO activity *in vitro* we embarked on a series of experiments to investigate – in comparative studies – tobacco smoke and tobacco leaf extracts. These experiments were done with a view to ascertain whether pyrolysis products of tobacco leaf products (as found in cigarettes) that form at high temperatures during the smoking process, will result in significantly different levels of MAO inhibition compared with products extracted directly from the tobacco leaf content. In Chapter 2 it was thus hypothesized that extracts of cigarette smoke will inhibit MAO more than extracts of tobacco leaves due to the presence of pyrolysis products formed in the process of smoking, and that these products are more active MAO inhibitors than products present in tobacco leaf. Acetone extracts of tobacco leaf were compared with cigarette smoke extracted in acetone using an *in vitro* MAO inhibition assay developed in our laboratory. Crude extracts of cigarette smoke applied to primate mitochondrial MAO preparations showed ~20 times more inhibition of MAO-B than inhibition measured for tobacco leaf extracts under identical experimental conditions. This finding led to the conclusion that pyrolysis products formed in the process of smoking are likely to be the entities that impart more potent MAO-B inhibition to smoke than found in extracts of unburnt tobacco leaf. These studies also revealed that there is a difference in inhibition of the two forms of the MAO enzyme upon challenge with tobacco smoke (and leaf) extracts. Cigarette smoke extracts

inhibited MAO-A 85% less than measured for MAO-B. Tobacco leaf extracts also were more potent in inhibiting the B form of the enzyme but this difference was not as pronounced, likely due to the overall lesser effect of the leaf extracts on MAO enzyme inhibition generally. The latter findings led us to conclude that:

- both MAO-A and -B are inhibited by tobacco leaf extract and cigarette smoke extract;
- MAO-A is less susceptible to inhibition by components of tobacco leaf and cigarette smoke extracts than MAO-B;
- tobacco (cigarette) smoke extract had significantly more pronounced MAO inhibitory effects – both in the -A and in the -B enzyme – than tobacco leaf extract;
- and that the difference in MAO inhibitory effects between tobacco smoke and leaf extracts likely is related to the formation of pyrolysis products formed during the smoking process that are present in smoke extracts but absent from leaf extracts.

To further investigate the influence of burning the contents of recreational smoking devices other than conventional cigarettes on MAO inhibition, several unique studies were performed on non-tobacco containing cigarette products, and tobacco-containing cigarette products that heat, rather than burn the tobacco.

Magic® cigarettes are recreational smoking products that do not contain any tobacco but a blend of different herbs and flavoring agents. These products are claimed to be "safe" and non-addictive alternatives to conventional tobacco cigarette products. Extracts of Magic® cigarette smoke and "leaf" (i.e. the unburnt dry contents of the cigarette) were tested for MAO inhibition. In an astonishing finding, we discovered that the smoke

extracts of Magic® cigarettes ($IC_{50} = 14 \mu\text{g/mL}$) showed *similar* potencies for inhibiting MAO-B than found for extracts of Marlboro® (a popular tobacco-containing cigarette brand) smoke ($IC_{50} = 14 \mu\text{g/mL}$). These findings led us to the conclusion that the combustion (through burning during recreational smoking) of plant materials, *other than tobacco*, lead to the formation of pyrolysis products that are *as active* in inhibiting MAO *in vitro* than those formed in burning tobacco. Furthermore, these compounds (the products of plant pyrolysis) showed the same potency for inhibiting MAO-B than the compounds found in tobacco smoke extract. Significantly, extracts of Magic® (non-tobacco containing) smoke were 38% more potent in inhibiting MAO-A than extracts of Marlboro® (tobacco containing) smoke. Even more compelling are results that show "leaf" extracts obtained from Magic® cigarettes to be *more potent* than Marlboro® tobacco leaf extracts in inhibiting *both* MAO-A and MAO-B. From these findings it can be concluded that there are components found in the "leaf" of Magic® cigarettes that have a *higher* potency for MAO inhibition than compounds found in cigarette tobacco leaf. Our findings also suggest that these components form pyrolysis products that have *better* MAO-A inhibiting properties than pyrolysis products formed during the burning of Marlboro® cigarette tobacco leaves.

Accord® cigarettes are an example of a new type of cigarette that was introduced primarily to heat tobacco, rather than burn it as found during regular recreational smoking. This product provided another avenue to investigate the influence of smoking-derived pyrolysis products on MAO inhibition. MAO inhibition by mainstream ("heated" but not burned) extracts of Accord® cigarettes were compared with Marlboro® cigarette smoke extracts. In addition, smoke extracts obtained from a low tar cigarette brand (Parliament®) were also tested and compared with Accord® heated mainstream and

Marlboro® cigarette smoke extracts for MAO inhibition. It was expected that mainstream extracts obtained from the Accord® cigarettes will show less MAO inhibition compared with extracts obtained from either Marlboro® or Parliament® cigarette smoke. Our results confirmed this expectation and showed that both MAO-B and MAO-A were significantly ($p < 0.05$) *less* inhibited by smoke extracts obtained from Accord® cigarettes than those obtained from Marlboro® or Parliament® smoke extracts. The high tar (Marlboro®) and low tar (Parliament®) tobacco cigarette smoke extracts did not differ statistically significantly in their potencies for inhibiting MAO.

These results led to two major conclusions. First, primarily *heating* a cigarette leads to an extract that is significantly less active than extracts obtained from *burning* cigarettes. It is as yet unknown whether *fewer* pyrolysis products active as MAO inhibitors are being formed in the heating-only process as opposed to burning a cigarette, or whether the compounds that are present in burning tobacco smoke are *more potent* than those found in the "heated only" extracts. However, it can be stated that the smaller the amount of pyrolysis products formed, or the less active they are – the less inhibition of MAO should be observed. Second, tar content (as represented by comparative data generated from experiments with "high tar" Marlboro® and "low tar" Parliament® cigarettes) does not appear to influence the ability of smoke extracts to inhibit MAO. Therefore, tar likely is not involved in constituting the pyrolysis products responsible for MAO inhibition.

In an effort to investigate the type of MAO inhibition observed in Marlboro®, Parliament® and Accord® cigarettes, we measured whether the observed inhibition was reversible or irreversible. Investigators (Yu and Boulton, 1987) had, in earlier work, extracted cigarette smoke into an aqueous buffer and demonstrated that this extract

irreversibly inhibited MAO-B. We hypothesized that smoke collected in our acetone system will also show irreversible inhibition. Acetone extracts of Accord®, Marlboro® and Parliament® cigarettes were tested for irreversible MAO inhibition in a gel filtration assay. It was hypothesized that the Accord® smoke extract would show less irreversible inhibition than the Marlboro® or Parliament® smoke extracts based on our previous results. We were able to show irreversible inhibition of MAO-B with acetone extracts obtained from Accord®, Marlboro® and Parliament® cigarette smoke. Some MAO-B activity recovered after gel filtration, suggesting that a reversible inhibitor component is present in these smoke extracts. Despite being significantly less potent in inhibiting MAO-B activity, Accord® smoke extracts did show irreversible inhibition of MAO-B. However, this inhibition was observed at a 5 times higher concentration of smoke extract than required for similar inhibition by Marlboro® and Parliament® smoke extracts. Proportionally the same amount of MAO-B activity was recovered after gel filtration in all the samples. Marlboro® smoke extract (160 µg/mL) was also tested in the gel filtration assay for irreversible inhibition of the -A enzyme. These data show a 48% recovery in MAO-A activity after gel filtration whereas MAO-B with the same smoke extract (at 20 µg/mL) showed only a 30% recovery of activity. Even though the experiment with MAO-A was not repeated in further studies these preliminary data show that MAO-A is less subject to irreversible inhibition by extracts of cigarette smoke than MAO-B. The conclusion reached from these experiments is that some components extracted from cigarette smoke by acetone inhibit MAO-B irreversibly. It has been stated that the "smoking" of Accord® cigarettes leads to the formation of fewer, or less active products due to the absence of pyrolysis reactions such as found during smoking (i.e. "burning") of conventional cigarettes. Even so, the products that do form in the heating process of Accord® cigarettes show proportionally the same *ratio* of reversible and

irreversible inhibition of MAO-B (however, only at a higher concentration of smoke extract) as did the pyrolysis products found in Marlboro® and Parliament® smoke extracts. In conclusion, all of our results support the findings published by Fowler and co-workers (1996a, 1996b), and others, that components of tobacco smoke may be responsible for the lower activity of brain and platelet MAO-B seen in human smokers.

5.2. The influence of cigarette smoking during pregnancy on placental MAO-A

In view of the results obtained in Chapter 3 of this thesis and reports in the literature on the inhibitory effect of cigarette smoking on MAO, we hypothesized that cigarette smoking may also impair placental MAO-A activity *in vivo*. Inhibited placental MAO-A activity implies that adequate turnover of serotonin in the placenta will be compromised and therefore lead to higher maternal and fetal serotonin concentrations. In order to test this hypothesis, the placental MAO-A activity of mothers who smoked and of mothers who did not smoke during pregnancy, was determined. In addition, the maternal and cord blood serotonin concentrations were measured in each group. Platelet MAO-B activity was also determined as a control for the influence of cigarette smoke on the MAO-B enzyme, as reported by other researchers.

From our results we conclude that cigarette smoking during pregnancy does not significantly inhibit placental MAO-A. Consequently, maternal serotonin concentrations expressed per platelet rich plasma volume also are not affected. It can however be concluded that cigarette smoking during pregnancy results in significant inhibition of maternal platelet MAO-B activity. Furthermore our data suggest that cigarette smoking during pregnancy has a profound effect on the number of platelets in maternal blood.

The maternal blood of cigarette smokers showed a marked increase in platelet counts and subsequently lower serotonin concentrations per platelet compared with non smokers.

The inhibition of placental MAO-A in cigarette smoking pregnant women was not sufficient in magnitude to lead to significant increases in cord blood serotonin concentrations (expressed per volume) in the smoking, compared with the non-smoking group. The serotonin measurements also confirm previous reports stating that – at the time of birth – cord blood normally shows higher serotonin concentrations compared to maternal blood. Our results support our hypothesis that no effect (such as inhibition) on placental MAO-A activity would result in no change in maternal or cord blood serotonin concentrations. When cord blood serotonin concentration is expressed per volume of platelet rich plasma, such a scenario is indeed reflected in this study.

When cord blood serotonin concentration is expressed per platelet there are measurable differences between smokers and non-smokers, even though none of these differences are significant. The large difference in serotonin concentrations expressed per platelet in the cord blood of smokers and non smokers is attributed to the platelet counts. In an effort to understand the values determined for the serotonin concentration per platelet as well as the platelet MAO-B activity a hypothetical platelet count was suggested for the cord blood of the non smoking group. Results based on the hypothetical platelet count were closer to previously reported values in the literature although it will not be scientific to make conclusive remarks based on these results. Therefore in this study the values determined in smokers and non smokers can only lead to the conclusion that maternal cigarette smoke does not have a significant effect on platelet count, serotonin concentrations or MAO-B activity in cord blood.

LIST OF ABBREVIATIONS

CSC	cigarette smoke condensate
CSE	cigarette smoke extract
DA	dopamine
DMSO	dimethylsulfoxide
5-HIAA	5-hydroxyindole acetic acid
HPLC-EC	high performance liquid chromatography coupled with electrochemical detection
5-HT	serotonin
K_m	substrate concentration that produces half-maximail velocity
MAO	monoamine oxidase
MAO-A	monoamine oxidase A
MAO-B	monoamine oxidase B
MMDP ⁺	1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium
MMTP	1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine
MPDP ⁺	1-methyl-4-phenyl-2,3-dihydropyridinium
MPP ⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
NA	noradrenaline
NMET	N-methyl-5-hydroxytryptamine
PET	positron emission tomography

PMSF	phenylmethylsulfonyl fluoride
PRP	platelet rich plasma
TLE	tobacco leaf extract
TMN	2,3,6-trimethyl-1,4-naphthoquinone
TPM	total particulate matter
V_i	initial velocity in an enzymatic reaction
V_{max}	maximum velocity of an enzymatic reaction

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APPENDIX

A.1.

PATIENT CODE:

Questionnaire for Study on The Effects of Smoking during Pregnancy on Monoamine Oxidase A and B

The following information is required in order to evaluate factors which could affect this study. Please answer all questions as accurately as possible. This information is kept confidential. This information will be compiled for statistical purposes and will be collated using only the Patient Code, therefore confidentiality will be assured.

Date_____

1. Demographic information:

1.1. Age_____

1.2. Marital status:

Single_____

Married_____

Divorced_____

Widowed_____

Living with partner_____

Living with a group_____

1.3. Ethnicity/Race:

Indicate your ethnicity/race_____ for example: African-American; Oriental Asian; Indian Asian; Hispanic (white/black/American); Caucasian; American Indian; Asian Pacific Islander; African black; Arab; Japanese & Mexican; Chinese & Caucasian; other.

1.4. Education:

Last grade completed?_____

Did you attend college?_____

Highest degree earned?_____

2. Tobacco intake:

Have you ever smoked tobacco cigarettes? No_____ Yes_____

smoked cigars? No_____ Yes_____

smoked pipes? No_____ Yes_____

chewed tobacco? No_____ Yes_____

If you have **never** smoked/chewed tobacco, please go to section 3.

Previous smokers/chewers, please go to section 2.1.

Current smokers/chewers, please go to section 2.2.

2.1. Previous smokers:

At what age did you begin smoking/chewing tobacco? _____

How long (many years/months) did you smoke/chew tobacco? _____

When was the last time that you smoked/chewed tobacco? _____

How much did you smoke/chew per day? _____

What type of tobacco did you use? Pipe _____ Cigar _____ Cigarette _____

Did you stop smoking more than once? _____

If so, please describe how long ago and for what period(s)? _____

Did you stop smoking before/during your pregnancy? _____

At what stage of your pregnancy did you quit? _____

Prior to pregnancy _____ 1st Trimester _____ 2nd Trimester _____ 3rd Trimester _____

2.2. Current smokers:

At what age did you begin smoking/chewing tobacco? _____

How long have you been smoking/chewing tobacco? _____

Have you ever stopped smoking? _____

How many times have you stopped smoking? _____

When did you stop? _____

For what length of time did you stop? _____

How many cigarettes do you smoke per day? _____

How many cigars do you smoke per day? _____

How many times do you chew tobacco per day? _____

3. Tobacco smoke exposure:

3.1. Do any of your household members smoke? Yes _____ No _____

How many of the other household members smoke in your presence? _____

How often do the other household member(s) smoke in your presence? (Circle)

never sometimes frequently

3.2. Are you exposed to tobacco smoke for long periods of time at your work or other environment? _____

Yes _____ No _____

If yes, please describe the duration and type of exposure per day (e.g. heavy smoking co-worker) _____

4. Caffeine intake per day:

4.1. List approximate number per day:

Cups of coffee _____, Cups of tea _____, # of soft drinks which contain caffeine _____

Name of soft drink(s) _____

5. Alcohol intake:

Weekends only _____ Amount & type _____
Occasionally _____ Amount & type _____
Daily _____ Amount & type _____
Never _____

6. Other: Please list health food supplements, herbal medicines, over the counter and prescribed medications used, approximate dose and frequency.

7. Please include any other information that you feel may be relevant to this study.

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