Chapter 7

General Discussion

Every 10 seconds, another person dies as a result of tobacco use. The death toll is steadily increasing and unless current smoking trends are reversed the mortality figure is expected to rise to 10 million deaths per year by the 2020s or early 2030s (WHO, 1997). Among men in developed countries, smoking is estimated to be the cause of 40-45% of all cancer deaths, 90-95% of lung cancer deaths in those aged 35-69 years. Of all diseases causally associated with smoking, lung cancer is the most well known, simply because in most populations almost all lung cancer deaths are due to smoking.

Though the tobacco smoke contains a complex array of potent carcinogens, tobacco-specific nitrosamines (TSNAs) are now getting more attention because the levels of TSNAs are rising substantially in tobacco smoke (Hoffmann et al., 1993). The carcinogenic nitrosamines are formed from *Nicotiana* alkaloids during tobacco processing, smoking most likely during chewing of betel quid (Hoffmann and Adams, 1981; Hoffmann et al., 1994) and most recently endogenous formation of TSNAs in rats has been reported (Carmella et al., 1997). Therefore these additional amounts of tobacco-specific nitrosamines formed endogenously doubles the risk of lung cancer in tobacco users.
Magee and Barnes (1967) reported a large series of nitrosamines carcinogenic in a wide range of organs of various species. The most interesting attributes of carcinogenic nitrosamines are their systemic action and organ specificity. There has been increasing interest in the possibility that nitrosamines can be formed in vivo and so give rise to tumours in individuals not apparently exposed to nitrosamines per se. Lijinski and Epstein (1970) suggested that human cancers might be caused by nitrosamines formed in the body from ingested nitrites and secondary amines. Tertiary amines are also reported to have the property of nitrosation similar to that of secondary amines (Hein, 1963). Further, Smith and Loepky (1967) observed dealkylation of tertiary amines, to form a carbonyl compound, a secondary nitrosamine and nitrous oxide, on reaction with aqueous nitrous acid. Presumptive evidence for the in vivo biosynthesis of nitrosamines has been provided by the demonstration of methylation of nucleic acids in the stomach, liver, and small intestine of rats, following simultaneous gavage with N[14C] methyl urea and sodium nitrite (Montesano and Magee, 1971). In addition, nitrosamines have been identified in the stomach of mammals and man, following feeding of their precursors (Sen et al., 1969).

It was Druckrey and Preussmann (1962) who first suggested that conditions might exist in the burning of tobacco that could cause the production of nitrosamines. The formation of nitrosamines, from secondary amines, however, requires an equimolar mixture of nitrogen monoxide and nitrogen dioxide (Neurath et al., 1965). Nevertheless, qualitative and quantitative estimation of the probable precursors of nitrosamines in tobacco smoke need to be taken into consideration for any conclusion of nitrosamine
formation. Also important is the know-how of the ambient condition for the nitrosation reactions both in vitro and in vivo.

It has been suggested that nitrate can be reduced in the stomach and can therefore provide nitrite ions for interaction with ingested amines (Sander et al., 1968). Moreover, Greenblatt et al. (1972) demonstrated in rats and rabbits the influence of nitrite to amine, on the ratio of nitrosation. Thus a definite increase in yield of nitrosamine with increasing nitrite concentration in the stomach of animals at a pH 3.5 establishes the risk of carcinogenic burden of endogenously produced nitrosamines.

Nitrosamine formation in humans has been conclusively established by Bartsch et al. (1989). Nair et al. (1987) reported that apart from the pre-formed N-nitrosocompunds (NOC) present in betel quid with tobacco (BQT) or without tobacco (BQ), a substantial fraction of TSNA and other NOC are synthesised in vivo in the oral cavity, thus increasing the body burden of NOC. Further more, it has been suggested that tobacco-specific nitrosamines could be formed endogenously from the tertiary amine, nicotine (Hoffmann and Hecht, 1985; Hecht and Hoffmann 1988) and Hoffmann et al. (1994). Hecht et al. (1978) reported that nicotine can be nitrosated under mild conditions to produce NNK, NNN and NNA. The nitrosation of nicotine, similar to that of other tertiary amines (Hein, 1963) is a low yielding and a slow process than that of the related secondary amines nornicotine or anabasine (Mirvish et al., 1977; Caldwell et al., 1991). According to Caldwell et al., adding thiocyanate, a catalyst present in physiological fluids especially of smokers, doubles the risk of N-nitrosation of nicotine. The rate limiting step is the formation of iminium intermediate (Figure 6).
Tannenbaum (1976) reported that salivary nitrite concentration in healthy individuals are 6-10 mg/l, but can increase to 500 mg/l after ingestion of nitrite.

A possible mechanism of endogenous nitrosation has been given by Ishiwata et al. (1975) that nitrate ingested in food is re-secreted in saliva, starting about 30 min after its ingestion and reaching maximum after 1 to 2 hrs. The re-secreted nitrate is reduced to nitrite by the oral microflora. The tertiary amines or secondary amines probably react with salivary nitrite to give N-nitroso compounds under the acidic conditions prevailing in the stomach. The inhibition of the formation of N-nitroso compounds by ascorbic acid or $\alpha$-tocopherol as demonstrated by Ohshima and Bartsch (1981). Hoffmann and Brunnemann (1983) indicate the sensibility of the endogenous
nitrosation. Here \(\alpha\)-tocopherol or ascorbic acid competes with proline for nitrite resulting in inhibition of N-nitrosoproline formation.

Human exposure to nitrite occurs through the diet, via dietary nitrate and from endogenously produced nitric oxide (NO) (Assembly of Life Sciences, 1988; Marletta, 1988). NO which is overproduced via the inducible form of nitric oxide synthase during inflammatory processes that accompany infection by bacteria, parasite, or viruses can increase the endogenous formation of nitrosamines (Ohshima and Bartsch, 1994). Studies in infected animal models (Wu et al., 1993; Leaf et al., 1991; and Liu et al., 1991) and in humans (Ohshima et al., 1987; Tricker et al., 1989; Srivatral et al., 1991) have shown that chronic infectious conditions yielded a higher amount of nitrosamines derived from nitric oxide (nitrosating species) and nitrostable amines. Further, the present study showed hepatic injury on administration of nicotine. This is evident from the liver function tests and also from the increased lipid peroxidation in liver of nicotine treated rats. Adding to this is the inflammatory responses and changes observed in the lungs and liver of nicotine treated rats. The decrease in the level of serum total protein accounts for derangement of normal functions of liver. Therefore the endogenous nitrosation potential of a non-smoker increases gradually. Apart from non-smokers, smokers have an additional risk of 0.6 mg of nitric oxide per cigarette (Williams, 1980).

The results of the present study provide the first evidence of carcinogenesis by the endogenously produced nitrosamines from purified nicotine in rat lung. It also establishes the endogenous nitrosation potential in non-smokers. The incidence of oxidants is a determinant factor contributing to the nicotine-carcinogenesis. These oxidants are likely to be generated either by nicotine and its various metabolites or by the dietary
component. The hepatic injury is triggered by nicotine and its metabolites and the organospecific metabolites thus formed may be transported to lungs and cause serious inflammation there. The chronic inflammation observed in the lungs clearly establishes this fact.

Carmella et al. (1997) observed that nicotine was nitrosated endogenously on addition of sodium nitrate (180 μmol/kg) to produce N'-nitrosonornicotine (NNN). The carcinogenic potential of NNN has already been discussed. Their experiment was for a short duration with a nicotine dose of 60 μmol/kg body wt. twice daily for four days. Whereas the group III rats in the present study received a total dose of nicotine (23 mg) with single doses of 2.5 mg/kg body wt. Interestingly well below, in single dose, compared to the above study (60 μmol/kg body wt. or ~10 mg/kg body wt.), by Carmella et al. (1997). But the 22 weeks duration of nicotine administration (5 days per week) may have accumulated the endogenously produced nitrosamines to result in cancer of the lung.

According to Hecht (1996), the possible mechanism is that NNK is metabolically activated by cytochrome P450 enzymes. The metabolic activation of NNK by α-hydroxylation results in methylation and pyridyloxobutylation of DNA. The DNA methylation and pyridyloxobutylation pathways are crucial in tumour induction by NNK and depends on the species and tissue involved.

The mechanism by which NNK selectively induces lung tumours in rodents is not crystal clear, but several aspects favour this result. The rodent lung contains cytochrome P450 that metabolically activate NNK by α-hydroxylation resulting in DNA binding. The DNA adducts formed such as O6 mG and HPB releasing adducts can be highly persistent in lung or in particular cell types due to depletion of the critical repair enzymes. These
methyl and pyridylloxobutyl DNA adducts are mutagenic leading to the permanent alterations in important genes.

But it is more likely that on the course of duration of the experiment nitrosamines other than NNN, viz. NNK and NNAL, also might be produced. The NOC thus produced, on metabolic activation by cytochrome P450 enzymes produce various metabolites in rat liver, lung and nasal mucosa. The oxidation products thus form adducts with DNA leading to the initiation of cancer of the lung in rats. The fate of nicotine, after endogenous nitrosation to produce TSNAs and their metabolites with the property of organospecificity to lungs form adducts with DNA, initiates tumours in lungs as suggested in Figure 7.

**Figure 7** Endogenous formation of nitrosamines and their possible mechanism of tumourigenesis (Adopted and modified from Hecht, 1998)
Apart from several potential DNA adducts, NNK induces single strand breaks upon generation of superoxide (Weitberg and Corvese 1993). An increased frequency of activation of Kras the GGT→GAT mutation in codon 12 have also been noted in hyperplasias in mouse (Devereux et al., 1991 and Belinsky et al., 1992). Other genotoxic effects of NNK and NNAL include induction of unscheduled DNA synthesis in rats hepatocytes, chromosome aberrations sister chromatid exchanges and micronuclei (Alaoui-Jamali, 1988; Liu et al., 1990). However, present study showed chromosomal aberrations including anaphase bridge, hypernuclei etc. which have already been discussed.

According to Hecht (1996), NNAL is not a detoxified metabolite of NNK, since its carcinogenic activity is similar to that of NNK. NNAL, the NNK carbonyl reduction product is distributed in rodent and human liver, as well as in human lung (Smith et al., 1992; Castonguay et al., 1983). But the oxidation of NNAL to NNK has not been characterised. This reaction however, may be important because the carcinogenic effect of NNAL would be due in part to re-conversion to NNK.

NNN is rapidly distributed and metabolised in tissues. NNN metabolites are excreted mainly in urine. Consistently metabolites resulting from α-hydroxylation, pyridine N-oxidation and denitrosation are observed in urine along with a small amount of unchanged NNN (Hecht et al., 1981). 2′ and 5′ hydroxylation of NNN are the only metabolic pathways known to lead to DNA damage (Hecht and Chen, 1979).

Studies with animals have shown that P450 enzymes are involved in the metabolic activation of TSNAs. But the roles of specific P450 enzymes in the catalysis in different tissues remain to be identified.
Although there are still important gaps in our mechanistic understanding of TSNAs induced tumourigenesis, the studies in rodents and primates have facilitated development of methods to assess TSNA bioactivation in humans which will be applicable to studies of lung cancer susceptibility and prevention (Hecht, 1997). Extensive studies have clearly shown that cytochrome P450 is mainly implicated in the bioactivation of NNK (Hecht, 1996). Though P450s are not major catalysts of NNAL formation carbonyl reductases mediate this reaction, reduction of NNK to NNAL. NNN is also metabolised by P450s (Adams et al., 1988).

There are different forms of cytochrome P450s involved in the biotransformation of TSNAs. It is interesting to note the structural similarities among NNN, nicotine and NNK relating to their metabolism by P450 2A6 (Patten et al., 1996; Nakajima et al., 1996; Patten et al., 1997).

Therefore, TSNAs are activated enzymatically to electrophiles that form carcinogen-DNA adducts. Detoxifying enzymes are competing with the activating enzymes in the metabolism of the procarcinogenic components. (Wynder and Hoffmann, 1994). Differences in carcinogen metabolic activation and detoxification are arguably can influence susceptibility, and such differences can be probed through genotyping and phenotyping approaches (Perera, 1997). Variation from person to person are known to exist in these two groups of enzymatic reactions, as well as in the repair reactions of DNA damage caused by TSNAs. These interindividual differences in metabolic activation and DNA repair reflect the acquired and inherited host factors that may influence the risk for a smoker of developing lung cancer. Au et al (1999) observed that inheritance of variant versions of certain polymorphic genes is frequently associated with the development of
lung cancer from cigarette smoking. The genetic risk for tobacco-related cancers is associated with polymorphisms of the CYPIA1 (cytochrome P450 1A1) GST M1 (glutathione-S-transferase M1) genes in terms of genotype frequencies and cigarette smoking dose have been well established (LeMarchand et al., 1998; Arai et al., 1999; Bennett et al., 1999; Salama et al., 1999; Sato et al., 1999; Smith et al., 1999). Soldan et al. (1999) identified 11-beta hydroxy steroid dehydrogenase type 1 as microsomal NNK carbonyl reductase in liver and lung and implicated its role in inter individual differences in the susceptibility of tobacco smoke related lung cancer. In agreement with the above observations present study also showed the escape of two rats each from Group III when the experiments repeated.

Another important observation made in the present study is the endogenous nitrosation and subsequent tumourigenesis of the lung in comparatively less dose of nicotine administered group. The effective daily dose of nicotine administered for 22 weeks duration is 2.5 mg/kg body weight of the rat. However, the higher dose groups, group IV and V received a daily dose of 5 and 7.5 mg of nicotine/kg body of weight respectively. But groups IV and V failed to produce tumours on lung whereas, hyperplasia in lungs and a significant hepatotoxicity were observed in the maximum dose group. This is quite similar in observation to that of Belinsky et al. (1990). They reported 6.7% lung tumour incidence and 16.4% incidence of hyperplasia in a group of 60 rats when NNK is administered with a lowest total dose 1.8 mg/kg body wt. In the same study a total dose of 6 mg/kg induced a 10% tumour incidence and a 15% incidence of hyperplasia. In a later study however, the 6 mg/kg dose induced significant pulmonary hyperplasia only (Boorman, 1994).
Clearly, the lung tumours resulted from systemic absorption of NNK. It should also be noted that in dose response studies, lung tumours are prevalent at lower doses where they are often observed to the virtual exclusion of other tumour types (Rivenson et al., 1988).

So the results observed clearly indicate the endogenous nitrosation processes taking place during subcutaneous administration of nicotine. The nitric oxide for this process may be formed during the inflammatory processes associated with nicotine cytotoxicity. As a result, carcinogenic nitrosamines are formed endogenously which in turn produces tumours of the lungs.

Nicotine substitution therapy is the most successful and widely used pharmacological approach in smoking cessation programmes and nicotine is being considered as a drug for other conditions (Gora, 1993). It has been conclusively established by Rose et al. (1993) that subjects using nicotine patch and nicotine gum concentrate nicotine in their saliva. Adding to this the fact that humans, like rats metabolize nicotine to nornicotine and the possibility of concentrating nicotine in saliva (Benowitz et al., 1994) where nitrite occurs in abundance create a favourable ambience for nitrosation reactions. That is when saliva is swallowed, the nicotine/nornicotine along with nitrate reaches stomach which provides a favourable pH for nitrosation. In the stomach nicotine and nornicotine will remain for a long time due to the inability of the nearby cells to absorb protonated nicotine and nornicotine. Therefore, as part of nicotine replacement therapy, the subjects are irrationally forced to endogenous nitrosation for a rational approach to quit cigarette smoking.

In a practical approach to explore the endogenous nitrosation of nicotine, the present study established endogenous nitrosation of nicotine as
well as the carcinogenic potential of the endogenously produced nitrosamines. It is also worthwhile to conclude from the present study, the feasibility of endogenous nitrosation potential. Together with this the potential of oxidants in smokers and the shortcomings of the antioxidant enzymes to resist the peroxidative damage of tissues mainly the macromolecules triggered by nicotine invites more attention to the problem of the hour. To conclude, it is fair to think that nicotine should be given full credit for its all round influence on the pathogenesis of lung cancer.