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Reproductive and Developmental Effects

Smoking among women of reproductive age is a critical risk factor for reproductive health problems including fetal and infant mortality and impaired fetal development. Cigarette smoking has numerous well-documented adverse effects on pregnancy and fetal health, including low birthweight, preterm delivery, perinatal morbidity, placental complications, and increased risk of sudden infant death syndrome (USDHHS, 1988, 1990). The harmful effects of cigarette smoke exposure during pregnancy have been well known for decades; nevertheless, a significant fraction of pregnant women continue to smoke and smoking continues to account for an estimated 10% of all fetal mortality (Kleinman et al., 1988). The percentage of women who smoke during pregnancy declined from 13.6% in 1996 to 12.9% in 1998 with rates being highest for non-Hispanic whites, American Indian, and Hawaiian women and for women of lower socioeconomic and educational levels (CDC, 1998, 1999, 2000). The number of cigarettes smoked per day has also steadily declined over the last decade with about a third of maternal smokers reporting smoking at least a half a pack per day in 1996 compared to more than 40% in 1990 (CDC, 1998). Among pregnant teenagers, however, the smoking rate increased from 18.8% in 1997 to 19.2% in 1998 (CDC, 2000).

Trends in maternal smoking behavior, based on data from the Behavioral Risk Factor Surveillance System, showed a decline in overall smoking initiation among women aged 18-44 to a reported rate of 38.2% in 1996, with no difference between pregnant and nonpregnant women. Reported rates of quitting in the same population have shown little change

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among pregnant and nonpregnant women, 25.2% and 14.4%, respectively in 1996 (Ebrahim et al., 2000). Even after learning that they are pregnant, 54% of women continue to smoke (Ebrahim et al., 2000). Additionally, maternal smokers who have experienced previous preterm delivery or small-for-gestational age infants do not show greater quit rates than smokers who have had uncomplicated deliveries (Cnattigius et al., 1999). According to the National Health Interview Survey (1992-1993), among female smokers in general, 72.5% reported that they wanted to quit smoking with 34% attempting to quit each year and 2.5% being successful.

The contribution of environmental tobacco smoke (ETS), including paternal smoking, to adverse reproductive health outcomes is uncertain, but ETS exposure is widespread among women of reproductive age. Data from the third National Health and Nutrition Examination Survey report a 32.9% prevalence of ETS exposure at home or at work among non-tobacco-using females age 17 and over.

FERTILITY IMPAIRMENT

Smoking has been associated with increased time to conception, decreased pregnancy rate in assisted reproduction, increased risk of ectopic pregnancy, and menstrual changes including early menopause. The risk of being unable to conceive within a year of trying is increased two- to threefold among smokers (Werler, 1997). Consistent with many previous studies, a recent cohort study of current and past smokers during assisted reproduction cycles suggested a dose-related decrease in ovarian function and a 50% reduction in pregnancy rates of current smokers (Van Voorhis et al., 1996). Also, a positive relationship (odds ratio, OR≅1.3-2.2) between cigarette smoke exposure at conception and during pregnancy and the risk of subsequent ectopic pregnancy has been documented, with mixed results regarding dose-response (Coste et al., 1991; Handler et al., 1989; Saraiya et al., 1998; Stergachis et al., 1991). Animal studies have suggested altered gonadotropin release, decrease in luteinizing hormone (LH) surge, inhibition of prolactin release, altered tubal motility, and impairment of blastocyst formation and implantation as possible mechanisms of fertility impairment among smokers (reviewed in Hughes and Brennan, 1996). Additional studies in rats have shown follicle destruction and oocyte depletion when exposed to benzo[a]pyrene (BaP), a tobacco smoke toxin (Cooper et al., 1999). Furthermore, an evaluation of the Women's Health Study found that current and former smokers, after adjusting for age, race, education, marital status, number of sexual partners, frequency of intercourse, history of gonorrhea, and current method of contraception, had a significantly increased risk of pelvic inflammatory disease, possibly related to impairment of immunity and altered tubal

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factors (Marchbanks et al., 1990; Scholes et al., 1992). Pelvic inflammatory disease is an independent risk factor for ectopic pregnancy and infertility.

The association of altered male fertility with cigarette smoking is less consistent. Studies have shown mixed results with respect to sperm and semen quality and have not supported detrimental effects on male fertility (reviewed in Hughes and Brennan, 1996). Several animal and human studies have established an increased risk of vascular erectile dysfunction associated with cigarette smoking (Juenemann et al., 1987; Shabsigh et al., 1991; U.S. DHHS, 1990), including a large survey of Vietnam-era veterans, age 31 to 49, which found a 50% increase in the risk of impotence in smokers after controlling for all other major risk factors for impotence (Mannino et al., 1994).

SPONTANEOUS ABORTIONS

Numerous studies suggest a positive, dose-related association between smoking and spontaneous abortions, with the risk reported to be increased 20-80% (Kline et al., 1977, 1983; Ness et al., 1999). A prospective study by Ness et al. (1999) of adolescent girls and women that presented to an emergency room found a strong correlation between the risk of spontaneous abortion and the presence of cotinine (threshold concentration=500 ng/ml) in the urine (OR=1.8). The association is found primarily in the second trimester and for chromosomally normal spontaneous abortions (Kline et al., 1995) thought to be associated with intrauterine growth retardation. Postulated causal mechanisms include fetal hypoxia mediated by carbon monoxide (CO) and placental and uterine vascular insufficiency or teratogenic effects mediated by nicotine (Kline et al., 1995; Ness et al., 1999). The effect is modified by alcohol consumption, caffeine use, and history of previous spontaneous abortions (Ness et al., 1999; Windham et al., 1992). Although recent large prospective study found no consistent evidence of an association between environmental tobacco smoke and spontaneous abortion (Windham et al., 1999), a few earlier studies have described such a relationship (Ahlborg and Bodin, 1991; Chatenoud et al., 1998; Windham et al., 1992).

PLACENTAL COMPLICATIONS

Findings have been consistent regarding the positive association of smoking with placenta previa (obstruction of the internal cervical os by the placenta), with a relative risk (RR)=1.3-2.6 and placental abruption (premature separation of the placenta from the uterus), RR=1.4-1.6 (reviewed in Andres and Day, 2000; Castles et al., 1999). These pregnancy complications cause at least one-fifth of all prenatal deaths (Ananth et al.,

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1996). Also, among smokers the perinatal death rate after placental abruption is two to three times higher than among nonsmokers (Werler, 1997). There has been consistent evidence that smoking is an independent risk factor for placenta previa and placental abruption after control for potential confounders including maternal age and parity, hypertension, preeclampsia, and alcohol use.

Studies have suggested a dose-dependent association between reported numbers of cigarettes smoked and placental complications, but the data have been inconclusive (Ananth et al., 1996; Handler et al., 1994; reviewed in Andres, 1996). Although the exact mechanism by which maternal smoking causes placental complications is unknown, the placentas of smokers exhibit anatomical and histological changes that suggest hypoxia and underperfusion (Voigt et al., 1990). The placentas of smokers have been found to be larger and heavier than those of nonsmokers (Christianson, 1979; Pfarrer et al., 1999). Pfarrer et al. (1999) found increased angiogenesis within the placental villi of smokers, thought to be a response to hypoxic stress caused by the components of cigarette smoke. Pfarrer and colleagues speculated that this adaptive response contributes to the large placentas of maternal smokers. Histologic studies have found necrotic and hemorrhagic changes of the decidua basalis, including calcification, hypertrophy, and thickening of the basement membrane (reviewed in Voigt et al., 1990). The hypoxic and ischemic changes in placental tissues are possibly due to damage of the endothelial cells of placental vessels resulting in decreased tissue perfusion and to the increase in carboxyhemoglobin resulting in decreased oxygen delivery (Ananth et al., 1996). It has also been postulated that smokers are more prone to placental inflammation and infection secondary to a smoking-related decreased immune response.

PRETERM DELIVERY

Preterm delivery is defined as delivery before 37 weeks gestation and is an important cause of perinatal mortality. Studies have suggested an increase in the risk of preterm delivery among maternal smokers but have been inconsistent regarding the magnitude of risk (20% to >100% increased risk). Evidence of a dose-response association has been demonstrated (Cnattingius, et al., 1999, reviewed in Shah and Bracken, 2000; Shiono et al., 1986). Risk of infant mortality was more pronounced for very preterm births for preterm delivery (≤32 weeks gestation) (Kyrklund-Blomberg and Cnattingius, 1998; Windham et al., 2000) and with spontaneous versus induced preterm births after controlling for complications of pregnancy such as placental abruption, placenta previa, premature

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rupture of membranes, and hypertension (Kyrklund-Blomberg and Cnattingius, 1998).

The literature has supported the reduction of risk of preterm delivery associated with smoking cessation. A large population-based cohort study of Swedish women by Cnattingius et al. (1999) found that among nonsmokers with a term first delivery, those who initiated smoking after the first pregnancy had a greater risk of subsequent preterm pregnancies compared to women who did not smoke during pregnancy. Maternal smokers with an initial term delivery reduced their risk of subsequent preterm deliveries by stopping smoking prior to the second pregnancy. An earlier prospective interventional trial by Li (1993) found no improvement of gestational age with smoking reduction but did show a significant improvement after smoking cessation.

The mechanism of smoke-attributed preterm delivery is not certain but may be related to intrauterine infections secondary to decreased immunity, structural abnormalities especially the loss of integrity of type III collagen, or the increase in production of prostaglandin (PGE_2), causing myometrial muscle contractions (Cnattigius et al., 1999).

LOW BIRTHWEIGHT

The detrimental effect of smoking on birthweight has been extensively studied and well documented. This effect has been labeled "fetal tobacco syndrome," which is described as maternal smoking of five or more cigarettes per day during pregnancy, no evidence of maternal hypertension during pregnancy, symmetrical growth retardation of newborn at term, and no other explicit cause of intrauterine growth retardation (Benowitz, 1991; Nieburg et al., 1985). Twelve percent of infants born to all mothers who are smokers weighed less than 2,500 grams (low birthweight) in 1998, and eleven percent of infants born to mothers who report smoking as few as one to five cigarettes per day have low birthweight (CDC, 2000). Among pregnant smokers the risk of low birthweight babies is doubled compared to nonsmokers, and about 20% of all low birthweight babies are attributable to smoking (U.S. DHHS, 1983). Infants born to mothers who smoke during pregnancy are on average 200 grams lighter and 1.4 cm shorter than infants of nonsmokers (Wang et al., 1997). The effect of smoking is particularly prominent if exposure occurs after the first trimester. The association persists even after controlling for confounding factors such as maternal age, maternal nutrition, socioeconomic level, education, maternal weight gain, and alcohol consumption (reviewed in ACOG, 1997). Long-term effects of maternal smoking on growth have not been described, but small-for-

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gestational-age infants have a higher incidence of certain illnesses and disabilities into childhood and adulthood, such as cardiovascular disease, non-insulin-dependent diabetes mellitus, and hypertension (reviewed in Barker, 1997, 1999; Foresen et al., 2000).

Environmental tobacco smoke has also been suggested to have a significant association with intrauterine growth retardation. A recent Swedish study found an OR of 2.4 for low birthweight infants among nonsmoking mothers exposed to ETS and an OR of 3.6 for smoking mothers exposed to ETS (Dejin-Karlsson et al., 1998). Paternal smoking has also been shown to have an adverse effect on infant birthweight. Martinez et al. (1994) report an 88-gram average reduction in birthweight of infants whose fathers smoked more than 20 cigarettes per day.

The dose-response evidence has been studied, extensively, and the findings suggest reversibility of risk with smoking reduction (Ahlsten et al., 1993; Hebel et al., 1988; Li et al., 1993; Sexton and Hebel, 1984). Li and his colleagues (1993) in a prospective intervention trial found that reduction as well as cessation of smoking (defined by serum cotinine levels) resulted in significantly increased birthweights compared to women who continued to smoke. Secker-Walker et al. (1998) reported that women who stop smoking before 20 weeks' gestation obtain maximum benefits of smoking reduction. Others have found that cessation even late in pregnancy led to normal-weight infants (Ahlsten et al., 1993; Hebel et al., 1988). Attempts have been made to quantify the relationship of cigarette consumption and fetal growth outcomes. One such study, which evaluated taking the average of serial cotinine measurements over the entire pregnancy, reported that for every 1,000-ng/ml increase in urine cotinine concentration, there was an associated 59 ± 9 gram reduction in birthweight, an ≅ 0.25-cm reduction in length, and an ≅ 0.12-cm reduction in head circumference (Wang et al., 1997). Maternal serum and urine cotinine levels have been reliable markers of maternal nicotine intake and cigarette use and useful tools in predicting infant birthweight (Haddow et al., 1987; Li et al., 1993). Cotinine levels in infant cord blood are highly correlated with maternal serum and urine cotinine concentrations, r=.91 and r=.72, respectively (Wang et al., 1997). Among African Americans, a few studies have noted differences in the relationship between cigarette consumption and cotinine concentrations. Specifically, these studies have shown higher cotinine levels for all cigarette dose levels among African Americans (English et al., 1994). This group also reported no significant difference in birthweight reduction per 1 ng/ml maternal cotinine among black infants compared to white infants, and Li et al. (1993) found that among mothers with high baseline cotinine levels (>200 ng/ml), the birthweights of black infants were less sensitive to smoking reduction than those of white infants.

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The pathogenesis of low birthweight secondary to smoking is not known but is generally thought to be multifactorial. The probable mechanisms involved include CO formation of carboxyhemoglobin in maternal and fetal circulation, causing decrease in oxygen delivery and resulting in tissue hypoxia and increased viscosity that may affect placental perfusion (Benowitz et al., 2000). Cyanide in tobacco smoke can decrease stores of vitamin B_{12}, a cofactor for fetal growth (Ness et al., 1999). Additionally, smoking-related maternal and fetal nutritional deficits caused by cigarette smoking have been postulated as mechanisms for fetal growth retardation. Reduction in uteroplacental blood flow due to the vasoconstrictive catecholamine-mediated effects of nicotine has been suggested as a possible mechanism of intrauterine growth retardation and has been studied in animal models (Bassi et al., 1984). These findings have not been fully supported by measurements of placental blood flow in humans (Benowitz et al., 2000).

SUDDEN INFANT DEATH SYNDROME (SIDS)

It is estimated that more than 50% of the risk of SIDS may be attributed to exposure to cigarette smoke (Dwyer et al., 1999). A positive association between maternal smoking and SIDS has been consistently supported in the literature (reviewed in Golding, 1997; Leach et al., 1999). Generally, the risk of SIDS increases two- to fourfold among infants of mothers who smoke during pregnancy, and the risks increase even further when combined with postnatal exposure to tobacco smoke (ACOG, 1997). However, it has been difficult to separate the effects of postnatal smoke exposure from the effects of prenatal tobacco smoke exposure (Golding, 1997; Spiers, 1999). A recent prospective study found an OR of 2-3 for the association between prenatal maternal smoking and risk of SIDS, and an OR of roughly 3 for postnatal maternal smoking; however, no significant effect was seen for smoke exposure from other household members or maternal smoking restricted to rooms without the infant (Dwyer et al., 1999). The mechanism of effect is not fully known. It has been suggested, based on animal models, that fetal nicotine exposure results in loss of the normal response of the adrenomedullary system to hypoxia (Benowitz, 1998; Slotkin et al., 1995, 1997). Furthermore, the increased susceptibility of infants of smoking mothers to respiratory infections may play a role.

CONGENITAL MALFORMATIONS

Oral clefts is the most extensively studied malformation thought to be associated with maternal smoking. Research has failed to show a consis-

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tent association, although a moderate association with cleft lip ± cleft palate (CLP) has been described (Lieff et al., 1999). A recent study found a strong positive association between cigarette smoking and CLP, with a dose-response effect. This study found an overall 55% increase in the risk of infant CLP among all pregnant smokers, with the risk almost 80% higher for women who smoked more than a pack a day compared to nonsmoking mothers (Chung, 2000). Results have also been inconsistent regarding the interaction of maternal smoking with the rare transforming growth factor-α (TGF-α) allele and association with CLP (Christensen et al., 1999).

COGNITIVE AND BEHAVIORAL DEFICITS IN CHILDHOOD

Tobacco use during pregnancy has been linked to neurological damage that may be expressed during childhood as intellectual deficits, behavioral problems, and poor school achievement. Studies have found significant differences in IQ scores and increased likelihood of mental retardation in children of mothers who smoked during pregnancy compared to children of nonsmokers (Drews et al., 1996; Olds et al., 1994). The differences are decreased but generally persist after control for environmental and parental factors that may influence intelligence. Attention deficit hyperactivity disorder (ADHD) has been associated with smoking during pregnancy. An OR of 2.7 has been found for the risk of children of mothers who smoked during pregnancy after controlling for socioeconomic status, parental ADHD, and parental IQ. Furthermore, conduct disorder and disruptive behavior have been found to have a weak dose-related association with maternal cigarette smoking after control for confounding social factors (Fergusson et al., 1993).

Postulated mechanisms of cognitive impairment involve effects of cigarette smoking on the fetus including chronic hypoxia, decreased nutrition, and direct toxicity to cortical tissue by toxins such as CO, nicotine, and lead in cigarette smoke. Small-for-gestational-age infants, a significant adverse effect of maternal cigarette smoking as discussed previously, have been independently linked in several studies to decreased cognitive abilities (measured by IQ scores) into childhood and adolescence, compared to children of normal birthweight (McCarton et al., 1996; Seidman et al., 1992; Sommerfelt et al., 2000). Animal models have linked fetal nicotine exposure to upregulation of nicotinic receptors causing hyperactivity in infant mice (Milberger et al., 1996). Also, fetal nicotine exposure has potentially detrimental effects on fetal brain development through premature stimulation of nicotinic cholinergic receptors in the fetal brain causing disruption of the development of cholinergic neurons, which

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affects numerous other neurotransmitters and hormones, including catecholamines, serotonin, and dopamine (Benowitz et al., 2000).

FETAL LUNG DEVELOPMENT

Maternal smoking during pregnancy has been demonstrated to be associated with abnormal effects on fetal lung function and development, possibly contributing to increased incidence of early childhood respiratory diseases, including bronchitis, pneumonia, and asthma (Joad, 2000; Morgan and Martinez, 1998). Based on a review by Joad (2000), in utero cigarette smoke exposure more than doubles the risk for disease of airway hyperresponsiveness in childhood, including wheezing illnesses and asthma. In a large cross-sectional study, Cunningham and colleagues (1994) found significantly lower measures of flow in pulmonary function tests among 8-12 year olds who were exposed to maternal smoking compared to children of nonsmokers. After controlling for prenatal exposure, no significant effect of current ETS exposure was found. This finding was further supported in a study by Tager et al. (1995) that found significantly lower expiratory flow measurements of infants up to 18 months of age among mothers who smoked during pregnancy. Lodrup Carlsen and colleagues (1997) found supporting evidence that flow parameters were diminished when measured within days of birth after in utero smoke exposure. This study also found an effect on volume measurements and a dose-response relationship to number of cigarettes smoked per day. A study of infants who were seven weeks premature (Hoo et al., 1998) suggests that the detrimental effects on respiratory function occur before the last trimester and further supports the predominant effects of in utero smoke exposure compared to postnatal ETS exposure.

Suggested mechanisms of airway dysfunction caused by cigarette smoke exposure during pregnancy include decreased airway compliance, poor bronchial tree development, and emphysema-like changes of the alveoli (Cunningham et al., 1994; Lodrup Carlsen et al., 1997). Several animal studies have shown impaired fetal lung growth, including decreased lung interstitium and elastic tissue (Maritz et al., 1993; Moessinger, 1989). It has been suggested that nicotine interferes with the synthesis of elastic tissue needed for stability of the alveoli and that other smoke constituents may promote protease function (Maritz et al., 1993). See Chapter 14 for further discussion of this topic.

SMOKELESS TOBACCO

Reproductive effects of smokeless tobacco have been less extensively studied than those of smoking. Many of the human data come from coun-

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tries in which consumption of tobacco in other forms is more widespread among women. Animal studies have shown significant weight reduction and perinatal mortality among rat fetuses of mothers exposed to smokeless tobacco, administered through gastric intubation of smokeless tobacco aqueous extract (Krishna, 1978; Paulson et al., 1994). Researchers in India have found a significant decrease in birthweight of infants born to mothers who use smokeless tobacco (Deshmukh et al., 1998; Krishnamurthy and Joshi, 1993; Verma et al., 1983). Changes in placental anatomy were also noted by Agrawal and colleagues (1983), who discovered the placenta of tobacco-chewing mothers to be on average 65.9 grams heavier than that of non-using mothers. It has been suggested that nicotine-induced vasoconstriction and decreased perfusion may be the primary mechanism leading to decreased fetal growth in mothers who use smokeless tobacco (Verma et al., 1983).

CONCLUSIONS

Exposure to cigarette smoking is a major cause of fetal and infant morbidity and mortality. This is particularly true for the association with low birthweight and it consequences, as well as for preterm delivery and SIDs. For several important adverse reproductive effects of maternal smoking, a decrease in smoking has been found to decrease or be associated with a decrease in risks to the fetus and infant. The greatest benefit, of course, comes from smoking cessation. However, many women continue to smoke during pregnancy, despite knowledge of the harmful effects of smoking and personal experience with adverse fetal and infant conditions. Moreover, as current rates of smoking among adolescent women slowly rise, these adverse effects associated with tobacco smoke exposure while pregnant may worsen.

On average, infants exposed to maternal smoking in utero are 200 grams lighter and 1.4 cm shorter than those who are unexposed. A strong dose-response has been supported in numerous studies and a decrease in dose (number of cigarettes) in controlled studies has led to increased birthweights in a predictable pattern. What is known about the mechanism of effect of cigarette smoke on the fetus suggests a multifactorial etiology, with CO considered to play a major role in growth retardation through increased tissue hypoxia. Nicotine has also been thought to play a role through increasing vasoconstriction and decreasing perfusion through the placenta.

Although nicotine replacement products and bupropion SR are currently not approved by the Food and Drug Administration for use by

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pregnant women, the Agency for Healthcare Research and Quality's (AHCRQ) Clinical Practice Guidelines for Treating Tobacco Use and Dependence (Fiore et al., 2000) recommend that "Pharmacotherapy should be considered when a pregnant woman is otherwise unable to quit, and when the likelihood of quitting, with its potential benefits, outweighs the risks of the pharmacotherapy and potential continued smoking". It is generally thought that nicotine replacement therapy can reasonably be used with pregnant patients if prior behavioral modifications have failed and the patient continues to smoke at least 10-15 cigarettes per day (ACOG, 1997). There are no data regarding the efficacy of potential reduced-exposure products (PREPs) during pregnancy, but there is the presumption that the tobacco-related PREPs are likely to have toxic effects at some level and that, until further evidence is produced, existing guidelines concerning pharmacological PREPs still pertain.

RECOMMENDATIONS

Surveillance of Tobacco Use Patterns Among Pregnant Women

Central to understanding exposure to tobacco products is continuous population information on patterns of tobacco use among pregnant women. This may not be attainable by general population survey methods, due to inadequate sample sizes and insufficient representation of various geographic or demographic groups or of the earliest stages of pregnancy. Thus, surveys should be devoted specifically to pregnant women in all stages of gestation, irrespective of receipt of medical care. Survey content should include other known or putative causes of adverse maternal or fetal outcomes, as well as detailed product types and usage patterns as delineated in Chapter 6, in recommendations for general population surveillance.

Biochemical and toxicological exposure measures should be a routine part of surveillance for exposure to conventional products as well as PREPs. These will be necessary to conduct more precise, coordinated toxicological studies and also to assess actual exposure rates more accurately. For example, dose may be measured by maternal serum and urine cotinine levels, which have shown reliable correlations with maternal and consequently fetal tobacco smoke exposure. Self-reported smoking data can be unreliable, since pregnant women who have been advised to quit tend to under report tobacco use because of the stigma attached to smoking (Kendrick et al., 1995). Also, self-reports do not adequately account for differences in depth and frequency of puffs among smokers.

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Assessment of Fetal and Maternal Outcomes Associated with New Tobacco Product Exposure

To practically assess the health effects of PREPs, reliable measures of health outcomes that can be utilized in a relatively short time are desired. Among the reproductive outcomes of maternal smoking, intrauterine growth retardation resulting in low birthweight babies has been extensively studied, and a large body of evidence has supported a causal link with cigarette smoke exposure. The committee recommends, based on currently available scientific knowledge, that fetal birthweight and intrauterine growth retardation be used as the outcome measure in evaluating the harm reduction potential of the use of PREPs. Study designs might include repeated cohort or case-control studies of pregnant women with an appropriate distribution of exposures to both PREPs and conventional products, and suitable contrast groups. Concomitant, coordinated toxicological studies should be performed to provide biological correlations with clinical outcomes. Such outcomes as fetal birthweight and incidence of other reproductive and developmental health outcomes (e.g., fertility outcomes, placental complications, gestational age at birth, incidence of SIDS, spontaneous abortion, etc.) should be considered primary objects of study.

Findings in pregnant women exposed to PREPs may have value beyond maternal/fetal outcomes. The nature of adverse effects from PREP exposure will likely be determined much sooner in pregnant women (several months) than findings on chronic disease outcomes such as various cancers and cardiovascular disease in nonpregnant tobacco users. Should adverse findings become apparent, there may substantial implications for risk of chronic illnesses among nonpregnant adults, and coordinated pathogenic studies might allow conclusions on new tobacco product outcomes in advance of studies exploring longer "incubation periods."

Studies on the Component Exposures of PREPs

The committee recommends that further basic research be undertaken to elucidate the components of cigarette smoke that are primarily responsible for adverse health outcomes. In order to evaluate the safety of many PREPs, it is important to understand the effect of smoke components, especially nicotine and CO, on the pathogenesis of intrauterine growth retardation, spontaneous abortions and other health outcomes. In addition, better understanding of the risks of bupropion SR use by pregnant women (i.e., seizure risk) and the teratogenic effects of nicotine on the central nervous system, including dose-response and periods of vulner-

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ability during gestation, is needed for adequate risk-benefit analysis of the harm reduction potential of these products.

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16

Other Health Effects

This chapter briefly reviews some of the health outcomes of tobacco consumption not covered in previous chapters. Although these outcomes by themselves are not as prevalent or threatening as cardiovascular disease, respiratory disease, or cancer, they do contribute significantly to the morbidity and reduced quality-of-life associated with smoking. In addition, cigarette smoking is associated with a small number of positive health outcomes in Parkinson's disease, ulcerative colitis, and preeclampsia, which are reviewed here. This is not intended to be an exhaustive review of smoking-attributable disease but does include peptic ulcer disease, wound healing, inflammatory bowel disease, rheumatoid arthritis, oral disease, dementia, osteoporosis, ocular disease, diabetes, dermatological disease, schizophrenia, and depression.

PEPTIC ULCERS

Cigarette smokers have been found to have an increased risk of peptic ulcer disease, increased rate of relapse after treatment, and increased risk of the complications associated with ulcer development (Kato et al., 1992; Smedley et al., 1988). A prospective study by Kato et al. (1992) is consistent with previous findings suggesting a positive association between cigarette smoking and gastric and duodenal ulcers, with relative risks (RR) of 3.4 and 3.0, respectively. This association was maintained after controlling for alcohol use. Ma et al. (1998) reviewed potential mechanisms of the effect of smoking on ulcer formation and healing. These

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mechanisms include increased levels of oxygen-derived free radicals, which are known to injure gastric mucosa; decreased mucosal blood flow mediated by decreased levels of nitric oxide (NO; a potent vasodilator); increased neutrophil infiltration; reduced epithelial cell proliferation and granulation tissue formation; and decreased prostaglandin synthesis. Ma and his colleagues (1999) demonstrated grossly the slowing of ulcer healing and an increase in ulcer size associated with smoking in rat models, which were attributed to reduced blood flow at the ulcer margins and decreased constitutive NO synthesis. Cigarette smoking has also been found to augment nonsteroidal anti-inflammatory drug (NSAID) and alcohol-attributable peptic ulcer disease (Guo et al., 1999; Ma et al., 1998).

Ulcer healing has been found to improve with cessation and reduction of smoking. Presenting results consistent with many other studies, Tatsuta et al. (1987) described the course of healing of patients who were advised to reduce their smoking (by at least 50%) or to stop. They found that after 12 weeks of treatment, 91.7% of the ulcers had healed in patients who reduced or stopped smoking, while only 25% had healed in patients who continued to smoke. Furthermore, about 23% of ulcers recurred in patients who stopped or reduced their smoking during the six-month follow-up, while 75% recurred in those who continued to smoke.

SURGICAL WOUND HEALING

Cigarette smoking and its adverse effects on wound healing have been well established in animal models and in surgical patients (Mosely et al., 1978). Surgical complications experienced by smokers include increased failures of amputations, skin flaps, and atrioventricular (AV) shunts (Kwiatkowski et al., 1996). Mechanisms suggested for the adverse effects on wound healing include the cutaneous vasoconstrictive properties of nicotine, poor oxygen delivery due to an increase in carboxyhemoglobin levels leading to cellular hypoxia and tissue ischemia, and possibly interference with cellular respiration and metabolism due to hydrogen cyanide (HCN) (Campanile et al., 1998; Jensen et al., 1991; Kwiatkowski et al., 1996; Silverstein, 1992). Further detrimental effects on wound healing attributed to smoking are decreased microcirculation due to increased platelet aggregation and microclot formation and reduction of epithelial cells, fibroblasts, and macrophages, which are important in scar formation (Campanile et al., 1998; Silverstein, 1992).

INFLAMMATORY BOWEL DISEASE

Smokers with Crohn's disease, especially women, have increased risk of developing severe disease and have a greater risk of requiring surgery

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and of having postsurgical complications (Thomas et al., 2000). Studies, reviewed by Rhodes and Thomas (1994), have attributed a three- to five-fold higher risk of developing Crohn's disease to smoking. Ex-smokers were found to have less risk, and nonsmokers or those who had quit for at least ten years were found to experience the least risk. Several studies have found a decrease in the need for surgery after smoking cessation and a decrease in recurrence after surgery (Cosnes et al., 1996; Yamamoto and Keighly, 2000).

In contrast, smoking has been shown to have a protective effect for ulcerative colitis (UC), with odds ratios (ORs) between 0.34 and 0.48, and smokers with UC exhibit a better clinical course (reviewed by Calkins et al., 1989). The literature indicates a negative association (pooled OR=0.41) of current smoking with the development of ulcerative colitis, a significant positive association among ex-smokers, and a positive or equivocal risk among nonsmokers (Thomas et al., 2000). Researchers have found a positive influence of smoking on the clinical course of UC, with a lower rate of recurrence found among smokers (Fraga et al., 1997). Studies have revealed an earlier age of onset of ulcerative colitis in lifetime nonsmokers, and among a sample of patients who quit smoking, almost 70% developed UC during the first year postcessation (Rhodes and Thomas, 1994; reviewed in Thomas et al., 1998). The pathological process is involved in the relationship between smoking and inflammatory bowel disease is unknown. It is speculated that nicotine affects cellular immunity, especially the levels of immunoglobulin A (IgA) that are important in the defense of mucosal tissue (Rhodes and Thomas, 1994). It is also postulated that nicotine is involved in the reduction of blood flow and ischemic changes that may contribute to the changes seen in Crohn's disease.

RHEUMATOID ARTHRITIS

Environmental risk factors are known to be important in the pathogenesis of rheumatoid arthritis (RA). The few studies that have evaluated smoking as a risk factor indicate a positive link between cigarette smoking and the development of rheumatoid arthritis (Uhlig et al., 1999). This relationship has been identified among men (Heliovaara et al., 1993), with a more modest association found among women smokers (Karlson et al., 1999). Smoking has also been more closely associated with seropositive RA compared to rheumatoid factor (RF) negative disease (Silman et al., 1996). Evidence of a dose-response relationship has not been consistently upheld. It is unclear whether smoking plays a causal role in the etiology or progression of rheumatoid arthritis or the formation of rheumatoid factor or whether it is linked to other unidentified factors that predispose patients to RA.

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ORAL DISEASE

Research has consistently found that cigarette smoking is a major risk factor for periodontal disease (Tomar and Asma, 2000); studies report 30-75% of periodontitis linked to cigarette smoking. Smoking has been linked to increased frequency of diseased sites, reduced periodontal height, and a weak association with gingival bleeding (Bergstrom et al., 2000). A dose-related relationship has been described. In a review by Haber (1994), he noted that smokers had earlier onset of disease, resistance to treatment, and faster disease progression. Smoking cessation has been found to improve gingival health, and there is evidence of a decrease but not a complete reversal in the severity and prevalence of periodontitis among former smokers. Hypothesized mechanisms of action include smoking-induced impairment of immunity, reduced epithelial cell and fibroblast function involved in the healing process, decreased bone mineralization, and local nicotine-mediated gingival vasoconstriction leading to decreased blood flow and tissue oxygen delivery (Christen, 1992; Haber, 1994; Palmer et al., 1999).

Increased risk of oral lesions in immunocompromised smokers has been described. These lesions include hairy leukoplakia, oral candidiasis, and human papillomavirus (HPV) lesions; on the other hand, smoking has been consistently found to decrease the risk of aphthous ulcers (Palacio et al., 1997).

Smokeless tobacco use has been extensively linked to oral disease. The prevalence of leukoplakia, a white mucosal plaque that cannot be attributed to any other disease process, among smokeless tobacco users is very high (at least 50% by some reports), and it is generally accepted that smokeless tobacco use is an important cause (Spangler and Salisbury, 1995). The site of plaque formation in smokeless tobacco users corresponds to tobacco contact with the mucosa. The prevalence of tobacco-associated leukoplakia exhibits a dose-response relationship, and there has been evidence of reversal of these changes upon cessation of tobacco use (Grady et al., 1990). A review by Walsh and Epstein (2000) found the rate of malignant transformation of tobacco-attributable leukoplakia to range between 4 and 17.5%.

The dentitia and gingiva adjacent to the location of tobacco placement in the mouth are at a high risk of oral problems ranging from gingivitis and gingival recession to halitosis (Spangler and Salisbury, 1995). A study involving professional baseball players by Robertson et al. (1990) found that the only significant periodontal changes associated with smokeless tobacco use are gingival recession, loss of gingival attachment, and mucosal lesions at the area of tobacco placement. The evidence has been

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uncompelling regarding the risk of dental caries and gingivitis among smokeless tobacco users (Walsh and Epstein, 2000).

DEMENTIA

Many previous studies have suggested an inverse relationship between smoking and Alzheimer's dementia (Brenner et al., 1993; Forbes and Hayward, 1993; reviewed in Lee 1994). More recent studies, however, have suggested no relationship or possibly a positive association (Debanne et al., 2000; Doll et al., 2000; Merchant et al., 1999; Ott et al., 1998). In a large prospective study of British doctors, Doll et al. (2000) found no significant association of current or former smoking with the risk of Alzheimer's disease or other types of dementia including vascular dementia. In another large prospective study (the Rotterdam study) by Ott et al. (1998), it was found that smoking significantly increased the risk of vascular and Alzheimer's dementia and was linked to a younger age of onset. Smokers with the apolipoprotein-E4 (APOE4) allele have shown a reduction in dementia risk, consistent with many other studies (Carmelli et al., 1999; Merchant et al., 1999; Ott et al., 1998;). The association persisted when factors such as educational status, alcohol use, and ethnicity were controlled.

Nicotine, however, has been shown to have beneficial effects on cognition in human and animal studies. Evidence exists to suggest that nicotinic cholinergic receptors are decreased in Alzheimer's patients, and the receptors have been found to increase with chronic exposure to nicotine. Nicotine has also been found to decrease neuron loss in animal models of Alzheimer's disease (reviewed in Lee, 1994). Nicotine has been shown to acutely ameliorate recall, attentional, and visuospatial abilities (Leibovici et al., 1999; Newhouse et al., 1997) and to improve performance of memory-related tasks in animal studies (Buccafusco et al., 1999; Newhouse et al., 1997).

Attempting to reconcile the contradictory studies, investigators have postulated numerous reasons for the inconsistent findings. Investigators have raised the possibility that the protective effect of smoking in Alzheimer's disease (AD) may be mediated by the APOE4 allele (Ott et al., 1998; Van Duijn et al., 1995). Many of the earlier studies that pointed to a negative relationship between smoking and AD were case-control designs in which it is necessary to match cases and controls by age and other variables. In this design, there may be an increased effect of selective mortality among smokers, thus decreasing the proportion of smokers available (Debanne et al., 2000; Riggs, 1993). In addition to selection bias, other sources of bias that may have been a factor in the early studies include problems inherent in the design of retrospective studies such as

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recall bias and the use of surrogate informants for case patients (Doll et al., 2000) and, generally, poor control of confounding variables.

ORTHOPEDIC CONSEQUENCES

Cigarette smoking has been linked to adverse orthopedic consequences including osteoporosis, hip fracture, and delay in bone healing. A dose-response effect has been reported for the increased risk of hip fracture and decreased bone mineral density (BMD) in many but not all studies (Cornuz et al., 1999; Hoidrup et al., 1999; Hollenbach et al., 1993; la Vecchia et al., 1991;). Based on a meta-analysis by Law and Hackshaw (1997), one out of eight hip fractures is attributable to smoking. Smoking is associated with loss of bone mineral density, and this association increases with age especially in postmenopausal women. The study goes on to conclude that the risk of hip fracture is 17% greater in smokers at age 60, 41% greater at age 70, 71% greater at age 80, and 108% greater at age 90. Reversal of the risk for hip fractures has been described 10-20 years postcessation (Cornuz et al. 1999; la Vecchia et al., 1991). There have been few studies examining BMD and hip fracture risk among male smokers. An evaluation of the National Health and Nutrition Examination Survey (NHANES) I Epidemiologic Follow-up Study found a nonsignificant increased risk associated with smoking (Mussolino et al., 1998). A review by Kwiatkowski et al. (1996) stated that bone mineral content of smokers was 10-20% lower among men and 20-30% lower among women. Hypothesized mechanisms include impairment of osteoblastic function and consequently decreased bone formation, reduced calcium absorption, vasoconstrictive effects of nicotine that prevent revascularization of healing bone, and decreased body weight due to smoking, which is an independent risk factor for osteoporosis.

OCULAR DISEASE

Cigarette smoking has been associated with numerous diseases of the eye. Smoking can induce tobacco smoke-mediated vasoconstriction, atherosclerosis, hyperviscosity, hypercoagueability, and decreased oxygen availability in ocular tissues. There is a strong association with ischemic diseases including amaurosis fugax, retinal infarction, and anterior ischemic optic neuropathy (Solberg et al., 1998). Smoking has also been consistently associated with two of the most important causes of vision loss, cataracts and age-related macular degeneration. A dose-dependent relationship between cigarette smoking and risk and severity of cataracts has been established (Hankinson et al., 1992; West et al., 1995). A large prospective study by Delcourt et al. (2000) found an OR of 1.9 for nuclear-

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type cataracts in current smokers and a two- to fourfold increase in the rate of cataract surgery among current and former smokers. Most studies have found the increased risk limited to nuclear-type cataracts, although there has been evidence of an increased risk of posterior subscapular cataracts as well (Solberg et al., 1998). The risk of cataract formation among smokers appears to be related to lifetime cumulative cigarette dose, with less reduction in risk found among heavy smokers compared to moderate and light smokers after cessation (Christen et al., 2000; Hankinson et al., 1992). The mechanism of smoking-associated cataract formation is thought to be due to direct and indirect oxidative damage to the lens and exposure to heavy metals in tobacco smoke.

Age-related macular degeneration induced by free-radical formation and oxidative stress associated with cigarette smoking has also been well supported in the literature. Many studies have found a two- to threefold increase in risk of macular degeneration among smokers (reviewed in Solberg et al., 1998).

DERMATOLOGIC CONDITIONS

In many retrospective and prospective studies, cigarette smoking has been associated with premature wrinkling independent of age, sun exposure, pigmentation, and sex (Ernster et al., 1995; Smith and Fenske, 1996). Kadunce et al (1991) found a dose-response relationship associated with pack-years of exposure. Smokers with a 0.9 to 49 pack-year history were twice as likely to be wrinkled compared to nonsmokers. The risk increased to 4.7 among 50+ pack-year smokers. Tobacco smoke toxins are believed to have a detrimental effect on collagen and elastin integrity and on the microvasculature of skin, to increase oxidant stress free-radical activity, and to have possible antiestrogenic effects (reviewed in Frances, 1998; Kadunce et al., 1991; Leow and Maibach, 1998; Smith and Fenske, 1996).

Psoriasis also has been found to be associated with cigarette smoking, especially in women (Mills, 1993; Naldi et al., 1999; Poikolainen et al., 1994). A recent case-control study by Naldi et al. (1999) found an OR of 1.5-2.4 among smokers and exhibition of a dose-response relationship.

A few studies have also suggested a positive association of cigarette smoking with a variety of dermatological changes such as psoriasis palmoplantar pustulosis, atopic dermatitis, and yellowing of fingers and nails (reviewed in Smith and Fenske, 1996). These findings are not discussed in detail here.

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DIABETES

Several studies over the last decade have indicated a positive dose-related association between cigarette smoking and risk of non-insulin dependent diabetes mellitus (NIDDM) (Nakanishi et al., 2000; Rimm et al., 1993, 1995; Simon et al., 1997; Uchimoto et al., 1999). In a Nurses' Health Study cohort, Rimm et al. (1993) found a positive, dose-related association between cigarette smoking and development of NIDDM that persisted after controlling for age, body mass index (BMI), family history, physical activity, alcohol, and so forth. Simon et al. (1997), in another large prospective study, found a significant link between smoking and increased waist-to-hip ratios and an odds ratio of 1.38 for the relationship of smoking more than ten cigarettes per day and the prevalence of reported diabetes mellitus. The odds ratio was modified when data were adjusted for waist-to-hip ratio, suggesting a relationship between smoking-associated body fat distribution and the occurrence of diabetes. Investigators have consistently suggested that smoking is an independent risk factor for increased insulin resistance, measured by insulin clamp test and oral glucose challenge, among both diabetics and nondiabetics (Facchini et al., 1992; Targher et al., 1997), and increased transient blood glucose levels during oral and intravenous glucose tolerance tests (Janzon et al., 1983). Muhlhauser (1994) in his review of smoking and diabetes discusses the epidemiological evidence suggesting increased risk of diabetic nephropathy and retinopathy and increased overall mortality among smokers.

RENAL DISEASE

Smoking has been linked to abnormal renal function in diabetics and more recently in nondiabetics (Cirillo et al., 1998; Halimi et al., 2000; Pinto-Sietsma et al., 2000; Ritz et al., 2000). Research has consistently established the association of cigarette smoking with a two- to threefold increased risk of microalbuminuria and proteinuria and an increased rate of progression to diabetic nephropathy and ultimately end-stage renal disease in type I and type II diabetics in a dose-responsive manner (reviewed in Orth, 2000; Ritz et al., 2000). Other studies have found an independent association of smoking with increased risk of developing renal failure in patients with autosomal dependent polycystic kidney disease, lupus nephritis, and glomeruloephritis (Ritz et al., 2000).

Recently, consistent with previous findings, in a cross-sectional study of nondiabetic patients by Pinto-Sietsma and colleagues (2000), current smokers were found to have a dose-responsive increased rate of high-normal albuminuria and microalbuminuria (markers of some degree of

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renal damage). Current smokers also had significantly abnormal glomerular filtration rates (increased or decreased) compared to nonsmokers. Former smokers had a risk for both events that fell between that of current smokers and nonsmokers, suggesting some degree of reversibility of the effects of smoking. Another large population-based, cross-sectional study (Halimi et al., 2000) of subjects without known renal disease showed that current and former smokers, even after adjusting for diagnoses of diabetes and hypertension, had greater risks for proteinuria by dipstick testing compared to nonsmokers (RR=2-3). Current male smokers in this study also had a higher creatinine clearance than nonsmokers, suggesting a degree of glomerular hyperfiltration.

Many mechanisms of smoking-attributable nephrotoxicity have been postulated including increased sympathetic nervous system activity, transient blood pressure elevation, endothelial cell damage and dysfunction of renal vasculature, direct toxic effects on tublar cells, and oxidative stress (Orth, 2000; Pinto-Sietsma et al., 2000).

SCHIZOPHRENIA

It has been reported that up to 80% of patients with schizophrenia smoke cigarettes (McCreadie and Kelly, 2000; Simpson et al., 1999). The rate of smoking among schizophrenics is also higher than rates among other mentally ill patients (Leonard et al., 2000; Tidey et al., 1999). Many different reasons for the high rate of smoking among schizophrenics have been postulated including "self-medication" of the symptoms of schizophrenia, attenuation of the adverse effects of antipsychotic medication, social factors of schizophrenic patients that predispose them to smoke, or genetic vulnerability for both conditions (reviewed in Levin and Rezvani, 2000; Tidey et al., 1999). Nicotine may attenuate the negative symptoms of schizophrenia by stimulating the release of dopamine and glutamate (Dursun and Kutcher, 1999). Nicotine has also been found to stabilize sensory deficits found in schizophrenia including smooth eye tracking movements and auditory gating, possibly through decreased expression of the α-7 nicotinic acetylcholine receptor (Durson and Kutcher, 1999; Leonard et al., 2000). Furthermore, cigarette smoking has been found to reduce monoamine oxidase activity, which is thought to increase vulnerability to the development of schizophrenia (Fowler et al., 1996; Simpson et al., 1999).

DEPRESSION

Depression has consistently been linked with smoking (Breslau and Johnson, 2000; Glassman, 1993). Studies have demonstrated that a history

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of major depression is associated with a greater prevalence of smoking and less success in smoking cessation (Anda et al., 1990; Balfour and Ridley, 2000; Kinnunen et al., 1996). Kinnunen and colleagues, looking at data from a randomized interventional trial, found that significantly fewer depressed smokers were able to remain abstinent three months after cessation compared to nondepressed smokers. The same study concluded that depressed patients were more responsive to the use of nicotine for cessation. Investigators have suggested that depressive symptoms may be a part of the withdrawal syndrome in those with a history of depression, presenting an obstacle to successful cessation (Glassman, 1993). It has also been proposed that depression increases the intensity of nicotine dependence (Breslau et al., 1998; Carton et al., 1994). A prospective study by Breslau and colleagues (1998) with a five-year follow-up found that baseline major depression tripled the risk for progression to daily smoking. Smokers with a history of depression or depressive symptoms are thought to "self-medicate" with nicotine and its antidepressant properties (Carton et al., 1994; Lerman et al., 1996). Other studies have hypothesized that smokers are predisposed to develop depression secondary to chronic nicotine central nervous system (CNS) effects. Investigators have suggested that certain genetic or environmental factors may predispose patients to depression and the tendency to smoke independently (reviewed in Breslau et al., 1998).

WEIGHT CHANGE

Weight loss is a commonly cited reason for smoking, especially among young females (reviewed in Perkins, 1993), and fear of weight gain has been an obstacle to successful cessation (Emont and Cummings, 1987; Froom et al., 1998). It has been reported that around 80% of smokers gain weight after cessation (Perkins, 1993). The average weight gain after smoking cessation is 3-4 kg. This weight gain has been found to peak within the first few weeks or months of cessation, and smokers often return to the weight range of nonsmokers. A recent large prospective study, however, found that significant weight gain continued five years after cessation (O'Hara et al., 1998). The amount of weight gain, however, is subject to many individual differences in baseline BMI, age, physical activity, genetic predisposition, and so forth (reviewed in Froom et al., 1998; O'Hara et al., 1998; Pomerleau et al., 2000; Williamson et al., 1991).

The physiologic effect of nicotine has been described as a combination of a reduction in caloric intake and an increase in caloric expenditure. Although the evidence has been inconsistent, short-term increases in intake postcessation occur, peaking within weeks or months (Froom et al., 1998), and decreases in intake upon relapse or initiation have been noted.

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The research has supported acute changes in metabolic rate associated with changes in smoking, but chronic changes have not been as well supported. A further hypothesis for the weight change seen with change in smoking habits is the speculation that cigarette smoke or nicotine changes the weight set point of the smoker (reviewed in Perkins, 1993).

Investigators have found that weight changes upon smoking initiation and cessation are nicotine associated (Emont and Cummings, 1987; Grunberg et al., 1986). Consistent with many previous studies, Doherty et al. (1996) found that nicotine replacement during smoking cessation suppresses weight gain. In their randomized control study, they describe a linear relationship between nicotine dose and postcessation weight gain, with placebo users gaining the most weight. This relationship was maintained when smoking was biochemically validated by serum cotinine.

DRUG INTERACTIONS

Exposure to cigarette smoke affects the metabolism of many categories of drugs, which may make the action of a certain dose of a drug unpredictable. Certain constituents of tobacco smoke can affect drug action through pharmacokinetic (changes in absorption, distribution, metabolism, and elimination) and pharmacodynamic (changes in drug action or response) actions (Schein, 1995). Among the best-understood agents that affect enzymes of drug metabolism are polycyclic aromatic hydrocarbons (PAHs) and nicotine—though less defined—selectively induces various cytochrome P-450 (CYP) enzymes and uridine 5-diphosphate (UDP) glucuronosyltransferases, while carbon monoxide and heavy-metal constituents have been found to inhibit or decrease certain CYP enzymes (Zevin and Benowitz, 1999).

The effect of cigarette smoke exposure on drug metabolism can influence the action of many commonly used drugs, including certain antidepressants, antipsychotics, heart medications such as β-blockers and antiarrhythmics, anticoagulants, alcohol, caffeine, theophylline, and others (D'Arcy, 1984; Schein, 1995; Zevin and Benowitz, 1999). Many of the drug interactions are of unknown clinical significance, but others may necessitate the adjustment of medication dose among smokers.

Although not considered to be due to an interaction of cigarette smoke and oral contraceptive pills, it is important to note the significantly increased risk of cardiovascular and cerebrovascular events in female smokers using oral contraceptives.

FIRE SAFETY

Cigarettes are the leading cause of accidental fires in the United States, causing about 20-25% of all fire deaths and resulting in about 1,000 deaths

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and 2,500 injuries annually (Barillo et al., 2000; Brigham and McGuire, 1995). Often, fires are caused by the ignition of upholstered furniture and mattresses by lit cigarettes (Achauer and McGuire, 1989). Many fires also involve the use of alcohol by the smoker (Botkin, 1988). The fire hazard of cigarettes stems from the fact that a cigarette continues to burn until completely consumed.

There has been a push from the public health community to force cigarette manufacturers to modify cigarettes to reduce their combustibility (Chapman, 1999). In response to pressure from various organizations to decrease the danger of improperly discarded cigarettes, the Cigarette Safety Act of 1984 was signed. This act created the Technical Study Group in 1987 to study the technical and commercial feasibility of developing a fire-safe cigarette. This group was comprised of representatives of different medical organizations, federal agencies, the furniture industry, fire service, and four of the top tobacco companies (Barillo et al., 2000). The report of this group stated that it was technically and commercially feasible to develop fire-safe cigarettes and that a reduction of up to 90% in morbidity and mortality could be reached if such modifications were made (Achauer and McGuire, 1989). The main characteristics of cigarettes that were found to significantly decrease flammability included decreased packing density, smaller cigarette circumference, decreased paper porosity, and reduction of citrate—a paper-burning additive (Achauer and McGuire, 1989). The Fire Safe Act was passed in 1990 and mandated further study of the issue based on the recommendations of the Technical Study Group. The Technical Advisory Group was formed which developed cigarette fire-safety test methods. No federal legislation has been passed up to this time that has set a fire-safety standard for cigarettes. In June 2000, however, the state of New York passed a cigarette fire-safety bill that would require all cigarettes sold in New York to meet flammability standards by 2003 (Perez-Pena, 2000).

PARKINSON'S DISEASE

Parkinson's disease is a neurological disease affecting 1-2% of the population that is characterized by motor dysfunction and, in severe cases, cognitive dysfunction caused by loss of dopamine and other neurotransmitters through the destruction of neurons in the substantia nigra. Over the last decade there have been many epidemiological studies suggesting a biologically protective effect of cigarette smoking on the risk of Parkinson's disease. Numerous population based case-control and prospective studies have indicated an inverse dose-response relationship between smoking and Parkinson's disease (Checkoway and Nelson, 1999; Gorell et al., 1999; Grandinetti et al., 1994; Tzourio et al., 1997). In a large 26-year

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prospective follow-up of the Honolulu Heart Study, Grandinetti et al. (1994) found the relative risk of Parkinson's disease among smokers to be 0.39 with a dose-response relation to pack-years of smoking. Postulated mechanisms of the neuroprotective traits of smoking include direct neuronal effects of nicotine on dopamine release in the substantia nigra, reduction of the action of monoamine oxidase B, or reduction of free radical injury (Checkoway et al., 1998; Court et al., 1998; Grandinetti et al., 1994; Kelton et al., 2000). Critics of the protective association between smoking and Parkinson's disease have attributed the results to an artifact of selective mortality of smokers that would have acquired Parkinson's, diagnostic errors, cause-and-effect bias, or another unrecognized confounder (Morens et al., 1996; Riggs, 1996).

PREECLAMPSIA

Studies have described an inverse relationship between cigarette smoking and the risk of preeclampsia. Many studies have reported around a 30-70% decrease in risk for preeclampsia among smokers, using reported daily cigarette use and serum cotinine levels, when compared to nonsmokers. A finding of a dose-response effect has been less consistent (reviewed in Conde-Agudelo et al., 1999; Klonoff-Cohen and Savitz, 1993; Lain et al., 1999; Lindqvist and Marsal, 1999). A population-based study by Cnattingius and colleagues (1997) found a decreased incidence of preeclampsia among smokers but also found that smokers who did develop preeclampsia tended to have worse pregnancy outcomes. In a case-control study by Marcoux et al. (1989), the protective effect of smoking for preeclampsia was maintained only among pregnant smokers who continued to smoke throughout pregnancy or quit after 20 weeks' gestation. The postulated mechanisms for this protective effect of cigarette smoking includes the nicotine-mediated inhibition of fetal thromboxane A, stimulation of NO production, increased levels of thiocyanate, endothelial damage secondary to the oxidative stress effect of cigarette smoke, and altered immune responses (Cnattingius et al., 1997; Conde-Agudelo et al., 1999; reviewed in Lindqvist and Marsal, 1999).

SUMMARY

Several important diseases and conditions of adults, in addition to cardiovascular disease, chronic obstructive pulmonary disease, and various cancers, have been associated with tobacco use. Some of the associations are supported by substantial scientific evidence, and a causal linkage is likely. These illnesses must ultimately be subjected to the same evaluation with regard to changing risks and outcomes associated with

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potential reduced-exposure products (PREPs), because they are common and clinically important, even if not often as fatal as the diseases considered earlier in this report. Further, each of the conditions for which the association with tobacco use is substantial also offers the opportunity to address pathogenic mechanisms related to the varying constituents of PREPs, as well as the impact on disease incidence of coordinated behaviors and exposures such as alcohol use, various dietary elements, and certain medications.

RECOMMENDATIONS

Surveillance

The committee recommends that selected conditions, reviewed in this chapter, be part of a comprehensive, population-based surveillance program, as outlined in Chapter 6. This will allow determination of trends in occurrence for these tobacco-related conditions and assessment on a national basis of whether changes in tobacco product use have an effect on these important health problems. Based on surveillance findings, more specific population, clinical, and basic research studies can be directed and justified, in order to pursue causal mechanisms and suggest more effective interventions.

Applying Selected Conditions as Indicators of Clinical Harm Reduction

Some of the conditions reviewed in this chapter may be applied as indicators of the general biological effects of new tobacco products. For example, cigarette smoking has been consistently found to be an independent risk factor for an adverse clinical course of both peptic ulcer disease and wound healing. The effects of smoking on ulcer formation and healing have been clearly described clinically and in animal models. Peptic ulcers have been found to be larger, slower to heal, and more likely to recur among smokers and to exhibit clinically improved healing upon cessation. Surgical and traumatic wounds heal more slowly in cigarette smokers. The committee recommends that rigorous clinical studies be designed and executed to determine whether variations in ulcer and wound healing rates are related to different categories of tobacco products, including those with claims of harm reduction. This may offer the opportunity to define some clinical outcomes that have clinical relevance in their own right and to identify potential indicators of harm alteration that could be obtained much sooner after the introduction of PREPs than would be possible when evaluating heart disease and cancer.

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Other candidate diseases for such evaluation might include periodontal disease and Crohn's disease. Here the outcomes to assess would be the effects of various conventional tobacco products and PREPs on the natural history of the condition, including intermittancy, progression or regression, and longitudinally collected biomarkers of disease severity. As noted above, PREPs that alter the history and outcomes of these conditions could be further evaluated for specific constituent exposures that are associated with this altered history. This may lead to a more refined understanding of pathogenic mechanisms as well.

There is also room for clinical and basic research on intermediate clinical outcomes. For example, as noted in this chapter, the risk of osteoporosis has also been strongly linked to cigarette smoking. In controlled observational studies, bone mineral density has been found to be significantly lower among cigarette smokers, which contributes to a higher risk of osteoporotic fractures among older populations. While the effects of smoking on fracture rates may take a few decades or longer to detect, it is possible that surveillance for bone mineral density among those using PREPs and conventional products may prove valuable in a shorter time period and thus serve to detect important outcomes over an interval in which tobacco policy and clinical preventive interventions may have their greatest effects.

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Appendixes

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A

Presentations and Submissions

The committee would like to thank the following people who shared their expertise.

PRESENTATIONS

December 15, 1999, Washington, D.C.

Presentation of the Charge to the Institute of Medicine Committee
Mitch Zeller, Food and Drug Administration (current affiliation, American Legacy Foundation
Bern Schwetz, Food and Drug Administration

March 2, 2000, Washington, D.C.

Harm Reduction and Lessons Learned: Historical Perspective and Policy Advice
John Slade, University of Medicine and Dentistry at New Jersey School of Public Health
Harm Reduction and Lessons Learned: Low Tar and Nicotine Story
Don Shopland, National Cancer Institute
Tobacco Toxicology Review: Emphasis on Carcinogenesis
Steve Hecht, University of Minnesota
Exposure Assays
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April 25, 2000, Washington, D.C.

Risk Perception and Decision Making
Paul Slovic, Decision Research, University of Oregon
Epidemiology of Low-Tar Cigarettes
David Burns, University of California, San Diego
Clinical Trial Design Considerations
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B

Committee Biographical Sketches

Stuart Bondurant, M.D. *(chair),* is professor of medicine and dean emeritus at the University of North Carolina School of Medicine. Previously, Dr. Bondurant was director of the Center for Urban Epidemiologic Studies in New York City, chairman of the Department of Medicine at Albany Medical Center Hospital, and dean of Albany Medical College. He has been an active member of professional organizations including president of the Association of American Physicians and American College of Physicians, member of the Committee on Smoking and Health for the American Medical Association, and chairman of the Association of American Medical Colleges. He has served on numerous scientific advisory committees including the Directors Advisory Committee of the National Institutes of Health (NIH), the Special Medical Advisory Group of the Veterans Administration, and the Scientific Advisory Board of the U.S. Air Force. As a member of the Institute of Medicine (IOM), Dr. Bondurant has served as acting president as well as vice-chair of the IOM Council. He was chair of the IOM Committee on the Safety of Silicone Breast Implants, co-chair of the Committee on Public Health, and a member of the Committee on Science, Engineering and Public Policy. Dr. Bondurant's research interests have been in the area of cardiovascular and pulmonary medicine as well as issues concerning medical education and public health.

J. Richard Crout, M.D., is president of Crout Consulting, a company that provides regulatory and drug development advice to pharmaceutical

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and biotechnology companies. Dr. Crout formerly served as the director of the Bureau of Drugs at the Food and Drug Administration (FDA) (1973-1982) and as vice president of medical and scientific affairs at Boehringer Mannheim Pharmaceuticals Corporation (1984-1993). Dr. Crout was an IOM scholar-in-residence in 1994 and has served on various Institute of Medicine committees including the Committee to Study Medication Development and Research at the National Institute on Drug Abuse (NIDA) and as adviser to the Project on the Medical Use of Marijuana. He has published extensively in the area of clinical pharmacology and drug regulatory policy. Dr. Crout's awards include the Distinguished Service Medal of the U.S. Public Health Service and the Oscar B. Hunter Award in Therapeutics from the American Society for Clinical Pharmacology and Therapeutics.

Garret FitzGerald, M.D., is chair of the Department of Pharmacology, professor of medicine and pharmacology, and director of the Center for Experimental Therapeutics at the University of Pennsylvania. Before coming to the University of Pennsylvania, Dr. FitzGerald was professor and chairman of medicine and experimental therapeutics at University College in Dublin, Ireland, and professor of medicine and pharmacology and director of the Division of Clinical Pharmacology at Vanderbilt University School of Medicine. He has received many honors and appointments for his work, including the Robinette Foundation Professor of Cardiovascular Medicine and appointment as chair of the Arterosclerosis, Thrombosis, Vascular Biology Council of the American Heart Association. Dr. FitzGerald is on the editorial board of *Circulation* and was formerly on the board of the *Journal of Biological Chemistry*. His principle area of research involves isoeicosanoids as indices of oxidant stress, pharmacology of cyclooxygenase-2 and eicosanoid receptors and pathways, and development of antithrombotic and anti-inflammatory medications.

Adi Gazdar, M.D., is professor of pathology and deputy director of the Hamon Center for Therapeutic Oncology Research at University of Texas Southwestern Medical Center, Dallas. Prior to coming to Dallas, Dr. Gazdar worked at the National Cancer Institute (NCI) where he served as head of the Tumor Cell Biology Section and deputy head of the NCI-Navy Medical Oncology Branch. His research focuses on the molecular and genetic analysis of cancer, especially cancers of the lung, breast, and cervix, in order to further cancer diagnosis, prognosis, and treatment. Dr. Gazdar manages an extensive cancer tissue specimen data bank and a collection of in vitro developed tumor cell lines. He is also developing molecular and cellular techniques for early cancer detection.

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Gary Giovino, M.S., Ph.D., is senior research scientist in the Department of Cancer Prevention, Epidemiology, and Biostatistics at Roswell Park Cancer Institute, Buffalo, New York. He was previously chief of the Epidemiology Branch (1991-1998) at the Office on Smoking and Health of the Centers for Disease Control and Prevention (CDC). Dr. Giovino serves on multiple professional organizations that are concerned with tobacco issues, including Adviser to the American Cancer Society on Epidemiology and Surveillance Research and expert consultant on tobacco issues for the National Household Survey on Drug Abuse, of the Substance Abuse and Mental Health Services Administration. He is a senior editor of *Tobacco Control: An International Journal* and currently serves as a core group member in the Research Network on the Etiology of Nicotine Dependence and as director of the State Tobacco Research Component of Project ImpacTeen, both funded by the Robert Wood Johnson Foundation. Dr. Giovino has earned numerous awards for his tobacco-related work and has published extensively in the areas of patterns, determinants, consequences, and control of tobacco use.

Dorothy Hatsukami, Ph.D., is professor in the Department of Psychiatry, adjunct professor in the Department of Psychology, and adjunct professor in the Division of Epidemiology at the University of Minnesota. She is director of the Tobacco Research Use Center at the University of Minnesota. Dr. Hatsukami is former president of the Society for Research on Nicotine and Tobacco and president-elect of the College on Problems of Drug Dependence. She serves on numerous scientific advisory boards and committees on substance abuse and addiction. Dr. Hatsukami recently served as an adviser for the IOM Project on Medical Use of Marijuana: Assessment of the Science Base. Her research interests include nicotine addiction and treatment, smoking behavior, smokeless tobacco use, and gender and age differences in tobacco use. She has received much recognition for her work, including being a co-recipient of the Ove Ferno Award for contributions in the area of tobacco dependence.

Rogene Henderson, Ph.D., is deputy director of the National Environmental Respiratory Center and senior biochemist and toxicologist at the Lovelace Respiratory Research Institute. Dr. Henderson also holds appointments as clinical professor of pharmacy at the University of New Mexico, Albuquerque, and as adjunct professor in the Department of Veterinary Microbiology, Pathology, and Public Health in the School of Veterinary Medicine, Purdue University. She has chaired the National Research Council (NRC) Subcommittee on Toxicological Hazard and Risk Assessment, Subcommittee on Pulmonary Toxicology, and standing Com-

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mittee on Toxicology for six years, and she is currently a member of the Board on Environmental Studies and Toxicology. She has served as a member of the advisory council of the National Institute of Environmental Health Sciences and the U.S. Environmental Protection Agency's (EPA) Science Advisory Board, Environmental Health Committee. Dr. Henderson has received numerous appointments on scientific advisory committees, including her current appointment as a member of the Health Effects Institute Research Committee. Dr. Henderson has done extensive research in the areas of lung biochemistry, the pharmacokinetics of inhaled toxins and their metabolites, and biological markers of exposure.

Peter Reuter, Ph.D., is professor of public policy and of criminology at the University of Maryland, where he also directs the public policy Ph.D. program. In July 1999, he became editor of the *Journal of Policy Analysis and Management*. Dr. Reuter previously held the position of codirector of the Drug Policy Research Center and senior economist at the RAND Corporation. His research has included an examination of the organization of illegal markets and alternative approaches to controlling drug problems. Dr. Reuter's policy analysis of harm reduction approaches to illegal drug use has produced numerous articles on the subject and a forthcoming book: *Drug War Heresies: Learning from Other Vices, Times and Places* (Cambridge University Press with Robert MacCoun). He is a member of the NRC Committee on Law and Justice and served on the IOM Committee on the Federal Regulation of Methadone.

David J. Riley, M.D., is professor in the Division of Pulmonary and Critical Care Medicine at the University of Medicine and Dentistry of New Jersey (UMDNJ)-Robert Wood Johnson Medical School. Dr. Riley holds additional academic appointments as adjunct professor of physiology and biophysics, member of the Joint Graduate Program in Toxicology, and affiliate member of Environmental and Occupational Health Sciences Institute at the University of Medicine and Dentistry of New Jersey (UMDNJ)-Robert Wood Johnson Medical School. He has received numerous awards for his work in the field of pulmonology including the Sir William Osler Humanitarian Award from the New Jersey Thoracic Society and a Pulmonary Academic Award from the National Heart, Lung, and Blood Institute (NHLBI) of NIH. Dr. Riley has served on many scientific advisory committees, including several for the NIH, the Review Committee of the Health Effects Institute, chairman of the Pulmonary Disease Study Section of the Tobacco-Related Research Program, University of California at San Francisco (1993-1994), and a variety of committees of the American Thoracic Society. Dr. Riley holds NIH-supported grants for his research on vascular and interstitial diseases of the lung.

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Peter Shields, M.D., is professor of medicine and oncology at Georgetown University Medical Center, where he is director of the Division of Cancer Genetics and Epidemiology in the Department of Oncology. Dr. Shields is associate director for population sciences at the Lombardi Cancer Center at Georgetown University. Prior to his position at Georgetown, Dr. Shields was a tenured investigator and chief of the Molecular Epidemiology Section of the Laboratory of Human Carcinogenesis in the Division of Basic Sciences at the NCI. Dr. Shields serves on the editorial board or as reviewer for a number of scientific journals including *Journal of the National Cancer Institute*, *Journal of the American Medical Association*, *Carcinogenesis*, *Cancer Epidemiology Biomarkers and Prevention*, and others. He has been a member of various professional and scientific committees, including the Tobacco Research Implementation Group for NCI (1998). Dr. Shields was the first elected chairperson of the Molecular Epidemiology Group of the American Association of Cancer Research. His research activities involve identifying and using biomarkers of cancer risk and gene-environment interactions to enhance risk assessment. More specifically, he is examining the relationship between tobacco and alcohol use as environmental elements in predicting cancer risk in susceptible subgroups of the population.

Robert Wallace, M.D., M.Sc. *(vice-chair)*, is professor of epidemiology and internal medicine at the University of Iowa Colleges of Public Health and Medicine. He was formerly head of the Department of Preventive Medicine at the University of Iowa College of Medicine and director of the University of Iowa Cancer Center. He was a member of the U.S. Preventive Services Task Force, Second Panel. Dr. Wallace's research interests include cancer epidemiology and prevention; the causes and prevention of chronic, disabling diseases among older persons; women's health issues; and risk factors for cardiovascular disease. Dr. Wallace received the Irene Ensminger Stecher Professorship in April 1999 for cancer-related research. He is currently a member of the IOM Board on Health Promotion and Disease Prevention. He recently served as a member of the Committee on Leading Health Indicators for Healthy People 2010 and the NRC Panel on a Research Agenda and New Data for an Aging World.

James T. Willerson, M.D., is President of the University of Texas Health Sciences Center at Houston. He is the Edward Randall III Professor and chairman of the Department of Internal Medicine at the University of Texas Medical School. Dr. Willerson serves as chief of medical services at Memorial Hermann Hospital and Lyndon B. Johnson General Hospital. He is also medical director, chief of cardiology, director of cardiology research, and codirector of the Cullen Cardiovascular Research

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Laboratories at the Texas Heart Institute. He is a member of numerous professional and scientific organizations including the Council of Clinical Cardiology of the American Heart Association, a fellow of the American College of Cardiology, editor-in-chief of *Circulation* (1993-2004), and a member of the IOM. Dr. Willerson was a member of the IOM Committee to Provide Review of the Fialuridine Clinical Trials. He is the author of multiple textbooks and hundreds of articles on a wide variety of cardiovascular issues including gene therapy, basic mechanisms involved in the development of acute coronary syndromes, and detection of vulnerable atherosclerotic plaques.

Liaisons

Kenneth E. Warner, Ph.D., is the Richard D. Remington Collegiate Professor of Public Health in the Department of Health Management and Policy at the University of Michigan School of Public Health. He is also director of the university's Tobacco Research Network and associate director of the university's Robert Wood Johnson Foundation Clinical Scholars Program. Dr. Warner's research has focused on economic and policy aspects of tobacco and health. Dr. Warner served as senior scientific editor of the twenty-fifth anniversary Surgeon General's report on smoking and health. He is on the editorial board of numerous journals and chairs the board of *Tobacco Control*. He serves on the Board of Directors of the American Legacy Foundation and on the Council and Membership Committee of the IOM. Among his numerous professional awards, Dr. Warner has been honored twice by Delta Omega, the national public health honorary society, for "outstanding achievement in public health." He received the Surgeon General's Medallion from Dr. C. Everett Koop in 1989. Dr. Warner was named to the first class of fellows of the Association for Health Services Research in 1996; was elected to the Institute of Medicine in 1996; and received the Excellence in Research Award from the University of Michigan School of Public Health in 1997.

Richard Bonnie, LL.B., is the John S. Battle Professor of Law at the University of Virginia School of Law and director of the Institute of Law, Psychiatry and Public Policy at the University of Virginia. Dr. Bonnie's professional expertise is in law and public policy pertaining to public health and mental health, including prevention and treatment of addiction and substance abuse. He has written extensively about regulation of alcohol, tobacco, and controlled substances and has served as a member of the National Advisory Council on Drug Abuse and on the Board of Directors of the College on Problems of Drug Dependence. Also, Professor Bonnie has written about psychiatry and the law and the rights of

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mental health patients, and serves as an adviser to the American Psychiatric Association's Council on Psychiatry and Law. He is a member of the Institute of Medicine and serves on the Board on Neuroscience and Behavioral Health. Professor Bonnie has served as chair of the IOM Committee on Opportunities in Drug Abuse Research and Committee on Injury Prevention and Control, and as a member of the Committee on Prevention of Nicotine Dependence in Children and Adolescents. He is currently a member of the NRC Committee on Data and Research for Policy on Illegal Drugs.

Institute of Medicine Staff

Kathleen R. Stratton, Ph.D. (senior program officer), received a B.A. in natural sciences from Johns Hopkins University. She received her Ph.D. in neurotoxicology from the University of Maryland at Baltimore. After completing postdoctoral fellowships in neuropharmacology at the University of Maryland School of Medicine and in neurophysiology of second-messenger systems at the Johns Hopkins University School of Medicine, she joined the IOM staff in 1990. Dr. Stratton has worked in the IOM Divisions of Health Sciences Policy, Biobehavioral Medicine and Mental Disorders, and Health Promotion and Disease Prevention, as well as the NRC Board on Environmental Studies and Toxicology. She has worked on projects in environmental risk assessment, neurotoxicology, the organization of research and service programs in the Public Health Service, vaccine safety, fetal alcohol syndrome, and vaccine development. She directed the Division of Health Promotion and Disease Prevention from 1997 until 1999.

Padma Shetty, M.D. (program officer), finished her B.A. in Psychology at Northwestern University and her M.D. at the Ohio State University College of Medicine in 1998. After completing an internship in Internal Medicine, she joined the IOM staff for the Committee to Assess the Science Base of Tobacco Harm Reduction in 1999.

Ann W. St. Claire is senior project assistant for the IOM Committee to Assess the Science Base for Tobacco Harm Reduction. Ms. St. Claire joined the IOM Board on Health Promotion and Disease Prevention in 1999 and has worked on the IOM study, *Reducing the Burden of Injury* (1999). Ms. St. Claire graduated from Mount Holyoke College in 1999 with a degree in politics and a certificate in the culture, health and science program.

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C

Time Line of Tobacco Events

Science	Products	Policies or Regulation
	1700s • Cigarettes are first made from scraps of cigars	
1761 • Dr. John Hill reports in *Cautions Against the Immoderate Use of Snuff* on two case histories and observed that ("snuff is able to produce swellings and excrescences") in the nose, and he believed these to be cancerous		
1807 • Cerioli isolates the "essential oil" or "essence of tobacco"		
1828 • Posselt and Reimann isolate nicotine from tobacco		
1843 • Melsens describes nicotine's chemical empirical formula		

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Science	Products	Policies or Regulation
	1854-1856 • Cigarette popularity grows between 1854 and 1856 during the Crimean War **1881** • James Bonsack of Virginia patents the first cigarette-making machine.	
1893 • Pictet and Crepieux synthesize nicotine **1900** • Brosch appears to be the first investigator involved in experimental tobacco carcinogenesis. He applies tobacco ("juices") to guinea pigs, observing epithelial proliferation		
	1913 • Camel brand is produced by R.J. Reynolds in 1913	
1928 • Lombard and Doerring find an association between heavy smoking and buccal cancer **1938** • Pearl, a statistician and biometrician at Johns Hopkins, publishes the first statistical analysis comparing the health of smokers and nonsmokers and finds that individuals who smoked could expect shorter lives		
1950 • Epidemiological studies report that lung cancer is particularly prevalent among cigarette smokers	**1950** • Filters introduced	

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Science	Products	Policies or Regulation
1954 • Doll and Hill publish The Mortality of Doctors and Their Smoking Habits in the *BMJ*	**1954** • Winston by RJR features a filter (cellulose acetate) and reconstituted sheet tobacco • Tobacco Industry Research Committee is formed **1956** • RJR introduces Salem as its first filter-tipped menthol cigarette **Early 1960s** • More porous cigarette paper is introduced	**1954** • Industry faces first liability lawsuit by lung cancer victim claiming negligence and breach of warranty. Suit is dropped 13 years later **1960** • Ban on advertising tar and nicotine levels as less harmful 1960-1966
1962 • Royal College of Physicians reports that lung cancer is prevalent among smokers in *Smoking and Health* **1964** • Surgeon General's report concludes that smoking causes cancer and other serious diseases		**1964** • Public Health Service establishes the National Clearinghouse for Smoking and Health (NCSH), later to become the Office on Smoking and Health **1965** • Federal Cigarette Labeling and Advertising Act is passed requiring warning labels on all cigarette packs but not on advertisements. The act also requires the Secretary of Health, Education, and Welfare to issue annual reports to congress on the health consequences of smoking

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Science	Products	Policies or Regulation
		• Federal excise tax on smokeless tobacco products is repealed.
1966 • The Cambridge Filter method is ultimately adopted by the Federal Trade Commission		
1967 • Surgeon General's report concludes, "Cigarette smoking is the most important of the causes of chronic non-neoplastic bronchiopulmonary diseases in the United States." The report also identifies measures of morbidity associated with smoking	**Late 1960s** • Expanded or "puffed" tobacco appears in cigarettes	
	1968 • National Cancer Institute begins work on a safer cigarette by establishing the Less Hazardous Cigarette Working Group	
1969 • Surgeon General's report makes solid conclusions regarding the relationship between maternal smoking and infant low birthweight. It also defines evidence of increased incidence of prematurity, spontaneous abortion, stillbirth, and neonatal death • Use of chlorinated pesticides begins to be faded out		

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Science	Products	Policies or Regulation
	Early 1970s • Ventilated filter tips are used and modified to be longer with increased efficiency • Introduction of puffed, expanded, and freeze-dried tobaccos	
1971 • Surgeon General's report finds smoking associated with cancers of the oral cavity and esophagus		**1971** • Broadcast ads for cigarettes banned
1972 • Surgeon General's report studies immunological effects of tobacco and tobacco smoke, and identifies carbon monoxide, nicotine, and tar as smoke constituents the most likely to produce health hazards from smoking		**1972** • Officials declare that airlines must create nonsmoking sections
1973 • Surgeon General's report presents evidence on the health effects of smoking pipes, cigars, and "little cigars"		
1975 • Surgeon General's report issues further evidence regarding health effects from involuntary (passive) smoking, especially the relationship between parental smoking and rates of bronchitis and pneumonia in children's first year of life	**1975** • RJR introduces NOW with lower-tar and nicotine while preserving tobacco taste	
1977–1978 • Surgeon General's report focuses on health effects of smoking on women, noting in particular the effects of oral contraceptives and smoking on the cardiovascular system		

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Science	Products	Policies or Regulation
1979 • Surgeon General's report addresses the role of adult and youth education in preventing smoking habits. Report also reviews health effects of smokeless tobacco • Bandury conference and report		
1980 • Surgeon General's report projects that lung cancer in women will surpass breast cancer as the leading cause of cancer mortality in women. Report also notes prevalence of smoking by adolescent females		
1981 • Surgeon General's report examines the health consequences of lower-tar and nicotine cigarettes. Concludes that lower-yield cigarettes decrease the risk of lung cancer, but have little effect on rates of cardiovascular disease, chronic obstructive pulmonary disease, and fetal damage. The report also reviews risks related to various additives and their combustion. The Surgeon General reinforces the fact that there is no safe cigarette		**1981** • Insurers offer discounts on life insurance premiums to nonsmokers

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Science	Products	Policies or Regulation
1982 • Surgeon General's report releases epidemiological evidence from a study of nonsmoking wives and their smoking husbands, finding that the risk of lung cancer in wives was not causal, but a possible serious public health problem. Report notes possible low-cost smoking cessation interventions		
1983 • Surgeon General's report evaluates health consequences of smoking for cardiovascular disease, declaring cigarette smoking as one of the three primary causes of coronary heart disease		
1984 • Surgeon General's report examines health effects of smoking on chronic obstructive lung disease (COLD). Smoking accounts for 80-90% of COLD deaths in the United States	**1984** • Nicotine-based chewing gum approved as an aid to quitting	**1984** • Warnings strengthened on cigarette packages and ads • San Francisco requires business to accommodate nonsmokers
1985 • Surgeon General's report focuses on smoking and hazardous substances in the workplace. Smoking alone is found to be a greater risk than the average workplace environment. Workplace exposure to asbestos and other such substances is found to compound health risks		

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Science	Products	Policies or Regulation
1986 • Surgeon General's report states that "Involuntary smoking is a cause of disease, including lung cancer, in healthy nonsmokers." Report further notes the health of children of smokers and nonsmokers, as well as the exposure to smoke of passengers in smoking and nonsmoking sections of airplanes • A special report of the advisory committee appointed by the Surgeon General examines the health effects of smokeless tobacco, concluding that it too leads to nicotine addiction and can cause cancer		
	1987 • Tobacco Institute Testing Laboratory (TITL) assumes cigarette-testing responsibilities from the Federal Trade Commission Test Center using its approved methodology	
1988 • Surgeon General Koop's report states that nicotine (cigarettes and other forms of tobacco) are addicting		**1988** • Government bans smoking on short domestic airline flights
1989 • Surgeon General's report reports that cigarette smoking is a major cause of cerebrovascular disease (stroke). Report also addresses the future of nicotine addiction in light of new nicotine delivery systems test marketed in 1988	**Late 1980s** • RJR Premier is developed and introduced to the public	

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Science	Products	Policies or Regulation
1990 • Surgeon General's report identifies the health benefits of smoking cessation: "Smoking cessation has major and immediate health benefits for men and women of all ages." Report examines life expectancy, smoking-related diseases, and reproductive health issues of smokers and former smokers		**1990** • Smoking banned on interstate buses and domestic airline flights of six hours or less
	1992 • Nicotine patches introduced • 97.5% of cigarettes in the U.S. have filters	
		1993 • Vermont bans smoking in all indoor public places
1994 • Surgeon General's report looks at "preventing tobacco use among young people." Report examines and discusses age at first initiation, issues or problems encountered with youth cessation, tobacco as a "gate-way drug," effect of advertising, and school-based tobacco use prevention programs	**1994** • Major U.S. cigarette companies release a list of 599 additives used in the manufacture of cigarettes	**1994** • Executives of seven largest U.S. tobacco companies swear in congressional testimony that nicotine is not an addictive and deny manipulating nicotine levels in cigarettes • Amtrak bans smoking on short-and medium-distance trips • Brown and Williamson documents provide evidence that tobacco executives discovered smoking risks before the surgeon general made declaration • Mississippi files first of 24 state lawsuits seeking to recoup millions from tobacco companies for smokers' Medicaid bills

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Science	Products	Policies or Regulation
	1996 • RJR Eclipse test marketed in United States, Germany, and Sweden	**1996** • Liggett Group settles claims with five state attorneys-general and promises to help them against other companies **1997** • Federal judges rules that government can regulate tobacco as a drug, but industry is allowed to continue advertising • Landmark settlement, subject to congressional approval, calls for restrictions on cigarettes and on tobacco maker's liability in lawsuits. Industry is required to spend $368 billion over 25 years, run antismoking campaigns, issue bold health warnings on packs, decrease advertising, and pay fines if youth smoking doesn't drop significantly • Mississippi is first state to settle, agreeing to $3.6 billion deal with tobacco companies • Florida settles at $11.3 billion

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Science	Products	Policies or Regulation
1998 • Surgeon General's report examines tobacco use among U.S. racial and ethnic minority groups: African Americans, American Indians and Alaska Natives, Asian Americans and Pacific Islanders, and Hispanics. Concludes that cigarette smoking is one of the major health hazards among different racial and ethnic groups. Tobacco use and patterns of use vary among these groups as well	**1998** • Phillip Morris Accord presented at a poster presentation at the Society of Toxicology in Seattle	**1998** • Texas settles with industry at $15.3 billion over 25 years • Tobacco executives testify before Congress that nicotine is addictive under current definitions of the word and smoking may cause cancer • Minnesota and Blue Cross/Blue Shield of Minnesota settle at $6.6 billion with the tobacco industry • Senate vetoes a proposal of $1.50 tax increase per pack on cigarettes • McCain Universal Tobacco Settlement Bill dies in congressional filibuster. Bill addressed tobacco product regulation of ingredients, sales, and advertising. It also addresses education and nicotine addiction prevention • 46 states welcome a $206 billion settlement with the tobacco industry over health care costs for treating sick smokers
	1999 • Star Tobacco introduces new cigarettes with low-nitrosamine tobacco and activated-charcoal filter, but is unable to make health claims	**1999** • Justice Department sues the tobacco industry to recover billions of government dollars spent on smoking-related health care, accusing cigarette makers of a "coordinated campaign of fraud and deceit"

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Science	Products	Policies or Regulation
		• Government and tobacco industry lawyers present oral argument to the Supreme Court over whether the Food and Drug Administration (FDA) can regulate tobacco as a drug and crack down on cigarette sales to minors
2000 • National Institute for Environmental Health Sciences publishes ninth report on Carcinogens including ETS as one of the known human carcinogens • Surgeon General's Report, *Reducing Tobacco Use,* published	**2000** • Phillip Morris introduces cigarettes with "safer" paper • Star Tobacco—new cigarettes produced in Virginia and Kentucky with low-nitrosamine tobacco, but without health claims	**2000** • Tobacco farmers sue cigarette makers for $69 billion, claiming they conspired to undo the federal system that regulates tobacco prices • Supreme Court rules, 5-4, that FDA lacks authority to regulate tobacco as an addictive drug • New York state imposes fire-safety standards on cigarettes

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Effects of nicotinic stimulation on cognitive performance

Paul A Newhouse*, Alexandra Potter and Abhay Singh

Recent advances in studies of nicotinic agents in humans have begun to more carefully define cognitive operations that can be influenced by nicotinic stimulation and/or blockade. Careful separation of the cognitive domains affected by nicotinic stimulation has identified attentional performance as the most likely candidate to be positively influenced by nicotinic receptor activation. Studies of the effects of nicotinic systems and/or nicotinic receptor stimulation in pathological disease states such as Alzheimer's disease, Parkinson's disease, attention deficit/hyperactivity disorder and schizophrenia show the potential for therapeutic utility of nicotinic drugs. In contrast to studies in pathological states, studies of nicotine in normal-non-smokers tend to show deleterious effects. This contradiction can be resolved by consideration of cognitive and biological baseline dependency differences between study populations in terms of the relationship of optimal cognitive performance to nicotinic receptor activity. Although normal individuals are unlikely to show cognitive benefits after nicotinic stimulation except under extreme task conditions, individuals with a variety of disease states can benefit from nicotinic drugs. Attentional function/dysfunction may serve as an endophenotypic therapeutic target for nicotinic drug development.

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Abbreviations

AD	Alzheimer's disease
ADHD	attention deficit/hyperactivity disorder
CNS	central nervous system
fMRI	functional magnetic resonance imaging
MCI	mild cognitive impairment
nAChR	nicotinic receptor
PD	Parkinson's disease
PET	positron emission tomography
VSWM	visuospatial working memory

Introduction

Neuronal nicotinic receptors (nAChRs) are found throughout the central nervous system (CNS). These receptors are composed of two types of subunits, α and β, of which nine α (α2–α10) and three β (β2–β4) have been found in vertebrates [1–3]. Nicotinic innervation of the hippocampus, amygdala and frontal cortex has been demonstrated to be vital to memory function [4•].

Nicotine is one of over 4 000 compounds found in tobacco smoke, among which is found a variety of carcinogens as well as other toxic compounds such as carbon monoxide, heavy metals and cyanide. However, nicotine administered independent of tobacco appears to be relatively safe [5]. There is a large amount of literature showing that nicotine skin patches are safe and have very low abuse liability [6,7].

The behavioral effects of nicotine are complex and belie a simple classification of nicotine as either a stimulant or depressant. Determinants of the effect of nicotine on human behavior include pharmacological variables (e.g. dose, route of administration) and subject differences (e.g. gender, personality variables). Whether, and how, subjects control the administration of nicotine appears to have significant impact on its cognitive effects. Such effects need to be taken into consideration when considering the use of novel nicotinic agonists for possible beneficial behavioral effects.

This review focuses on recent advances in studies of the cognitive effects of nicotine in smokers and normal volunteer populations, and contrasts those studies with trials of nicotinic stimulation in pathological disease states. These disease states represent the most likely targets for nicotinic drug development and include Alzheimer's disease (AD), mild cognitive impairment (MCI), Parkinson's disease (PD), schizophrenia and attention-deficit/hyperactivity disorder (ADHD). In an attempt to resolve the conflicting results of generally negative studies in normal volunteer populations and positive studies in pathological disease states, we focus on an analysis of cognitive and neurobiological baseline differences between study populations. Finally, the concept of attentional function and dysfunction as an endophenotype for nicotinic drug development is introduced, and we offer the hypothesis that this target is orthogonal to disease diagnosis and offers the best target for the therapeutic effects of nicotinic agents.

Nicotinic receptor stimulation as a cognition-enhancing strategy in 'normals'

Studies in humans have spanned several decades and consist mostly of experiments utilizing cigarettes to administer nicotine, usually to smokers deprived of cigarettes for some period of time. Although nicotine might

'improve' performance in deprived smokers, it appears that this improvement is usually limited to restoring pre-deprivation performance, which clearly declines during cigarette withdrawal [8]. Enhancement of normal non-deprived smokers and nonsmokers with nicotine has been more difficult to demonstrate. In studies with humans, nicotine has been shown to improve performance in smokers on attentionally and cognitively demanding vigilance tasks [9–11]. Recent work has shown that attentional improvements are seen even in the absence of nicotine withdrawal effects [12].

Recent studies of the effects of nicotine and/or smoking on cognitive performance in smokers have attempted to mitigate or minimize many of the problems associated with cognitive studies utilizing drug-dependent individuals. Bell, Taylor, Singleton, Henningfield and Heishman [13] confirmed that smoking deprivation impairs cognitive performance and that re-administering cigarettes briskly relieves this performance decrement. Utilizing electrophysiological assessment, nicotine administration to smoking-deprived smokers improved power indices of electroencephalogram arousal, with shortened reaction times increasing P300 (evoked potential occurring 300 msec after stimulus presentation) amplitudes [14]. Under conditions of suboptimal alertness, smokers who were administered nicotine showed improved and constant performance during a sustained choice reaction time task, suggesting that nicotine has an enabling effect on sustained cognitive effort, at least in this population [15]. Utilizing the strategy of administering a transdermal nicotine patch to smokers participating in a smoking withdrawal study, Cook, Gerkovich, Graham, Hoffman and Peterson [16] did not find that nicotine administration mitigated the cognitive effects of smoking cessation and hypothesized that performance decrements noticed by smokers during cessation may be related to affective disturbances rather than cognitive impairment. Utilizing non-deprived smokers, Sakurai and Kanazawa [17] compared smokers with nonsmokers in a variety of memory, calculation and executive function tasks after the smokers had received one or two cigarettes. They found no intergroup differences and suggest that a daily dose of nicotine has little effect on performance. Ernst, Heishman, Spurgeon and London [18] found that smoking history appeared to interact with performance effects in individuals who had been administered nicotine gum, with abstinent smokers performing most poorly and 'never smokers' performing best on a working memory task. Heishman and Henningfield [19] administered nicotine gum across a range of doses to normal nonsmokers both acutely and chronically. Nicotine administration increased the rate of responding and decreased response time, but impaired accuracy on working memory tasks; accuracy also was impaired on visual scanning attention and gross motor coordination.

Functional neuroimaging studies of the effects of nicotine utilizing functional magnetic resonance imaging (fMRI) or positron emission tomography (PET) have suggested that performance enhancement is associated with increased task-induced fronto-parietal-thalamic activation, areas associated with visual attention, arousal and motor activation [20•], or altered lateralization accompanied by reduced cerebral blood flow [21]. A PET study of cerebral glucose metabolism in normal volunteers showed that nicotine administration reduced global glucose metabolism by approximately 10% across most brain regions [22]. Finally, in an intriguing set of studies, Mumenthaler *et al.* [23••] compared the effects of nicotine and the anticholinesterase inhibitor donepezil on the performance of experienced pilots in a flight simulator device. In this highly demanding and stressful cognitive situation, both nicotine and donepezil improved performance substantially with roughly the same effect size, particularly on tasks that required sustained visual attention. Interestingly, a single dose of nicotine improved performance almost as effectively as one month of donepezil administration.

Psychopathological conditions

nAChRs play important roles in the functional impairments of certain neurodegenerative diseases, including AD [24,25]. These associations, although in some cases indirect, are impressive for their longevity and because the associations have been made both by many different investigators and by using more than one approach. The association between AD and nAChRs, although also indirect, suggests that these receptors may mediate important signals that influence the course of the disease. Thus, epidemiological evidence suggests that smokers have a significantly lower incidence of symptoms and diagnosis of AD [26,27]. This inverse relationship even extends to populations with high risk factors for early-onset AD, such as apolipoprotein E gene status [28]. In addition to a strong epidemiological association, many studies have found a decrease in the density of nAChRs in the autopsied brains of AD patients [29–33]. The receptors lost in AD brains are predominantly of the $\alpha4\beta2$ subtype, which binds nicotine and other nicotinic agonists with high affinity and is one of the major nAChR subtypes in mammalian brain [34,35]. This decrease in nAChRs is now well-enough established as part of the findings in autopsied AD brains (>10 studies) that efforts are underway in several laboratories to develop neuroimaging ligands for possible use in early diagnosis or for following the course of the disease [36–42].

Newhouse, Potter, Kelton and Corwin [24] recently reviewed the evidence supporting the importance of nAChRs in the development of AD. In summary, a loss of nicotinic binding sites has been demonstrated in AD patients to be linked to the pathological hallmarks of plaques and tangles. The loss of nAChRs appears to be associated with decreased cerebral perfusion in AD.

Mild cognitive impairment

MCI is defined as a subjective and objective decline in cognition and function that does not meet criteria for a diagnosis of dementia [43–45] and that represents a transitional state between the cognition of normal aging and mild dementia [46]. Furthermore, recent evidence indicates that people with MCI are at high risk for subsequently developing dementia [47,48]. Amnestic MCI [46] appears to represent the condition most likely to progress to AD, whereas individuals who have multiple domains or non-memory domains impaired might progress to AD and/or other types of dementias. By utilizing criteria for amnestic MCI, long-term follow-up studies have suggested that these individuals progress to dementia at a rate of approximately 12% per year [46]. Stimulation of CNS nAChRs with nicotine could be a promising strategy to ameliorate symptoms of MCI and/or slow or prevent progression to frank dementia. In a recent study of cognitive performance in patients with MCI, White and Levin [49] studied 10 subjects with MCI in a double-blind, placebo controlled, crossover study consisting of two four-week periods separated by a two-week washout period. Subjects were given nicotine patches to wear for 16 hours a day up to 10 mg per day. Nicotine significantly improved ratings of overall performance on the clinical global impression scale, as well as objective tests of attentional function on the Connors Continuous performance test and on the neuropsychology test battery, compared with placebo. Intriguingly, nicotine treatment improved the decision-making portion of reaction time to a greater extent than it did the simple psychomotor speed. This was accompanied by improvements in error performance on the Connors Continuous performance test task. Such results are encouraging and have prompted the launching of a NIA-funded multicenter trial to test the efficacy of one year of transdermal nicotine therapy in MCI. In many respects, MCI might be the optimal diagnosis for which to test the efficacy of nicotinic therapy. The likelihood of relatively large numbers of preserved nAChRs and the relative preservation of attentional, acquisition, encoding and retrieval mechanisms, taken together with the evidence for substantial neuroprotective effects of nicotinic stimulation, make MCI an ideal diagnosis to test the efficacy of long-term, low-dose nicotinic stimulation. The large number of controlled efficacy smoking cessation trials in healthy and diseased subjects and their accompanying enormous safety database provide confidence that this treatment will be well-tolerated and safe.

Nicotinic treatment of cognitive impairment in Alzheimer's disease

In clinical studies, Newhouse *et al.* [50] first showed evidence of improved cognitive functioning (decreased errors) following intravenous injection of nicotine in AD subjects. Nicotine administration by subcutaneous injection was then shown to improve attention-related task performance in AD [51,52]. Two weeks of nicotine skin patch treatment was found to significantly improve cognitive function in AD patients by Wilson *et al.* [53]. These investigators found that the major effect of nicotine was to reduce error performance on the new learning phase of the repeated acquisition test — the same parameter on the same task (although performed differently) that Newhouse, Potter, Corwin and Lenox [54] found to be specifically impaired after the nicotinic antagonist mecamylamine. A four-week trial of transdermal nicotine in AD was performed by White and Levin [55], who showed significant improvement in attentional performance, as measured by a continuous performance task, with consistent improvements in omission errors and improved consistency of reaction time. Another nicotine patch study did not find any differential improvement in short-term memory with nicotine compared with placebo [56]. However, there were significant practice effects in that study which could have limited sensitivity. In a unique and not previously reported combination, subjects that were treated with the cholinesterase inhibitor tacrine were administered nicotine; they showed decreased auditory-evoked potential latencies and increased visual-evoked potential amplitude, suggesting improved sensory detection, attention and/or processing [57]. In the only example of treatment with a novel nicotinic agonist, Potter *et al.* [58] showed significant dose-related enhancement of learning and memory in AD patients after acute treatment with the nicotinic agent ABT-418.

In addition to direct stimulation of nAChRs, nicotine might provide cascading effects through stimulation of the release of a variety of transmitters involved in cognitive function, including acetylcholine, dopamine, norepinephrine, serotonin and glutamate [59]. Augmentation of the activities of the remaining nAChRs might provide therapeutic benefit. However, the loss of nAChRs in AD could also limit the potential for nicotinic therapy. This might underlie the lack of nicotine effect in AD seen by Snaedal, Johannesson, Jonson and Gylfadottir [56] and the limitations in the extent of improvement in studies of nicotine in AD. Nicotine treatment might be more effective in older adults with less severe cognitive impairment and more nAChRs, such as those individuals with MCI. The documented neuroprotective effects of nicotine [60,61] could help attenuate this decline.

Effects of chronic nicotine

An important consideration in the development of treatments for cognitive impairment is that they do not lose effectiveness with chronic treatment. It is often the case that chronic administration of agonists causes downregulation of their target receptors and the development of tolerance; this is not the case with nicotine. nAChRs actually show an increase in number with chronic nicotine administration [62,63]. This might be due to the fact that nAChRs have a desensitization response that prevents

chronic overstimulation. However, there might also be downstream accommodations in neural systems that receive nicotinic innervation, which would result in tolerance. Therefore, one cannot make a complete argument on the basis of the known mechanisms of action. Much more convincing are the data that show continuing nicotine-induced cognitive improvement with chronic administration. With mild to moderate AD, nicotine skin patch-induced attentional improvement did not diminish over four weeks of administration [55].

Parkinson's disease

Changes in cholinergic systems in the CNS have also been shown to occur in the brains of patients with PD. In particular, a similar loss of cholinergic cells in the basal forebrain nuclei to that occurring in AD has been described in PD [64]. Studies have shown a marked reduction in cortical nAChR binding that parallels the degree of dementia in PD and increasing age [33,65]. There is similarity between the cortical nicotinic binding site loss in PD and AD, as well as similar changes in other cholinergic markers. The loss of presynaptic [66] cortical nAChRs might reflect degeneration of cortical projections from subcortical structures, notably the nucleus basalis, pedunculo-pontine and lateral-dorsal tegmental nuclei. In PD, loss of striatal nicotine binding appears to occur early but is not associated with a loss of α4 subunit immunoreactivity. Accumulating evidence both in rodents and in primates suggests that α6-containing nAChRs are present on nigrostriatal dopaminergic neurons, and that these receptors may be the most vulnerable to nigrostriatal damage, at least in primates. nAChR ligands that activate this receptor might be particularly useful in PD therapeutics [67••]. Newhouse and colleagues proposed that nicotinic augmentation might be a useful therapeutic strategy for both the motor and cognitive symptoms of PD [68,69].

Several studies have shown that smokers have a lower than expected incidence of PD, suggesting a protective effect of nicotine [70,71]. Tobacco use is associated with greater numbers of dopaminergic neurons in the substantia nigra pars compacta [72].

Nicotine was examined as a treatment for PD as early as the 1920s [73], although these patients suffered from a form of secondary parkinsonism caused by encephalitis lethargica. Some patients in the study showed improvement in rigidity but there were also severe side effects, including seizures, after large doses of nicotine. Fagerström, Pomerleau, Giordani and Stelson [74] reported a detailed study of two patients who had nicotine gum and patch added to their PD therapy. Using a single-subject placebo-control reversal design, improvement was associated with nicotine dosing and involved diminished tremor and disorganized thinking in one patient, and lessened bradykinesia and increased energy in the other. Kelton, Kahn, Conrath and Newhouse [75] examined the acute and chronic effects of nicotine in PD patients over several weeks. Positive effects of nicotine appeared in all motor performance tasks including the pronation/supination task, the stand/walk/sit task and the finger dexterity task. There was a statistically significant improvement on the choice reaction time task in the motor component. Particularly interesting was the persistence of some of the effects of nicotine even several weeks after the cessation of drug administration. These results suggest that nicotine administration has positive effects on certain cognitive and motor aspects of PD, particularly in the area of attention and motor speed. However, a randomized, double-blind placebo-control study of transdermal nicotine added to standard antiparkinsonian therapy for three weeks did not find statistically significant improvements in motor performance on clinical rating scores [19]. In contrast, studies of nicotine gum in PD patients have shown positive effects [76,77].

In addition to nicotine, other novel nicotinic agonists have been examined for effects in PD. SIB-1508 and its racemate, SIB-1765F, are subtype-selective nicotinic agonists particularly to α4β2-containing nAChRs [78]. These compounds appear to have greater efficacy than nicotine at releasing dopamine from striatal slices. SIB-1765F potentiated the positive locomotor effects of L-dopa in a reserpine model of PD in rats [79] with a rapid onset of action. The compound produced a small improvement in locomotion when administered alone; however, the effect was much greater when combined with L-dopa. SIB-1508Y, an isomer of SIB-1765F, was more potent in this model and has also shown positive activity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkey model of PD [80]. A dose of SIB-1508Y which was inactive by itself caused significant improvement in cognition and motor aspects of task performance when administered simultaneously with L-dopa.

Schizophrenia

Research in normal smokers and in patients with schizophrenia has indicated that cigarette smoking or nicotine administration may improve cognitive functioning, including memory, attention and spatial perception [81]. Epidemiological studies have shown a higher rate of smoking among schizophrenic patients (90%) compared with the general population (20–30%), and a lower rate of smoking cessation among schizophrenic patients [82]. It has been hypothesized that the high rate of smoking among schizophrenic patients might result, in part, from the ability of nicotine to ameliorate some of the cognitive deficits associated with schizophrenia [83].

Schizophrenic patients exhibit an auditory sensory gating deficit characterized by diminished suppression of P50 auditory-evoked response to repeated stimuli. Nicotine

transiently corrects the diminished gating response of P50 auditory-evoked potential in schizophrenic patients and their non-smoking relatives [84]. Clozapine, but not typical antidopaminergic antipsychotic drugs, improves gating of the P50-evoked response [85], and it has been suggested that modulation of smoking by clozapine treatment may, in part, be caused by similar mechanisms. The fact that these attentional abnormalities also occur in non-psychotic relatives of schizophrenic patients suggests a genetic basis for this deficit. The P50 inhibition deficit in schizophrenia has been linked to chromosome 15q13–14 in the region of the α7 subunit gene [86]. Postmortem studies have shown a reduction in the number of α-bungarotoxin-sensitive (α7-containing) nAChRs in the hippocampal region of schizophrenic patients [87], which appear to be secondary to polymorphisms in the α7 promotor [88•]. Another form of cognitive deficit associated with schizophrenia is latent inhibition, in which pre-exposure to a stimulus inhibits conditioning to that stimulus [89]. It has been suggested that smokers have enhanced latent inhibition, which is dependent upon the pre-exposure parameters [90].

Schizophrenic patients have impairments in other cognitive domains, including deficits in visuospatial working memory (VSWM), which is partly mediated by dopamine in the prefrontal cortex. Smoking abstinence differentially alters VSWM in schizophrenic versus control smokers, and cigarette smoking has beneficial effects on VSWM in schizophrenic, but not control, smokers [91]. Higher doses of nicotine patch improved reaction time, but not accuracy, in a spatial rotation task, and also improved performance on a visual-match-to-sample task in schizophrenic patients treated with haloperidol [92]. Recently, Smith, Singh, Infante, Khandat and Kloos [93•] have shown increased performance on two-choice reaction time, spatial rotation (accuracy/reaction time) and visual-match-to-sample tasks from the automated neuropsychological assessment battery (ANAM) with both high and denicotinized cigarettes in schizophrenic patients. Active nicotine nasal spray improved accuracy on a spatial organization task, and tended to improve some measures of verbal memory (paired words and short story from Randt memory test) and two-choice reaction time in schizophrenic patients. The differences in results between the above mentioned studies might be caused by different forms of smoking and/or to the fact that most of the patients in the Smith study [93•] were on atypical antipsychotic medications.

Attention deficit/hyperactivity disorder

Adults and adolescents who are diagnosed with ADHD smoke at significantly higher rates than comparable people in a community sample, and have lower quit ratios (percentage of ever-smokers who are ex-smokers) than the general population (23% versus 51.6%) [94]. In this study, there was a relationship between current smoking status and retrospective reports of ADHD symptoms, with current smokers recalling a greater number and greater severity of ADHD symptoms in childhood. A prospective study of tobacco smoking and substance dependence [95] found that, by age 17, 46% of adolescents with ADHD were smoking cigarettes daily compared with 24% of age-mate controls. This finding continued into adulthood where 35% of adult subjects with ADHD were smokers as compared with 16% of age-matched controls. These findings raise the possibility that adolescents with ADHD may be using cigarette smoking to relieve some of the symptoms of ADHD.

There is an emerging body of literature examining the therapeutic effects of nicotinic stimulation on the symptoms of ADHD. Levin *et al.* [96] studied the acute effects of transdermal nicotine and placebo in adults with ADHD (both smokers and non-smokers). They reported significant improvements in self-rated vigor and concentration and observer-rated illness severity for both subject groups (i.e. smokers and non-smokers). In addition, they found improvements in speed of responding for both the smokers and non-smokers, and a reduction in variability of reaction time for the smokers. In a second study, Levin, Conners, Siliva, Canu and March [97] studied the effects of chronic (four week) nicotine administration compared with methylphenidate treatment, placebo treatment and a combination of nicotine and methylphenidate in 40 adults with ADHD. They found nicotine to significantly reduce clinician ratings of severity of symptoms, and to decrease self-reported symptoms of depression. They found sustained improvement in the variability of reaction times on a continuous performance task. Wilens *et al.* [98] studied a novel cholinergic channel activator (nicotinic agonist), ABT-418, in 32 adults with ADHD for treatment of their symptoms. This study employed a crossover design with each subject receiving two double-blind three-week treatment periods with placebo and ABT-418. Significant improvements in subjective ratings of attentiveness and observer-rated illness severity on a clinical global impressions scale were seen following treatment with ABT-418.

The cognitive deficits in attention in ADHD are not in the areas of information processing or in perceiving information, but are seen in motor inhibition, motor control and in anticipating events [99,100]. Current views of ADHD hold failures of cognitive/behavioral inhibition as the central deficit of this disorder [101]. Potter and Newhouse [102] have recently examined changes in behavioral inhibition following nicotine administration to eight non-smoking adolescents with ADHD. In this study, subjects were administered acute transdermal nicotine (7 mg for 60 min), oral methylphenidate or placebo on three separate study days. Nicotine was associated with significant improvements in stop signal reaction time (a measure of the speed of inhibition). This

effect was comparable to the size of change seen after a single dose of oral methylphenidate and was not associated with general improvements in performance on this task, as overall speed and accuracy were not different from placebo. This study also found improved cognitive/behavioral inhibition associated with nicotine administration on the Stroop task, with a smaller Stroop effect (the cost of inhibiting) following nicotine, but not methylphenidate or placebo, treatment. In addition, nicotine was associated with decreased subject ratings of irritability and observer ratings of anxiety.

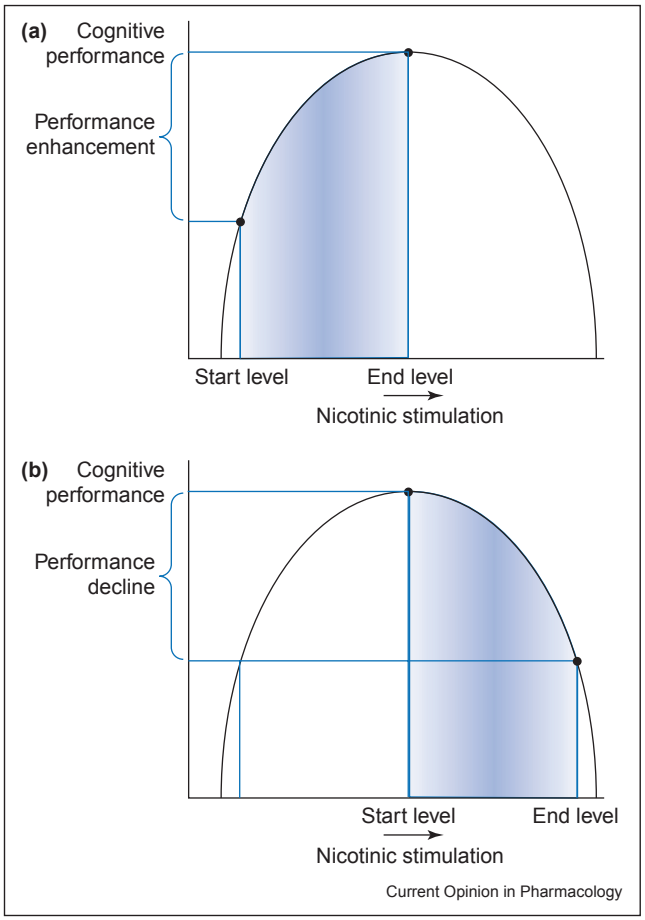
These initial studies indicate that nicotinic treatment may be useful for the symptoms of ADHD. The well-known effects of nicotine on attention might include positive effects on cognitive/behavioral inhibition, which is the core cognitive deficit of ADHD. Current standard pharmacological treatment for ADHD consists mainly of psychostimulants (e.g. methylphenidate), which are presumed effective via their effects on dopamine. Nicotine has been shown to increase the release of several neurotransmitters, including dopamine [103]. nAChRs might serve to regulate dopamine release in both striatal and mesocortical pathways [104,105]. Levin, McGurk, Rose and Butcher [106] have performed an extensive series of studies suggesting complex interactions with several possible anatomical loci for the site(s) of interaction, including both limbic and hippocampal areas, as well as descending projections to dopamine-containing areas of the mesencephalon via the medial habenula. Thus, is it possible that nicotine is exerting positive effects on inhibition and sustained attention in ADHD by enhancing the dopaminergic tone of both the striatal-frontal and mesocortical dopaminergic systems. In support of this conclusion, Solanto [107] in a recent review concluded that the majority of cognitive symptoms of ADHD (including behavioral inhibition) is mediated by the prefrontal cortex, and that stimulant medication might affect cognition by acting at D1 and D2 receptor sites to optimize the neurochemical environment.

Does nicotinic stimulation enhance cognitive functioning or performance?

A review of the recently published works on the effects of nicotine on cognitive function and performance in humans could lead to some confusion because of the contradictory conclusions of these studies. There seems to be an almost equal number of studies that show either performance enhancement or impairment by nicotine. A careful look at the nature of these disparate studies reveals clues to understanding the seemingly contradictory nature of research in this area.

Studies that tend to show impairment generally use normal non- or never-smokers as subjects, even studies with chronic use. These studies tend to conclude that nicotine does not improve cognitive functioning and most often impairs it. In contrast, studies that tend to show improvement generally utilize smokers or clinical populations of subjects. These studies generally demonstrate and/or conclude that nicotine has cognitive-enhancing effects. These disparate results can be resolved by considering that the findings reflect the differing populations utilized for the experiments. These populations can be expected to show quite different responses to nicotine from the principles of rate dependency or baseline effects [108] of nicotine (e.g. the Yerkes-Dodson principal). This is illustrated in Figure 1. Cognitive performance can be

Figure 1



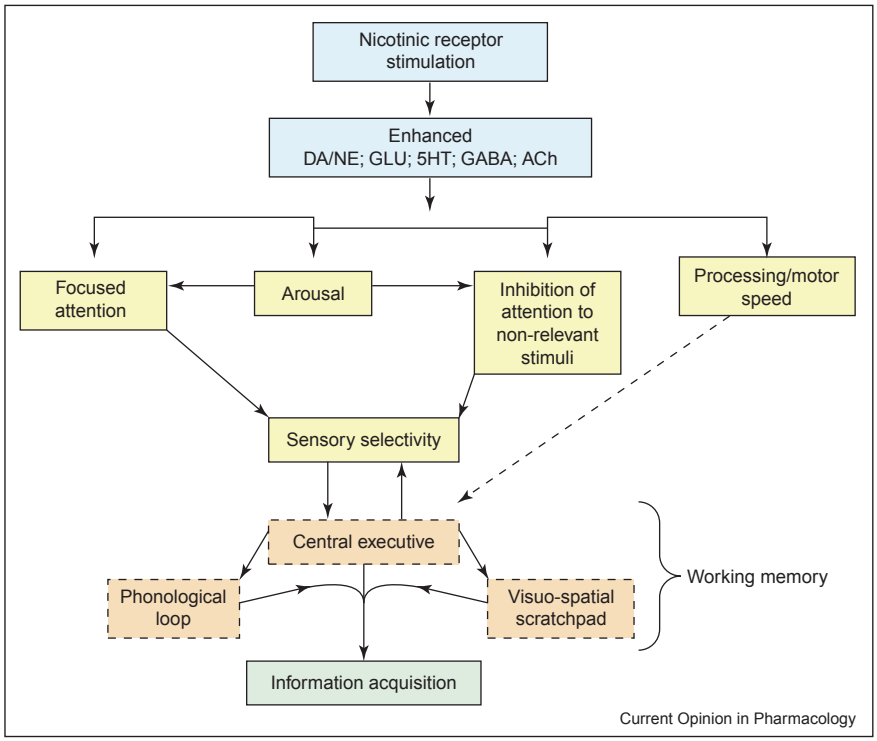
This figure illustrates two situations in which an equivalent degree of nicotinic stimulation produces opposite effects and illustrates the general principle that the results of nicotinic stimulation are a reflection of baseline performance level. The results of nicotinic stimulation, like many biological systems, can either increase or impair function (Yerkes-Dodson principal). **(a)** Illustrates an example of an individual whose cognitive performance is impaired or for whom task demands do not match the level of ongoing nicotinic stimulation. The nicotine administration or nicotinic receptor stimulation produces an improvement in cognitive performance, bringing performance to near optimal levels. **(b)** Illustrates a different scenario where the individual performance is already at or near optimum levels. In this case, the same degree of nicotinic stimulation as in **(a)** produces impairment of performance. This illustration might help to explain apparently paradoxical results of cognitive studies in differing populations.

envisioned as a curvilinear function related to nicotinic stimulation, with intermediate levels of stimulation producing optimal performance and either low or high levels of stimulation impairing performance. If an individual subject is performing suboptimally because of a disease state or impairment (e.g. AD), his performance will be enhanced by increased nicotinic stimulation (Figure 1a). However, if an individual subject is already performing at or near their optimal level of performance, increasing nicotinic stimulation will produce deterioration in cognitive functioning (Figure 1b). The same analysis can apply if the individual is normal but the task demands are severe. If the task is demanding enough in terms of attention, especially over a period of time, then the individual might move back to the left in terms of the performance curve, and optimal performance will require enhanced nicotinic stimulation.

Studies of normal volunteers, especially non- or never-smokers, are unlikely to show cognitive improvement with nicotinic stimulation because of the fact that these individuals are likely to be operating at or near their optimal level of performance, particularly in the setting of experimental paradigms with, for example, pre-training for cognitive tasks and financial rewards for participation. In addition, this might reflect differences between non-smokers and smokers in the underlying neurobiology and/or efficiency of the nicotinic system, and suggests that some of the reasons why subjects smoke may be, in part, related to the degree of improvement in cognitive performance that is seen with nicotinic stimulation.

The preponderance of evidence suggests that stimulation of nAChRs is most easily detected by effects on attentional systems and, to some extent, psychomotor speed. The most well-documented effect of nicotine is intensifying or sustaining attention to stimuli or tasks over a prolonged period of time. In addition, there is evidence from studies of individuals with disorders such as schizophrenia and ADHD that nicotinic stimulation enhances selective attention, sensory detection and inhibitional processes in attention. Positive effects of nicotine on learning or memory might be mediated by its effects on attentional functioning (Figure 2). Learning and memory require acquisition, encoding, storage and retrieval; however, attention is the 'front end' of this process, and adequate attentional functioning is a primary requirement.

Figure 2



Proposed model for nicotinic receptor stimulation effects on neurotransmitter function and attentional function. In this model, nAChR stimulation is presumed to lead to enhanced neurotransmitter release in particular brain areas relevant to arousal, sustained attention, inhibitional processes and processing/motor speed. Sensory selectivity is conceptualized as being secondary to improved attentional performance. These processes are thought to impinge on the 'central executive' component of Baddeley's model [110] for working memory leading to improved acquisition of information.

Conclusion: attention as a nicotinic endophenotype

In summarizing research done on nicotine and cognition, Warburton and Rusted [109] concluded that the most robust effects of nicotine are seen in tasks that have a high attentional requirement (i.e. memory enhancement might be a consequence of improved attentional functioning). Attention and related processes can be thought of as an endophenotype for nicotinic stimulation and consequently drug development. Attention, central processing impairment and executive dysfunction might be orthogonal to the underlying neuropsychiatric diagnosis and should be considered as an independent target for nicotinic drug development across diagnostic categories. Particular attentional deficits in different diagnoses could still respond to nicotinic stimulation; however, the parameters for assessing improvement might be quite different between disease states and will require careful attention to receptor subtype-specific agents, dosing regimens and outcome measures in clinical trials. Paying careful attention to the issue of baseline-dependency in treatment response will be vital to ensure appropriate interpretation of experimental results, both for studies of normal individuals and for those with disease states. Targeting specific populations that are already impaired is much more likely to reveal potential benefits of nicotinic stimulation. Studies of normal or unimpaired individuals with nicotinic drugs are unlikely to show cognitive benefit except under extreme task demands.

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Platelet monoamine oxidase, smoking cessation, and tobacco withdrawal symptoms

Jed E. Rose, Frederique M. Behm, Clay Ramsey, James C. Ritchie Jr.

Previous studies have found that constituents in tobacco inhibit both forms of the enzyme monoamine oxidase (MAO-A and MAO-B). This enzyme is important in the breakdown of the amine neurotransmitters, including dopamine, which is thought to mediate the reinforcing effects of nicotine and contribute to tobacco dependence. To further examine the relationship between cigarette smoking, smoking cessation and MAO, we measured platelet MAO-B activity in 16 smokers before and after being switched to smoking denicotinized cigarettes; in a subset of six subjects who subsequently quit-smoking, MAO-B activity was also measured at 1 and 4 weeks following cessation. Smoking cessation treatment was provided in an open-label format, and included nicotine skin patch treatment in conjunction with oral mecamylamine (a nicotinic antagonist) and neostigmine (a peripherally acting acetylcholinesterase inhibitor, administered to counteract constipation experienced from mecamylamine). Results showed that smoking behavior, indexed by expired air carbon monoxide levels, was negatively correlated with platelet MAO-B activity prior to smoking cessation. Moreover, MAO-B activity significantly increased by approximately 100% at 4 weeks after quitting smoking. However, little or no recovery occurred within the first week of abstinence, suggesting that the constituents in tobacco responsible for MAO inhibition may have half-lives of several days. Thus, if relapse to smoking is due in part to withdrawal from the MAO-inhibiting effects of tobacco, this effect likely occurs more than 1 week after quitting. Additionally, low baseline MAO-B activity significantly predicted the intensity of withdrawal symptoms reported upon switching to the denicotinized cigarettes as well as after smoking cessation. These results support the view that MAO inhibition from non-nicotine constituents in cigarette smoke is relevant to tobacco dependence and that continued investigation of the potential use of MAO inhibitors in smoking cessation treatment is warranted.

Introduction

A great deal of evidence has implicated nicotine as an important reinforcing agent maintaining tobacco use and dependence (USDHHS, 1988). However, results of several investigations have suggested that there may also be an important role for non-nicotine components of tobacco. In particular, tobacco constituents other than nicotine have been shown in *in vitro* studies to inhibit both forms of the enzyme monoamine oxidase (MAO-A and MAO-B), which play a critical role in the degradation of the biogenic amines norepinephrine, serotonin and dopamine (Castagnoli, Khalil, Steyn, & Castagnoli, 2000; Mendez-Alvarez, Soto-Otero, Sanchez-Sellero, & Lopez-Rivadulla Lamas, 1997). Cigarette smokers also have lower brain MAO-A and –B activity than non-smokers (Fowler *et al.*, 1996a,b). Moreover, it has been suggested that inhibition of MAO by endogenous tobacco compounds potentiates the reinforcing effects of nicotine, which include catecholamine release (Fowler *et al.*, 1996a). Dopamine release mediated by ventral tegmental neurons appears to be a substrate of nicotine reinforcement in a rodent model of nicotine self-administration (Corrigall, 1995). In view of the fact that dopamine is a substrate for MAO-A as well as MAO-B,

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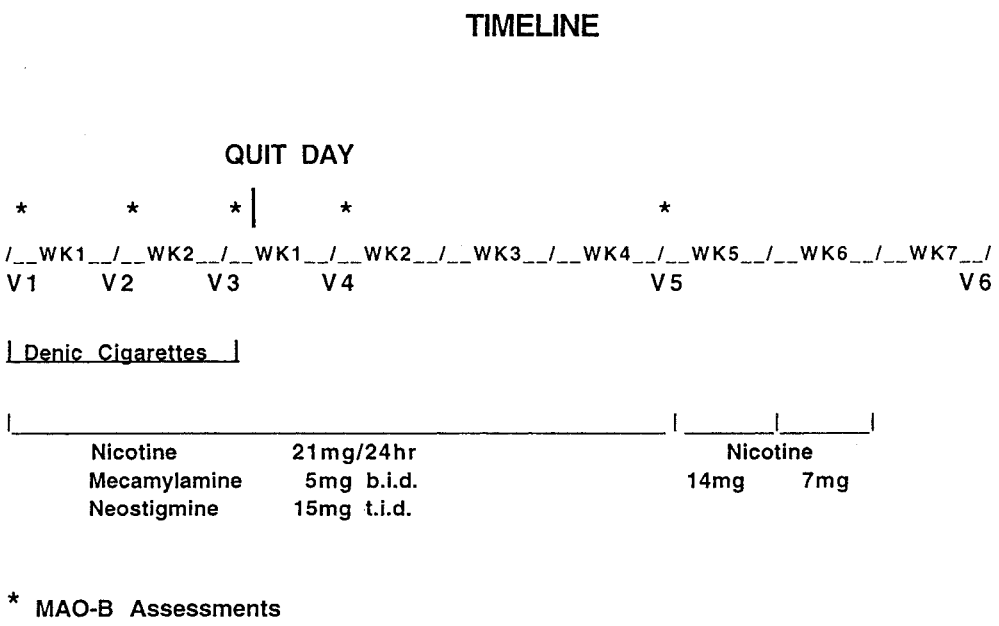


Figure 1. Timeline depicting the schedule of sessions (referred to as V1, V2, etc.), MAO-B assessments, cigarette brand-switching manipulation, and smoking cessation treatment.

both forms of MAO may be relevant to human cigarette addiction (Berlin *et al.*, 1995b). In a study of human smokers, Fowler *et al.* (Fowler *et al.*, 1996b) used positron emission tomography (PET) in conjunction with the radiotracer [11C]clorgyline, which binds irreversibly to MAO-A, and reported that smokers had an average reduction in MAO-A of 28%. In another study the same group showed that cigarette smokers have an approximately 40% reduction in brain MAO-B, based on PET measurements of the uptake of the radiotracer [11C]L-deprenyl-D2 (which binds irreversibly to MAO-B (Fowler *et al.*, 1996a)). Other studies have used peripheral measures of MAO activity to compare smokers, former smokers, and never-smokers (Berlin *et al.*, 1995b; Norman, Chamberlain, & French, 1987; Norman, Chamberlain, French, & Burrows, 1982). The results showed that former smokers have platelet MAO-B activity similar to non-smokers, and higher than smokers have, consistent with the hypothesis that tobacco components inhibit MAO.

Although several studies have documented smoker vs. non-smoker differences in MAO activity, little is known about the time course of MAO recovery after smoking cessation, or of the relationship between MAO activity, tobacco withdrawal symptoms, and smoking cessation outcome. The following study was conducted to provide further information on these issues, by measuring platelet MAO-B activity before smoking cessation and again 1 and 4 weeks after cessation. In addition, measures were taken before and after switching to denicotinized cigarettes for 2 weeks prior to quitting smoking, in order to determine whether components of smoke present in denicotinized cigarettes might sustain the reduction in MAO-B activity produced by conventional nicotine-containing cigarettes.

Methods

Design

This study was conducted as part of an open-label trial evaluating combination pharmacotherapy for smoking cessation. The treatment was based on previous studies in our laboratory, which have shown concurrent nicotine/mecamylamine administration to have considerable promise for smoking cessation treatment (Rose, Behm, & Westman, 1998; Rose *et al.*, 1994). However, one side-effect that has limited mecamylamine dose has been constipation, presumably due to mecamylamine's ganglionic blocking action. The current protocol also explored the use of neostigmine to counteract the constipation produced by mecamylamine. By enhancing cholinergic transmission at parasympathetic ganglia controlling gut motility, neostigmine was predicted to reduce the incidence and severity of constipation produced by mecamylamine (Moyer, Heider, & Dennis, 1957). The results of the study bearing on this issue have been reported elsewhere (Rose & Westman, 1999). In addition to receiving pharmacological treatment, subjects were instructed to switch from their usual nicotine-containing brands to denicotinized cigarettes (see below) for 2 weeks prior to their target quit-smoking dates. Figure 1 depicts the timeline of the study.

Denicotinized cigarettes

We obtained denicotinized cigarettes (Phillip Morris, Inc., Richmond, VA) which contain tobacco from which the nicotine has been selectively extracted by supercritical carbon dioxide. The cigarettes have a taste and tar delivery (9 mg, when smoked by Federal Trade Commission criteria) similar to nicotine-containing brands of

cigarette. However, the nicotine delivery is extremely low (0.08 mg). Hasenfratz, Baldinger, and Battig (1993) measured smoking behavior and nicotine intake after smoking denicotinized vs. nicotine-containing cigarettes and found that plasma nicotine levels increased less than 2 ng/ml after smoking the denicotinized cigarette. We have verified that these cigarettes produce less than 2 ng/ml boosts in arterial blood nicotine concentration and also that they do not increase heart rate, a sensitive index of nicotine delivery in deprived smokers (Rose, Behm, Westman, & Coleman, 1999).

Subjects

To be included in the study, participants had to be 18–65 years old, smoke at least 20 cigarettes per day on average, express a desire to quit smoking within the next 30 days, and be in general good health, based on physical examination, EKG, serum chemistries, CBC and urinalysis. Additionally, subjects must have indicated a willingness to switch to denicotinized cigarettes. Exclusion criteria were hypertension (systolic>140 mmHg, diastolic>90 mmHg), hypotension (systolic<90 mmHg, diastolic<60 mmHg), coronary artery disease, cardiac rhythm disorder, history of urinary retention, prostatic hypertrophy, glaucoma, impaired renal function, history of skin allergy, active skin condition (psoriasis) within the last 5 years, other major medical condition, current psychiatric disease, pregnancy or nursing, current alcohol or drug abuse, current smokeless tobacco use, nicotine replacement therapy, or other smoking cessation treatment.

Procedure

Subjects received concurrent nicotine/mecamylamine treatment (21 mg nicotine/24 h+5 mg oral mecamylamine hydrochloride twice a day) for 2 weeks before the quit-smoking date, and for 4 weeks post-cessation. Weaning patch doses of 14 mg/day and 7 mg/day were provided for weeks 5 and 6 post-cessation. Subjects also swallowed tablets containing 15 mg neostigmine bromide three times per day, before each meal. Subjects came to the laboratory to assess dependent measures at 2 weeks, 1 week and 1 day prior to the quit-smoking date, and 1 week, 4 weeks and 7 weeks (1 week post-weaning) after the quit-date.

Dependent measures. Smoking behavior: cigarette smoking was assessed by self-report using daily diaries, and by expired air carbon monoxide (CO) measurements at each session (Vitalograph, Lenexa, KS). Saliva samples were also collected and assayed for nicotine and cotinine by Clinical Pharmacology Laboratory at the University of California, San Francisco.

Withdrawal symptoms: To assess subjective withdrawal symptoms, subjects completed a questionnaire, which consisted of seven-point item self-ratings ranging from 'not at all' to 'extremely'. Previous factor analytic studies (e.g., Shiffman & Jarvik, 1976), have shown the items to cluster into the following scales:

- *Craving* ('would have liked a cigarette', 'missed a cigarette', 'thought of cigarettes', 'urges to smoke', 'craved a cigarette' and, scored oppositely, 'would have refused a cigarette');
- *Negative affect* ('tense', 'irritable', and, scored oppositely, 'calm', 'content');
- *Arousal* ('able to concentrate', 'wide awake', and, 'trouble sleeping');
- *Appetite* ('eaten more than usual', 'craved sweets', 'craved salty foods');
- *Habit withdrawal* ('missed something to do with hands', 'missed something in the mouth');
- *Somatic symptoms*, which were subclassified based on factor analyses into respiratory symptoms ('sore throat', 'cough'), somatic anxiety ('hands shaky', 'fluttery feelings in the chest', 'heart beating faster than usual') and gastrointestinal complaints ('upset stomach', 'nauseated', 'dizzy').

Smoking motivation questionnaires: A questionnaire was administered to assess the importance of different smoking motives, which included scales assessing sensorimotor, habit, tension-reduction, stimulation and addictive smoking (Ikard, Green, & Horn, 1969). Participants were also administered the Fagerström Test for Nicotine Dependence (FTND) (Heatherton, Kozlowski, Frecker, & Fagerström, 1991).

Measurement of platelet MAO-B activity. Venous blood samples (18 ml) were collected in tubes containing 2 ml of ACD (acid-citrate-dextrose). A platelet-rich plasma (PRP) was prepared by sequential centrifugation at 175*g* and 300*g*. The PRP was removed and pooled after each centrifugation. The pooled PRP was recentrifuged at 10,000*g* and the plasma was decanted. The platelet pellets were frozen at –80°C. For assay the platelet pellet was thawed and resuspended in 1.5 ml of 0.05 M sodium phosphate buffer plus 0.15 M sodium chloride, at pH 7.0. MAO activity was determined by the method of Murphy *et al.* (1976). Briefly, 10 µl of the mixed MAO inhibitor pargyline (0.0004 M) or normal saline was incubated with 250 µl of platelet suspension in duplicate for 15 min in a 37°C water bath with shaking. Next, 250 µl of substrate solution was added and incubated for precisely 30 min at the above temperature. The substrate solution was a mixture of [^{14}C]benzylamine+unlabeled benzylamine (approximately 0.1229 µmoles total per 250 µl in 0.25 M Tris buffer, pH 9.1). The reaction was stopped by the addition of 50 µl of 6 M HCl. Reaction products (labeled and unlabeled benzaldehydes) were extracted into 3 ml of hexane. One milliliter of the hexane extract was mixed with scintillation cocktail, counted, and corrected to DPMs. All determinations were done in duplicate. Blanks were run for both the inhibitor and buffer and samples were corrected for the blank DPMs.

Results were expressed as nmoles benzylamine deaminating activity/mg platelet protein/h (nM/mg/h). Platelet protein concentrations were determined by the bicinchoninic acid microplate method (Smith *et al.*, 1985) available from Pierce (Rockford, IL, USA). A standardized platelet-rich plasma was run with every assay as a quality control. Using this preparation, the interassay coefficient of variance (CV) averages 11% at a level of 5.2 nM/mg/h and the intra-assay CV averages 4.5%.

Data analytic approach: To examine the potential relationship between smoking and MAO-B activity, analysis of variance (ANOVA) and linear regression techniques were applied, using SuperANOVA and Statview statistical software (SAS Institute, Cary, NC, USA; Abacus Concepts, 1989). With repeated-measures factors (e.g., when time was included as a factor to assess changes in MAO activity after smoking cessation), these statistical programs use a multivariate approach to repeated measures ANOVA, which appropriately takes into account correlations between dependent variables. Covariates were sometimes included in the analyses as noted below, and in these cases the statistical significance of independent variables was evaluated with covariates set to their mean values. The alpha criterion was taken to be 0.05, although trends are noted as well. Effect sizes are described in terms of percentage of variance in the dependent measure accounted for by a given factor or independent variable (ω^2 or r^2; Vaughan & Corballis, 1969). Because previous literature raised the question as to whether men show any relationship between smoking and MAO activity (Norman *et al.*, 1982, 1987), separate analyses were conducted for the subset of male smokers in our sample.

For some results (e.g., subject sample characteristics, compliance and rates of smoking abstinence) only descriptive statistics are presented below.

Results

Subject characteristics

Sixteen subjects (12 males, four females) provided blood for MAO determination at baseline. Subjects' mean age (±SD) was 43.4±10.04 years, and they smoked an average of 30.2±9.77 cigarettes/day, with an FTC nicotine yield of 0.64±0.35 mg. They had smoked on average for 24.1±8.91 years. Their mean expired air CO level was 39.7±12.73 ppm and mean score on the FTND was 6.2±1.97, indicating a moderate degree of nicotine dependence.

Test–retest reliability of MAO-B measurement

Platelet MAO-B activity assessed at the screening physical exam was highly correlated with the activity at session 1 (which was held between 2 and 28 days later), $F(1,14) = 21.77$, $p = 0.0004$, $r = 0.78$. Also, the mean values were nearly identical (2.8±1.63 nM/mg/h at screening; 2.7±1.92 nM/mg/h at session 1). This indicates that the measure was stable prior to the introduction of smoking cessation treatment.

Smoking behavior

Smoking declined somewhat after subjects were switched to the denicotinized cigarettes. Cigarettes/day (calculated as a weekly average based on daily diaries, and at baseline from retrospective assessment) declined significantly over the first 2 weeks ($F(2,30) = 10.30$, $p = 0.0004$, $\omega^2 = 0.06$) from 30.5±10.81 cigarettes/day to 23.7±13.5 cigarettes/day at the end of week 1 and 22.7±13.47 cigarettes/day at the end of week 2. Expired air CO also declined across sessions ($F(3,42) = 8.55$, $p = 0.0002$, $\omega^2 = 0.08$) from a baseline value of 39.6±11.08 ppm to 32.5±16.56 ppm at the end of week 1 and 28.2±19.72 ppm at the end of week 2. Compliance with switching to the denicotinized cigarettes was excellent based on self-report. Fifteen of the 16 subjects reported smoking only the denicotinized cigarettes after day 3 of being switched. The cotinine results were consistent with the self-report data; mean salivary cotinine was 348±132.4 ng/ml at baseline, 253±157.4 ng/ml at the end of week 1 and 212±84.0 ng/ml at the end of week 2. For the latter 2 weeks, the estimated cotinine levels from wearing nicotine patches, based on previous studies (e.g., Rose *et al.*, 1998) was 170 ng/ml. Thus, the cotinine derived from smoking can be estimated to be 83 ng/ml at week 1 and 42 ng/ml at week 2. The latter represents approximately 12% (i.e., 42/348) of the baseline value obtained from smoking the usual brands of cigarettes, which agrees well with the ratio of standardized nicotine deliveries of the denicotinized to nicotine-containing brands of cigarettes (0.08/0.64 = 12.5%).

After the quit-smoking date, abstinence was assessed by self-report, and confirmed by an expired air CO value ≤8 ppm. Continuous and point (7 days') abstinence were measured, and drop-outs were assumed to have relapsed to smoking. Rates of continuous abstinence were 60%, 40% and 35% at quit-date (self-report only), 1 week and 4 weeks post-quit date, respectively. Point abstinence rates were 40% and 42.1% at 1 week and 4 weeks, respectively.

Relationship between MAO and smoking

The relationship between smoking and MAO-B activity was assessed in two ways. First, we examined correlations between expired air CO and MAO-B activity at each time point (baseline, 2 weeks after switching to denicotinized cigarettes, 1 and 4 weeks after the target quit smoking date). In addition, the change in MAO-B activity over time was assessed within subjects in a repeated-measures analysis. The mean of screening and session 1 MAO activity values was used as a baseline against which to assess changes. Complete MAO data were available from eight subjects, and of these, two had high expired air CO values at 1 week post-quit date.

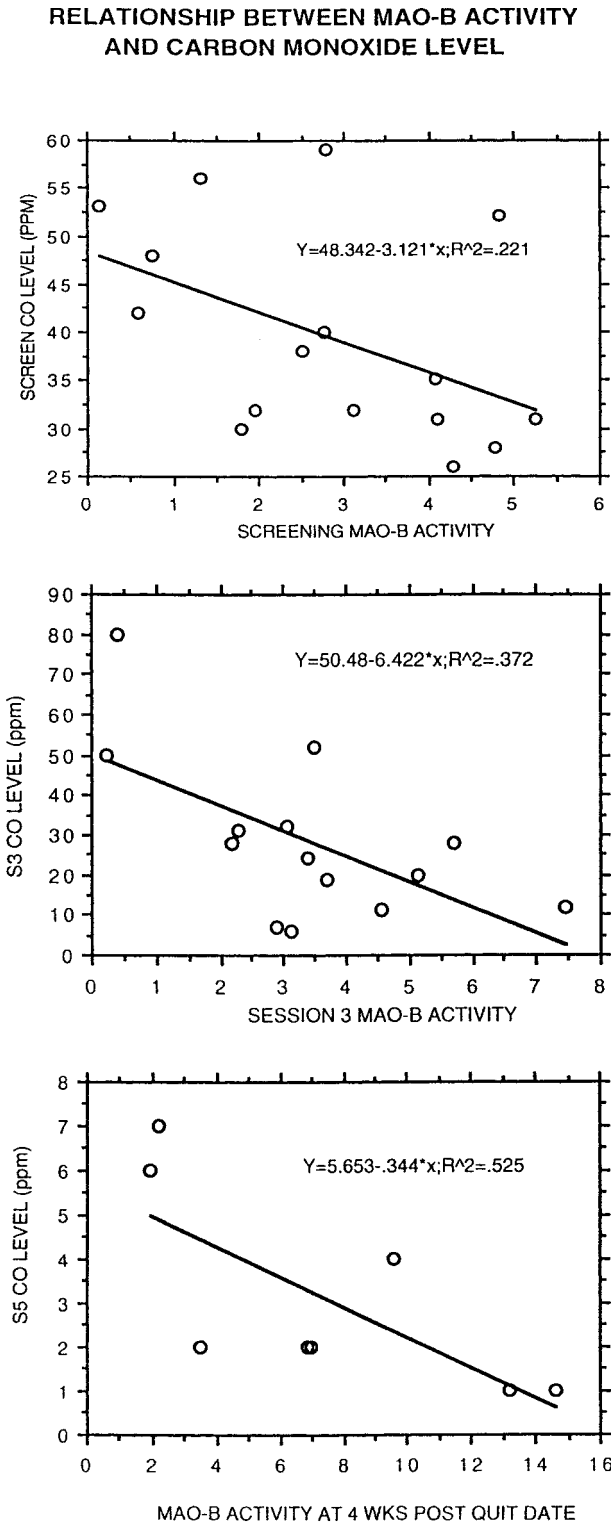


Figure 2. Relationships between platelet MAO-B activity and expired air carbon monoxide levels are shown for three time points in the study.

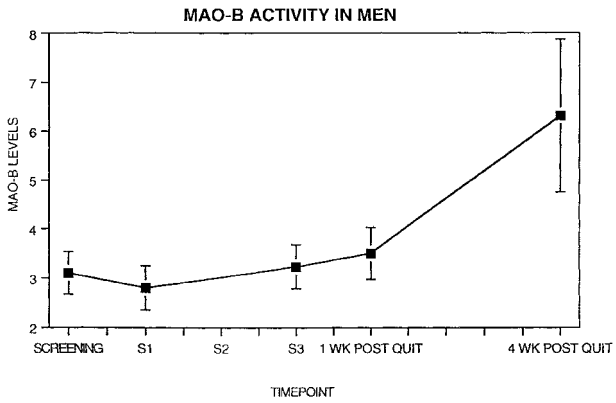


Figure 3. Time course of recovery of platelet MAO-B activity; mean (±SEM) values are shown for the subset of male subjects who were continuously abstinent from smoking at 1 and 4 weeks after the quit-smoking date.

These two were excluded from the analysis to more accurately estimate the recovery time of MAO during abstinence. Five of the remaining six subjects were completely abstinent at all time points, and in all cases expired air CO levels were less than 8 ppm at all points, indicating minimal smoking.

There were negative correlations between expired air CO and MAO-B at the following sessions: at baseline, $r^2 = 0.22$ ($F(1,14) = 3.97$, $p = 0.07$, $n = 16$); at session 3 $r^2 = 0.36$, ($F(1,12) = 7.10$, $p = 0.02$, $n = 14$); and at session 5 (4 weeks post-quit) $r^2 = 0.49$ ($F(1,6) = 6.62$, $p = 0.04$, $n = 8$). Figure 2 shows the regressions of MAO against CO for these time points.

The more sensitive repeated-measures analysis, based on six subjects, revealed a highly significant change in MAO-B activity over time ($F(3,15) = 4.92$, $p = 0.01$, $\omega^2 = 0.15$ for the main effect of time); subsequent contrasts showed that MAO-B activity at 4 weeks post-quit was significantly higher than baseline, ($F(1,15) = 11.43$, $p = 0.004$). Because of previous reports that the relationship between MAO activity and smoking might hold only for women, we conducted the same analyses for the two sexes separately, recognizing that statistical power would be quite limited in view of the small sample size. Nonetheless, the relationship between smoking and MAO-B activity appeared to hold for both men and women. For women, $r^2 = 0.81$ ($F(1,2) = 10.24$, $p = 0.08$) at baseline; $r^2 = 0.81$ ($F(1,1) = 7.40$, $p = 0.2$) at session 3; a correlation could not be computed for session 5 as only one woman was represented at that time point. For men, $r^2 = 0.09$ ($F(1,10) = 1.28$, $p = 0.3$) at screening; ($r^2 = 0.36$ ($F(1,9) = 4.44$, $p = 0.06$) at session 3, and $r^2 = 0.49$ ($F(1,5) = 4.18$, $p = 0.1$) at session 5. The repeated-measures analysis for men ($n = 5$) showed a significant increase in MAO-B activity over time ($F(3,12) = 6.43$, $p = 0.008$, $\omega^2 = 0.34$). Subsequent contrasts indicated a significant increase at 4 weeks post-quit relative to baseline ($F(1,12) = 15.15$, $p = 0.002$); see Figure 3. Although data were obtained at all sessions from only one female subject, her MAO-B activity did increase from 9.47 nM/mg/h (baseline) to 14.64 nM/mg/h at 4 weeks post-quit.

Baseline MAO-B activity was not significantly associated with point or continuous abstinence at any time point ($p>0.5$).

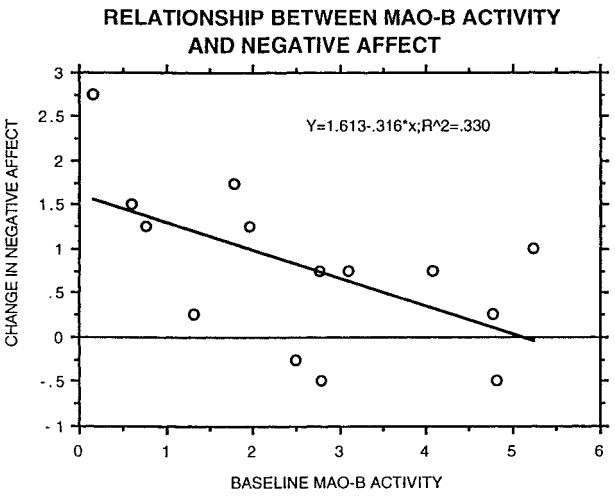


Figure 4. Relationship between baseline platelet MAO-B activity and the increase in negative affect following switching to denicotinized cigarettes.

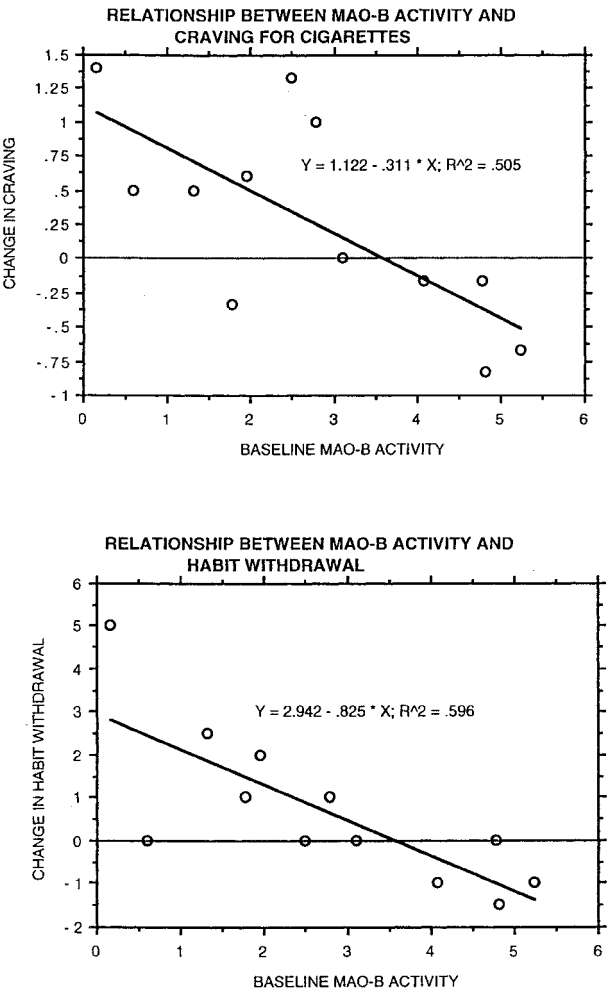


Figure 5. Relationships between baseline platelet MAO-B activity and the increase in craving for cigarettes and habit withdrawal symptoms on the quit-smoking day.

Relation of MAO activity to smoking motivation and withdrawal symptoms

Baseline MAO-B activity was correlated with the relaxation scale of the smoking motivation questionnaire (r^2 = 0.36, $F(1,14)$ = 9.17, p = 0.009, n = 16). Because both variables may have been associated with the degree of smoking, expired air CO was included as a covariate in the model; nonetheless, the association of MAO activity and the relaxation scale score remained significant ($F(1,13)$ = 5.12, p = 0.04).

Baseline MAO-B activity also predicted the intensity of certain smoking withdrawal symptoms. There were two points at which subjects displayed an increase in withdrawal symptoms: after switching from their usual brand of cigarette to the denicotinized cigarettes, and again after quitting smoking. The former was assessed by the change in symptoms from session 1 to session 2; the latter was assessed by the change from session 3 (day before quitting) to the quit-smoking day. There was a negative association between baseline MAO-B and the increase in negative affect at 1 week after being switched to the denicotinized cigarettes (r^2 = 0.33, F(1,12) = 5.91, p = 0.03), as shown in Figure 4. This association remained significant after including baseline CO and FTND scores as covariates ($F(1,10)$ = 10.94, p = 0.008). There was also a trend for an association between baseline MAO-B activity and the increase in habit withdrawal symptoms after switching to denicotinized cigarettes (r^2 = 0.25, F(1,12) = 3.09, p = 0.1), which became significant when the same covariates were incorporated in the model ($F(1,10)$ = 5.15, p = 0.047).

After quitting smoking, the increase in craving for cigarettes was significantly correlated with baseline MAO-B activity (r^2 = 0.51, F(1,10) = 10.19, p = 0.01); the increase in habit withdrawal was also highly correlated with baseline MAO-B activity (r^2 = 0.60, F(1,10) = 14.75, p = 0.003), as shown in Figure 5. These associations remained significant after including baseline CO and FTND scores as covariates ($F(1,8)$ = 6.43, p = 0.02 and F(1,8) = 9.84, p = 0.01 for craving and habit withdrawal, respectively).

Effects of study medications on MAO activity

To assess potential direct influences on MAO-B activity of the study medications (and in the case of nicotine, the main metabolite cotinine), *in vitro* tests of platelet MAO-B activity were conducted at levels comparable to, or exceeding, the expected physiological concentrations (200 ng/ml mecamylamine, 600 ng/ml neostigmine, 40 ng/ml nicotine and 140 ng/ml cotinine). No significant inhibition of MAO-B activity was detected for any of these substances (measures of activity remained within 12% of baseline levels).

Discussion

The main result of this study was that platelet MAO-B activity was highly correlated with smoking behavior, with a significant suppression in MAO-B activity

associated with more intensive smoking, as assessed by baseline expired air CO levels. Moreover, in our study MAO-B activity recovered substantially toward the levels of non-smokers (Murphy *et al.*, 1976) within the 4 weeks following smoking cessation. These results are consistent with findings reported by Norman *et al.* (1982) and Berlin *et al.* (1995a), who found an increase in MAO-B activity 1 month after smoking cessation. The Berlin *et al.* (1995a) study found an apparent trend for MAO-B activity to recover partially within 1 week of quitting smoking. However, there was no MAO assessment immediately prior to the quit date and no data were provided to assess smoke intake over the period between baseline assessment and the 1-week post-quit time point. Thus, recovery of MAO-B activity within the first week of smoking abstinence could not be evaluated. In our study there was no apparent recovery of MAO-B activity in the first week of abstinence (Figure 3). This time course does not correspond to the usual recovery of MAO-B levels following withdrawal of an irreversible MAO-B inhibitor (e.g., selegiline). In that case, MAO levels recover substantially within 1 week of ending treatment, corresponding to the production of new platelets (Heinonen & Lammintausta, 1991). The longer recovery time we observed after withdrawal of cigarettes suggests that the constituents in tobacco which inhibit MAO may have half-lives of at least several days, although a prolonged effect on MAO synthesis in platelet-generating megakaryocyte cells cannot be ruled out.

An important unresolved issue is to what extent peripheral measures of MAO activity reflect activity in brain. Whether recovery of platelet MAO-B activity is correlated with recovery of brain MAO-B activity may depend, in part, on the nature of inhibition, i.e., reversible or irreversible. In the case of an irreversible inhibitor, studies of synthesis of MAO-B in human brain using PET show a half-time of recovery of 40 days (Fowler *et al.*, 1994), much longer than platelet turnover time. In contrast, for reversible MAO inhibitors, such as have been identified in tobacco (Castagnoli *et al.*, 2000), platelet MAO-B recovery rates may parallel brain recovery more accurately.

Although earlier reports suggested that the relationship between smoking and MAO-B activity might only hold for women (Norman *et al.*, 1982, 1987), we did find a robust recovery of MAO activity upon smoking cessation in the subset of men included in our sample. One of the previous studies (Norman *et al.*, 1987) also found a significantly higher MAO-B activity in men 1 month after quitting smoking relative to baseline, even though no significant correlation between MAO-B and smoking (indexed by plasma thiocyanate) was detected prior to cessation. Possibly this results from the increased statistical power to detect a within-subjects effect accompanying the dramatic decrease in smoke exposure after quitting. This interpretation is consistent with results from a recent study by Berlin, Spreux-Varoquaux, and Launay (2000) that found a significant negative correlation between cotinine and platelet MAO-B activity in men, after partialing out age-related variability.

The observation that MAO levels remained essentially unchanged during the 2 weeks in which denicotinized cigarettes were provided, but then recovered after withdrawal of these cigarettes, is in accord with previous findings that nicotine is not the agent responsible for MAO inhibition. Smoking behavior did decrease by about 25% (based on CO measurements) during this time, but this reduced intake of tobacco smoke may have been sufficient to maintain the inhibition of MAO-B.

Although the measurements of MAO-B activity were conducted in the context of pharmacological treatment, it is not likely that this affected the time course of MAO recovery. The results of the *in vitro* testing showed that at physiological levels, the pharmacological agents administered (nicotine, mecamylamine and neostigmine) did not directly affect MAO activity, and furthermore the pharmacological treatment was held constant during the 6 weeks in which MAO activity was assessed.

An intriguing finding of the present study was that baseline MAO activity predicted the increase in negative affect accompanying switching from the usual brand of nicotine-containing cigarettes to denicotinized cigarettes. These results are consistent with the hypothesis that the inhibition in MAO activity from tobacco smoke augments the reinforcing effects of nicotine. Hence, when nicotine intake from cigarettes suddenly diminished (despite wearing of nicotine patches), individuals with the lowest MAO might have been expected to suffer the most intense withdrawal. On the other hand, the increase in craving for cigarettes and habit withdrawal symptoms on the quit-smoking day, elicited by withdrawal from denicotinized cigarettes, was also correlated with baseline MAO-B activity. This suggests that biogenic amines involved in nicotine reinforcement may potentially play a role in some of the conditioned reinforcing aspects of cigarette smoking as well.

Although most smokers in the study were able to make the transition from smoking their usual brand of nicotine-containing cigarettes to the denicotinized cigarettes (given concurrent pharmacological treatment), over half of the subjects relapsed within a week of quitting smoking altogether. This raises the question of what aspect of withdrawal from denicotinized cigarettes is most important in triggering relapse. The time course of recovery of MAO, which appears to be longer than 1 week, would indicate that loss of inhibition of MAO is not likely to be a factor precipitating early relapse after smoking cessation. However, one caveat is that the time course was assessed in subjects who returned at each visit, who tended to be the successful abstainers. This self-selection effect prevented us from evaluating the time course of MAO recovery in would-be relapsers. However, the fact that 40% of the subjects failed to abstain from smoking even on their target quit-smoking day suggests that recovery of MAO activity is not a likely explanation for early relapse. A more probable

cause of early relapse is the loss of the conditioned sensory and behavioral cues of smoking. Whether relapse to smoking after the first week could be triggered in part by withdrawal from the MAO-inhibiting effects of tobacco smoke is an open question. In view of our small sample size, too few relapses occurred after week 1 to address this issue.

In addition to the potential predictive value of MAO measurements, our results may have direct implications for smoking cessation treatment. The hypothesis that inhibition of MAO potentiates the reinforcing actions of nicotine would imply that it may be especially important to replace the effects of MAO-inhibiting components of tobacco when providing NRT. Indeed, a smoking cessation trial conducted by our group has recently found that the addition of the MAO-B inhibitor selegiline to NRT substantially boosted abstinence rates after cessation (Brauer, Paxton, & Rose, 2000). In another study, Berlin *et al.* (1995a) reported that moclobemide, a monoamine oxidase-A inhibitor, increased rates of smoking abstinence in highly dependent smokers, although nicotine replacement was not provided. Interestingly, in that study baseline MAO-B activity was a significant predictor of relapse. Based on these findings, the use of MAO inhibitors in smoking cessation treatment, with or without NRT, merits further investigation.

Acknowledgments

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Nicotine pharmacokinetics and its application to intake from smoking

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1 Five subjects were given 25 μg/kg nicotine intravenously over 1 min, before and after a loading period involving the smoking of six cigarettes.
2 Plasma nicotine concentrations declined in a biphasic manner, the half-lives of the initial and terminal phases averaging 9 min and 133 min respectively.
3 Terminal half-lives before and after the loading period were essentially the same suggesting the absence of saturation kinetics at nicotine concentrations that build up during smoking.
4 The plasma clearance of nicotine and the volume of distribution were very high averaging 915 ml/min and 173 l, respectively.
5 Two approaches were used to calculate the nicotine intake from smoking. The average dose of nicotine absorbed from one cigarette was 1.06 mg which was 82% of the standard machine-smoked yield of 1.3 mg.
6 To illustrate their potential use in 'nicotine titration' studies, these approaches were used to compare nicotine intake from smoking a high (2.4 mg) and low (0.6 mg) nicotine cigarette. The dose of nicotine absorbed averaged 1.14 mg and 0.86 mg per cigarette respectively, being 48% and 143% of the machine-smoked yields.

Keywords nicotine smoking pharmacokinetics

Introduction

Knowledge of the disposition and fate of nicotine in humans is important for understanding better the nature of the smoking habit and its effects on health. In two early studies the half-life of nicotine in plasma was found to be about 30 min (Armitage *et al.*, 1975) or less (Isaac & Rand, 1972). Both these studies derived their estimates from the first cigarette of the day in smokers who had abstained overnight and were based on blood concentrations from both initial and terminal phases which were not differentiated. We have shown that with repeated dosage accumulation of nicotine occurs with an apparent corresponding increase in half-life (Feyerabend & Russell, 1978; Russell *et al.*, 1977). This could be due to saturation of the metabolic enzymes or to the gradual elimination of the initial phase. This question was not considered in two further recent kinetic studies based on intravenous administration of nicotine. Moreover, the terminal plasma half-life of nicotine was somewhat different in these studies, averaging 0.8 h in one (Kyerematen *et al.*, 1982) and 2 h in the other

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(Benowitz *et al.*, 1982), but in the former case calculations were based on a less precise one-compartment model.

Most detailed kinetic studies involve the intravenous route of administration. This is because the dose reaching the systemic circulation is known. In the case of nicotine, however, knowledge derived from this form of administration needs to be applied to the natural form of intake, namely the inhalation of tobacco smoke. We report here an approach to estimate the dose of nicotine absorbed from a cigarette based on a principle, often used in bioequivalence testing, that the area under the plasma concentration *vs* time curve (AUC), during one dosing interval at steady state on repeated dosing is equal to the AUC to infinite time for a single dose.

We describe four experiments undertaken to answer these questions by investigating the pharmacokinetics of nicotine after intravenous injection and cigarette smoke inhalation in habitual smokers.

Methods

Experiment 1

The subjects were five healthy men, aged 24 to 41 years, who were habitual smokers. Their average cigarette consumption was 25 per day (Table 1). They were studied in the morning after at least 12 h abstinence from smoking. To standardise for the effect of urinary pH on nicotine excretion, the subjects were given 2 g ammonium chloride orally four times on the day before the experiment.

At the start of the experiment each subject received a dose of 25 μg/kg nicotine base made up in 10 ml of buffered normal saline. It was given intravenously over a period of 1 min. One hour after the intravenous dose of nicotine, the subjects smoked their usual brand of cigarette at a rate of one per 0.5 h to a total of six cigarettes, each one being smoked over a 5 min period. A second intravenous injection of nicotine was then given 25 min after the last cigarette, the dose being the same as for the first injection (Figure 1). Blood samples (10 ml) were taken predose and at 2, 5, 10, 15, 20, 25, 30, 45 and 60 min after the first intravenous dose of nicotine. The same sampling times, with the omission of the 25 min sample, were used after the second intravenous dose, with additional sampling at 90, 120, 150 and 180 min after the injection. Blood samples were also

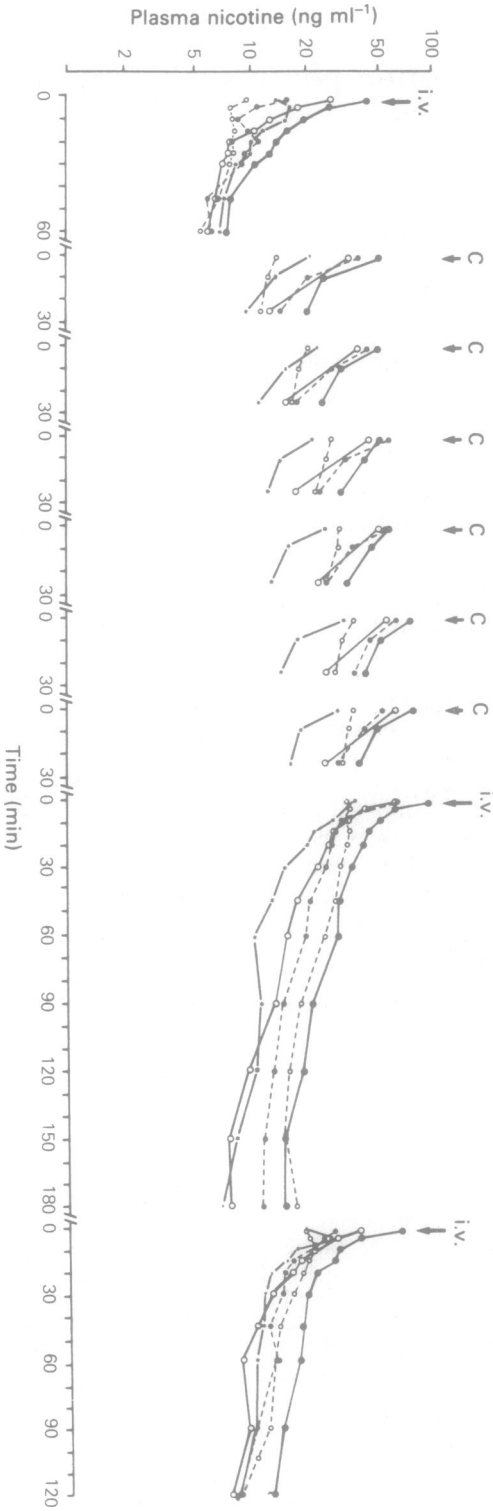


Figure 1 Plasma nicotine profiles of five subjects following three intravenous nicotine injections (i.v.) and smoking six cigarettes (C), plotted on a semi-logarithmic scale. Subject identification is as follows: ●—● PR, *—* BW, ○—○ JL, ●----● KK, ○----○ SS

taken at 2, 10 and 25 min after finishing each cigarette, apart from the first subject (JL) who was sampled at only 2 and 25 min. The same dose of nicotine was injected a third time 3 h after the second dose and blood samples were taken at the same times up to 2 h.

All blood samples were collected via an indwelling cannula inserted into a forearm vein which was never from the same arm as used for the intravenous injections. The blood samples were centrifuged and the plasma fractions stored at −20°C until analysed for nicotine. Saliva was collected continuously at 5 min intervals for the duration of the intravenous phases of the study. Total voided urine was collected at hourly intervals throughout the study period. The volumes and pH were measured immediately and aliquots were stored at −20°C until analysed for nicotine.

Experiment 2

Following the first intravenous dose in Experiment 1, the true log-linear decline of nicotine in plasma was not always reached within the 1 h sampling period. To enable the terminal half-life of nicotine to be determined more accurately the intravenous injection was repeated, on a different day, in four of the subjects and the sampling period was extended to 2 h. Conditions such as overnight abstinence and urine acidification were identical with Experiment 1. Blood sampling times were also the same over the first hour but at 75, 90, 105 and 120 min thereafter.

Experiment 3

To estimate the nicotine intake per cigarette when smoking heavily one of the subjects (PR) smoked his usual brand of cigarette (1.3 mg nicotine) over a 5 min period three times per hour for a total of 7 h. Blood samples were taken just before and 2 min after each cigarette.

Experiment 4

To compare nicotine intake from a high and low nicotine cigarette, a final experiment was done with one of the subjects (JL). After overnight abstinence and urine acidification as in Experiment 1, the subject smoked a low nicotine cigarette (0.6 mg) on one day and on a subsequent day a high nicotine cigarette (2.4 mg). Blood was sampled before and at 2, 5, 10, 15, 20, 25, 30, 45 and 60 min after the first cigarette. On each of the days the subject then smoked 12 further cigarettes at 0.5 h intervals. Blood was sampled at 2, 10 and 25 min after each cigarette, and at 2, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after the last one. Each cigarette was smoked over a restricted 5 min period.

Nicotine analysis

The plasma concentrations of nicotine were measured by gas chromatography as previously reported (Feyerabend & Russell, 1980) except that the internal standard was replaced by ethylnornicotine and the column packing by 2% Carbowax 20M/5% KOH on chromosorb W-HP 80/100 mesh. Sensitivity of this assay was 0.1 ng/ml with a coefficient of variation of 2% at a concentration of 5 ng/ml.

Pharmacokinetic analyses

Each of the plasma profiles of nicotine from the second intravenous dose (Experiment 1) and from the separate single intravenous dose (Experiment 2) were analysed by the method of residuals to fit a sum of exponentials (equation 1) to the data using linear regression. Curve-fitting of the saliva data was also attempted.

$$C = \sum_{i=1}^{n} C_i e^{-\lambda_i t} \qquad \text{Equation 1}$$

where C is the plasma concentration at time t, C_i and λ_i are the coefficient and exponential constants, respectively, to the ith term.

The half-life ($t_{1/2}$) of nicotine for each decline phase was calculated from the exponential constant using equation 2.

$$t_{1/2} = \frac{0.693}{\lambda_i} \qquad \text{Equation 2}$$

The area under the curve (AUC) calculations of the first intravenous dose and the area under the curve for the sixth cigarette (AUC_τ) were calculated by the trapezoidal rule.

The area under the curve measurements for the first intravenous dose were, in turn, used to calculate the plasma clearance (CL) of nicotine using equation 3.

$$CL = \frac{\text{Dose}}{\text{AUC}} \qquad \text{Equation 3}$$

The volume of distribution of nicotine based on area (V) and the initial distribution volume (V_1) were calculated from equations 4 and 5 respectively.

$$V = \frac{\text{Dose}}{\text{AUC}.\lambda_z} \qquad \text{Equation 4}$$

where z is the terminal exponential constant.

$$V_1 = \frac{\text{Dose}}{\sum_{i=1}^{n} C_i} \qquad \text{Equation 5}$$

Estimation of smoking dose

The calculation of the amount of nicotine inhaled and which reached the systemic plasma of each subject was determined using a modification of the calculation normally employed to determine absolute bioavailability (equation 6).

$$\text{Fraction of nicotine dose } (F) \text{ inhaled and absorbed} = \frac{\text{Dose of nicotine i.v.} \times AUC_\tau}{AUC} \quad \text{Equation 6}$$

where AUC_τ is the area under the curve for one dosing interval.

An alternative approach was based on the fact that at steady-state the rate of nicotine entering the body equals that leaving it (equation 7).

$$\text{rate in} = \text{rate out} = CL.C_{av} \quad \text{Equation 7}$$

where C_{av} is the mean steady-state plasma concentration.

The plasma clearance used was that calculated from the intravenous data. The C_{av} in the case of Experiment 1 was taken as the mean plasma concentration of the first three samples (2, 10 and 25 min) after the sixth cigarette. For Experiment 3, it was taken as the mean of the plasma concentrations over the plateau region of the plasma concentration *vs* time curve (from 3 h to 7 h). Where appropriate, statistical comparisons were made by a two-way analysis of variance.

Results

Intravenous administration

The plasma nicotine concentration 2 min after the first intravenous dose averaged 23 ± 14.7 ng/ml. The individual plasma nicotine profiles for Experiment 1 are shown in Figure 1 and the kinetic data are summarised in Table 1. After each intravenous dose of nicotine, the plasma concentrations declined in a biphasic manner. However, close inspection of the plasma profiles showed that the true log-linear decline of the compound from plasma had not always been attained within the 1 h period of sampling after the first dose and thus the terminal half-life of nicotine could not be accurately determined from these data alone. Experiment 2 was therefore undertaken to obtain the necessary terminal half-lives.

Curve-fitting analysis of the plasma data from the second intravenous dose, when the plasma levels were higher and the sampling was continued for longer (3 h), showed that for four of the five subjects, the plasma concentrations of nicotine declined in a biphasic manner. The initial decline was not observed in the fifth subject after any of the three injections and was also absent after each cigarette (Figure 1). The shapes of the curves following the three injections varied greatly between subjects but were consistent within subjects (Figure 1).

The half-life for the initial decline phase averaged 8.9, 9.0 and 9.1 min for the three injections respectively, and the terminal half-lives averaged 2.1, 2.2 and 2.2 h respectively. Very little of the drug was eliminated in the initial phase (6 ± 4%) as opposed to the terminal phase when determined from the ratio of the area under the curve for each respective phase to the total area under the curve. The values for the three injections were 6.0, 7.0 and 5.0%, respectively. The AUC for the first dose, measured to infinite time using the terminal half-life determined from the second intravenous dose of nicotine, averaged 32.07 ± 5.44 ng ml^{-1} h. This resulted in a high mean plasma clearance value of 915 ± 146 ml/min. The two measures of volume of distribution of nicotine, again determined from the first intravenous dose, were also comparatively high with mean ± s.d. values of 173 ± 23 l and 79 ± 45 l for volume of distribution based on area and the initial distribution volume respectively.

Nicotine intake from cigarettes

When six cigarettes were smoked at half-hour intervals starting 1 h after the first intravenous dose of nicotine, there was a steady accumulation of nicotine until, after approximately the fifth cigarette, steady-state conditions were reached (Figure 1). The average peak concentrations of nicotine at steady-state were 51.4 ± 20.0 ng/ml with trough levels of 28.6 ± 8.5 ng/ml. Using the area under the curve for the total 30 min period of the sixth cigarette, it was calculated that on average 1.04 ± 0.36 mg of nicotine was inhaled and absorbed into the systemic plasma of the subjects (Equation 6). The alternative method (Equation 7) gave almost identical values. These represented, on average, 82 ± 32% of the machine-smoked yields of the cigarettes (Table 1). The values for JL are a slight overestimate due to insufficient sampling (only two samples per cigarette) in his case.

In Experiment 3, after smoking three cigarettes per hour for 7 h, the plasma concentration of nicotine accumulated steadily until about 3 h when a steady-state was reached at an average concentration of 49 ng/ml (Figure 2). The dose

Table 1 Smoking habits, nicotine dosage and pharmacokinetic data of the five subjects

	PR	*BW*	*JL*	*KK*	*SS*	*Mean ± s.d.*
Daily cigarette consumption	25	25	30	30	15	25 ± 6.1
Nicotine yield of usual cigarettes (mg/cigarette)	1.3	1.3	1.2	1.3	1.4	1.3 ± 0.1
Body weight (kg)	71.0	80.2	71.2	62.0	62.0	69.3 ± 7.6
Dose of intravenous nicotine (mg)	1.78	2.01	1.78	1.55	1.55	1.73 ± 0.19
Initial $t_{1/2}$ (h)	0.12	0.16	0.15	0.15	—	0.15 ± 0.02
Terminal $t_{1/2}$ (h)	1.99	2.56	1.77	2.51	2.23	2.21 ± 0.34
% elimination in first phase	6	8	10	6	0	6 ± 4
% elimination in terminal phase	94	92	90	94	100	94 ± 4
AUC intravenous (ng ml^{-1} h)	37.78	37.25	25.72	31.85	27.75	32.07 ± 5.44
AUC_T (cigarette) (ng ml^{-1} h)	26.56	10.22	21.64	20.44	16.31	19.03 ± 6.13
CL (ml/min)	783	897	1153	811	931	915 ± 146
V_1 (l)	29	103	43	77	141	79 ± 45
V (l)	135	199	176	177	180	173 ± 623
*F (mg) (Equation 6)	1.25	0.55	1.50	1.00	0.91	1.04 ± 0.36
(Equation 7)	1.31	0.58	1.49	1.01	0.92	1.06 ± 0.35
% of cigarette yield (mean)	98	43	125	77	65	82 ± 32

*F is the systemic availability of nicotine inhaled from a cigarette.

of nicotine absorbed was calculated from the clearance and steady-state plasma concentrations of nicotine (Equation 7) to give a value of 0.77 mg per cigarette. This is less than the values of 1.25 mg and 1.31 mg per cigarette calculated for this subject in Experiment 1 and is also less than the machine-smoked yield of his cigarette (1.3 mg).

In Experiment 4 one of the subjects (JL) smoked a high nicotine cigarette (2.4 mg) on one day and a low nicotine cigarette (0.6 mg) on another day. The plasma nicotine concentrations are shown in Figure 3 and can be compared with this subject's data from Experiment 1. It can be seen that the concentrations on the low nicotine cigarette tended to be lower but the differences are small compared with the four-fold differences in machine-smoked yields. The plasma nicotine concentrations and AUCs for the first hour after the first cigarette can be compared with each other and with those after the first intravenous injection (1.78 mg nicotine) in Experiment 1. The amount of nicotine absorbed from the first cigarette on each occasion was 1.01 and 0.75 mg for the high and low nicotine brands respectively (Figure 3). Using the two methods described for Experiment 1 (but applied to the seventh cigarette in this case) the estimated nicotine intakes were 1.17 and 0.90 mg (Equation 6) and 1.24 and 0.95 mg (Equation 7). Using the average steady-state value based on all the data from 3 to 7 h the average nicotine intake per cigarette was 1.13 and 0.82 mg for the high and low brands, respectively (Equation 7). Averaging all the measures, the nicotine intake from the high yield cigarette was 1.14 mg compared with 0.86 mg from the low yield cigarette, 48% and 143%, respectively, of their machine-smoked yields.

Urinary excretion

By controlling the urine at an acid pH, the urinary elimination of nicotine was maximised. Nevertheless, at an average of 4.18 ± 1.39 μg

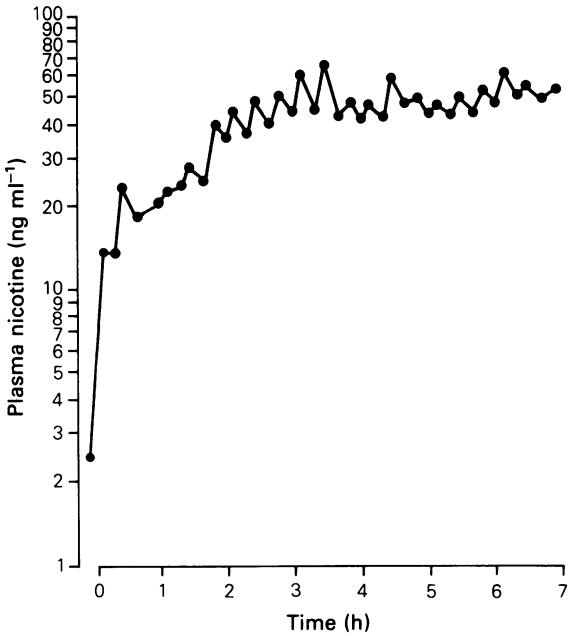


Figure 2 Plasma nicotine concentrations in one subject (PR) just before and 2 min after each cigarette when smoking three cigarettes per hour for 7 h, plotted on a semi-logarithmic scale.

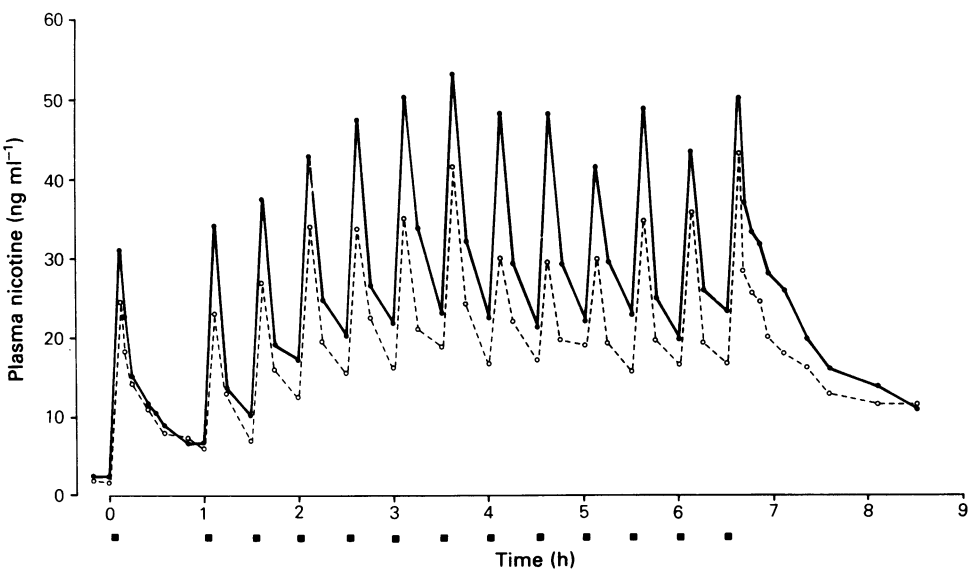


Figure 3 Plasma nicotine concentrations of one subject when smoking high (2.4 mg, •) and low (0.6 mg, ○) nicotine cigarettes. The smoking period for each cigarette was restricted to 5 min as shown (■).

over the 7 h period it was a small proportion of the administered dose. Moreover due to the frequent changes in dosing conditions (intravenous dosing followed by cigarettes then by another intravenous dose), combined with the lag between clearance by the kidney and actual urine collection, it was not possible to calculate renal clearance reliably.

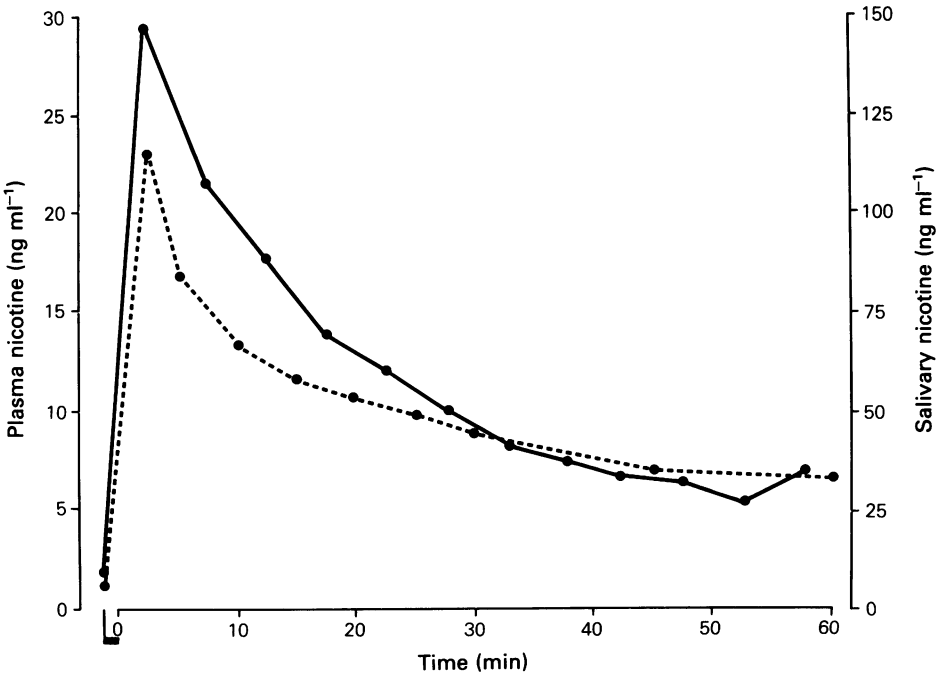


Figure 4 Average plasma (---) and salivary (—) nicotine concentrations of five subjects before and after the first dose of intravenous nicotine, 25 μg/kg given over 1 min.

Salivary excretion

Salivary concentrations of nicotine were much higher than those in plasma (Figure 4). The average ratio over the hour after the first injection ranged from 3.9 to 8.9 with a mean of 5.8. Although salivary concentrations closely paralleled the plasma concentrations for the first 30 min, accurate assessment of the terminal half-life was not possible due to the erratic decline in saliva concentrations after 30 min.

Discussion

The plasma profiles and half-lives of nicotine after the three intravenous injections were similar although the second injection was given after a steady-state had been attained by loading dosage from smoking. This suggests that saturation does not occur at nicotine concentrations that build up during smoking. There was no evidence that the initial phase was diminished or altered by repeated smoking. The initial phase half-life was similar after all three injections as was the proportion of the AUC attributable to the initial phase.

The decline of nicotine was biphasic. The average half-life of the initial phase was 0.15 ± 0.02 h and the terminal half-life averaged 2.21 ± 0.34 h. Although the elimination of nicotine was followed for only one half-life in the present study, our estimate is in good agreement with that of Benowitz *et al.* (1982), who employed longer sampling times. Attempts were also made to use saliva measurements of nicotine to determine its half-life after intravenous dosing. With the slightly acidic pH of saliva, levels of the compound would be expected to be considerably higher than those in plasma and should enable nicotine determinations to be carried on for longer. This was confirmed experimentally with a saliva to plasma ratio of approximately 6 and although initial results were encouraging (sampling times up to 30–60 min), the later samples proved to be rather variable and it was not possible to measure half-life with sufficient precision.

The average plasma clearance of nicotine (915 ± 146 ml/min) obtained in this study was similar to that reported by Rosenberg *et al.* (1980) and Benowitz *et al.* (1982). This high value might suggest that nicotine could be subject to first pass metabolism, since the clearance value approaches the hepatic blood flow. To confirm this, however, would require data on renal clearance and a correction factor for the distribution of nicotine into erythrocytes. The volume of distribution based on area (173

± 23 l) was fairly high indicating extensive distribution into tissues.

Many studies have demonstrated the plasma nicotine concentrations produced by cigarette smoking, but only one has estimated the actual dose of nicotine absorbed from a cigarette which averaged 1.04 mg nicotine per cigarette in subjects who were smoking an average of 35.6 cigarettes per day (Benowitz & Jacob, 1984a). We used two methods of estimating nicotine intake. They gave similar values which averaged 1.05 mg per cigarette in our five subjects. Both methods rely on steady-state conditions being achieved. One method used clearance and mean steady-state plasma concentrations of nicotine, while the other related area under the curve over one 'dosing' interval to the area under the curve of a known intravenous dose of nicotine. We have also shown that the half-life and plasma profiles after an intravenous dose are remarkably similar from one day to the next. This being so, once i.v. data have been gathered on given subjects, they could be used in 'nicotine titration' studies of smoking behaviour provided blood sampling was carried out in the afternoon when steady state conditions would apply.

Nicotine intake varied widely between subjects and bore little relation to the standard machine-smoked yields of their cigarettes. This was not unexpected since it is well-known that the plasma nicotine and cotinine concentrations of smokers also vary greatly and have little correlation with the nicotine yields of the cigarettes they smoke (Russell *et al.*, 1980; Ebert *et al.*, 1983; Benowitz *et al.*, 1983). When one subject smoked at a higher rate, three cigarettes per hour, he absorbed only 0.77 mg nicotine per cigarette compared with 1.28 mg per cigarette when smoking two per hour. Steady-state plasma nicotine concentrations were thus maintained at a similar level. Likewise, another subject, by 'undersmoking' a high nicotine cigarette absorbed only 48% of its machine-smoked yield and by 'oversmoking' and taking in 143% of the yield of a low nicotine cigarette, maintained fairly similar plasma nicotine concentrations despite a four-fold difference in the yields of the two brands.

These findings confirm previous work showing that smokers tend to regulate their nicotine intake by modifying their pattern of puffing and inhalation. In this way they tend to maintain a fairly constant intake whether the cigarettes they smoke have high or low nicotine yields (Benowitz & Jacob, 1984b). Previous nicotine titration studies have relied mainly on plasma nicotine concentrations as a valid dependent variable from which intake has been inferred. We have demonstrated how measurement of the actual dose of nicotine absorbed from a cigarette could be used in future nicotine titration studies. Such measurement could be especially useful as an intake marker of other smoke components of relevance to health that cannot be measured directly.

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Stereochemical Studies on the Cytochrome P-450 Catalyzed Oxidation of (*S*)-Nicotine to the (*S*)-Nicotine $\Delta^{1'(5')}$-Iminium Species

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Mammals metabolize the tobacco alkaloid (*S*)-nicotine primarily to the lactam (*S*)-cotinine by a pathway involving an initial cytochrome P-450 catalyzed two-electron oxidation at the prochiral 5′-carbon atom. The stereochemical course of this oxidation was examined with human microsomal preparations and the *E* and *Z* diastereomers of (*S*)-nicotine-5′-d_1. The metabolically generated $\Delta^{1'(5')}$-iminium ion intermediate was trapped and analyzed as the corresponding diastereomeric 5′-cyano derivatives by a capillary column GC–EIMS selected ion monitoring assay. The results of these studies established that this biotransformation proceeds with the stereoselective abstraction of the 5′-*pro-E* proton, that is, the C-5′ proton trans to the bulky pyridyl group. The observed stereoselectivity was independent of proton vs. deuteron abstraction. Additionally, the extent of (*S*)-cotinine formation was minor and did not influence the stereochemical composition of the metabolically derived α-cyano amines. Studies with male Dutch rabbit liver microsomal preparations gave similar results. These findings suggest that the structure of the complex formed between (*S*)-nicotine and the active site of cytochrome P-450 is highly ordered and dictates the stereochemical course of the reaction pathway.

The cytochrome P-450 catalyzed[1] overall two-electron oxidation of (*S*)-nicotine (**1**) at the prochiral C-5′ position to form the iminium intermediate **2** is the initial step in the biotransformation of this alkaloid to (*S*)-cotinine (**3**), the principal metabolite of (*S*)-nicotine in humans.[2] Since the two methylene protons at the C-5′ position are diastereotopic, this α-carbon oxidation may proceed by a process that could result in the selective abstraction of the 5′-*pro-E* (Ha) or 5′-*pro-Z* (Hb) proton. In order to understand better the detailed molecular mechanisms associated with the cytochrome P-450 catalyzed α-carbon oxidation of this and other aliphatic tertiary amines, we have undertaken studies to characterize the stereochemical course followed in this biotransformation.

The available experimental evidence relating to this type of transformation supports a stepwise reaction pathway involving an initial transfer of one electron from the lone pair of the pyrrolidine nitrogen atom to a heme-bound, electron-deficient oxygen atom.[3] The subsequent one-electron oxidation of the resulting radical cation **4** is the step that is accompanied by the net loss of hydrogen from the C-5′ position. This step may proceed directly to the iminium species **2** (loss of a hydrogen atom) or via the carbon-centered radical **5** (loss of a proton) and the carbinolamine **6**, which will be in equilibrium with **2**. The iminium intermediate, which in vivo is oxidized further to (*S*)-cotinine (**3**) in a reaction catalyzed by cytosolic enzymes,[4] may be trapped as the corresponding diastereomeric α-cyano adducts **7** and **8**.[1,5]

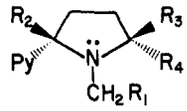
Several studies concerned with substrate diastereoselective biooxidations at prochiral methylene carbon atoms have been reported. For example, using cholesterol-7α-t_1 and -7β-t_1, Bergstrom et al. established that the 7α-hydroxylation of cholesterol in the rat proceeds with greater than 90% loss of the 7α-hydrogen atom.[6] A somewhat similar experiment showed that the 11β-hydroxylation of pregnanedione-*11α*, *12α*-t_2 by perfused bovine adrenal glands proceeds with complete retention of label.[7] On the other hand, the cytochrome $P\text{-}450_{cam}$ catalyzed oxidations of both camphor-*5-exo*- and *5-endo*-d_1 yield principally 5-*exo*-hydroxycamphor-*5-endo*-d_1.[8] According to our knowledge, analogous studies on aliphatic tertiary amines have not been reported.

This paper summarizes the results of our stereochemical investigations on the α-carbon oxidation of (*S*)-nicotine by human and male Dutch rabbit liver microsomal preparations with use of the specifically labeled (*E*)- and (*Z*)-5′-monodeuterio diastereomers **9** and **10**, respectively, of (*S*)-nicotine. The synthesis of **9** was achieved by the stereoselective[9] catalytic deuteriation of the corresponding $\Delta^{1'(5')}$-iminium species **2**. Similarly, catalytic hydrogenation of **2**-d_1 provided the corresponding *Z* diastereomer **10**.

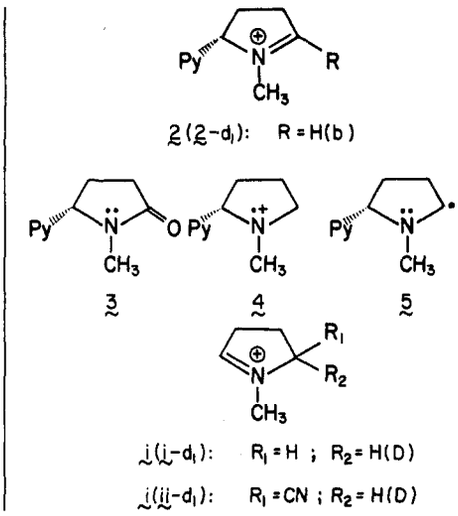
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Chart I



	R_1	R_2	R_3	R_4
1 (1-d_2)	H	H	Ha(D)	Hb(D)
6	H	H	H,OH	OH,H
7 (7-d_1)	H	H	CN	H(D)
8 (8-d_1)	H	H	H(D)	CN
9	H	H	D	Hb
10	H	H	Ha	D
11	CN	H	H	H
12	H	CN	H	H



2 (2-d_1): R = H(b)

3 4 5

i (i-d_1): R_1 = H ; R_2 = H(D)

ii (ii-d_1): R_1 = CN ; R_2 = H(D)

Analysis of the deuterium content of the metabolically generated iminium species was achieved by a capillary column GC–EIMS selected ion monitoring assay of the corresponding α-cyano amines **7** (**7**-d_1) and **8** (**8**-d_1) (Chart I).

Results

Chemistry. The synthesis of the $\Delta^{1'(5')}$-iminium species **2** was achieved by the two-electron reduction of (*S*)-cotinine with sodium dihydrobis(2-methoxyethoxy)aluminate (Red-Al).[10] The product was isolated as the corresponding (*E*)- and (*Z*)-α-cyano amine isomers **7** and **8**, respectively, by addition of potassium cyanide to the crude reaction mixture. Following column chromatographic purification, this diastereomeric mixture was treated with perchloric acid, which led to the formation of the crystalline bis-perchlorate salt of **2**. Deuteriation of this product over a platinum catalyst proceeded smoothly in a solution of deuterium oxide containing deuterium chloride. The resulting product was purified as its bis-*l*-tartrate salt.[11]

The deuterium content of the product was estimated by capillary column GC–EIMS analysis. Due to the presence of a strong $(M - 1)^+$ fragment ion in the EI mass spectrum of (*S*)-nicotine,[12] the base peak (fragment ion i) was monitored. This base peak in the spectrum of the reaction product appeared at *m/z* 85, corresponding to fragment ion i-d_1, consistent with the formation of (*S*)-nicotine-d_1. The ratios of the ^{13}C-satellite corrected ion intensities at masses 84:85:86 were found to be 2:88:10. Comparison of these ratios with the corresponding values obtained with standard (*S*)-nicotine led us to conclude that the product contained about 10% of a dideuteriated species, which presumably is formed via partial proton–deuteron exchange at C-4′ of the iminium intermediate.[13]

Analysis of the 240-MHz ^{1}H NMR spectrum allowed us to assign the structure of this product as the desired *E* diastereomer **9** of (*S*)-nicotine-5′-d_1. Previous ^{1}H NMR studies have established that the signal for the 5′-*pro-Z* proton (Hb) of (*S*)-nicotine appears at 3.3 ppm, 1.0 ppm downfield from the corresponding signal for the 5′-*pro-E* proton (Ha).[14] The ^{1}H NMR spectrum of the product obtained from the catalytic deuteriation of **2** (Figure 1, middle panel) differs from that of (*S*)-nicotine-d_0 (Figure 1, upper panel) in that the quartet corresponding to the signal for the 5′-*pro-E* proton (Ha) at 2.3 ppm is absent and the multiplet at 3.3 ppm for the Hb proton has simplified to a broad doublet, reflecting the loss of the geminal coupling with Ha. Careful and repeated integration of the spectrum indicated that the reduction had proceeded with ca. 87% stereoselectivity. Delay times greater than 5 times t_1 were used to allow the protons to relax completely between radiofrequency pulses to insure accurate integration of the signals.

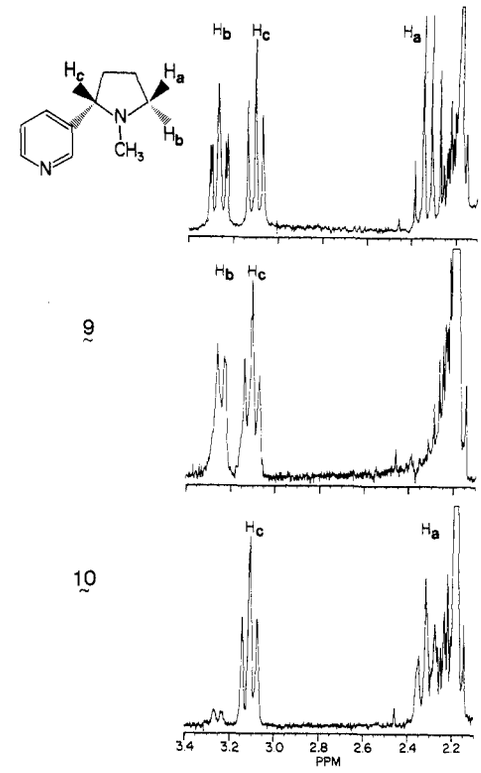


Figure 1. Relevant regions of the 240-MHz ^{1}H NMR spectra of (*S*)-nicotine-d_0 (upper panel) and the *E* (middle panel) and *Z* (lower panel) diastereomers of (*S*)-nicotine-5′-d_1 (**9** and **10**, respectively).

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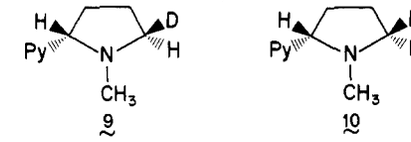
The synthesis of the *Z* diastereomer **10** of (*S*)-nicotine-5′-d_1 was achieved in a similar manner. The controlled reduction of (*S*)-cotinine with freshly prepared sodium aluminum deuteride[15] provided the required $\Delta^{1'(5')}$-iminium 5′-d_1 intermediate **2**-d_1, which was purified via the corresponding mixture of deuteriated α-cyano amines **7**-d_1 and **8**-d_1. Catalytic hydrogenation of **2**-d_1 gave **10**, which was obtained as its bis-*l*-tartrate salt. Unlike the *E* isomer, **10** was not contaminated with any multideuteriated species (*m/z* 84:85:86 = 5:94:1). The replacement of Hb with deuterium led to loss of the ^{1}H NMR signal at 3.3 ppm and to the simplification of the signal for Ha to a triplet due to loss of geminal coupling with Hb (Figure 1, lower panel). Integration of the ^{1}H NMR spectrum indicated that the reduction was 92% stereoselective.

Metabolic Studies. Previous studies have shown that rabbit liver microsomal enzymes catalyze the oxidation of (*S*)-nicotine to the chemically unstable iminium metabolite **2**, which, in the presence of 1 mM sodium cyanide, is converted to the corresponding α-cyano amines **7** and **8**.[1,5] In a preliminary study, capillary column GC analysis of human microsomal incubation mixture extracts of (*S*)-nicotine and sodium cyanide showed the presence of the diastereomeric 5′-cyanonicotine adducts and small amounts of (*S*)-cotinine. Neither the *N*-cyanomethyl isomer **11**, previously observed in rabbit liver microsomal incubation mixtures,[5] nor the 2′-cyano isomer **12** was observed in the GC tracings. These results suggested that the human cytochrome P-450 catalyzed carbon oxidation of (*S*)-nicotine is highly regioselective.

A capillary column GC–EIMS selected ion monitoring assay was developed to estimate the deuterium composition of the α-cyano amines **7** and **8** generated from **9** and **10** in liver microsomal preparations containing substrate and sodium cyanide. The capillary GC–EI mass spectra of a mixture of the synthetic (*Z*)- and (*E*)-α-cyano amines were essentially identical. As with (*S*)-nicotine, the molecular ion of this system was not useful for deuterium composition estimations due to the fragmentation that results in loss of a hydrogen atom. The base peak in both spectra, which appears at *m/z* 109, corresponds to the $(M - C_5H_4N)^+$ fragment ion ii. This ion and the corresponding deuterium-containing ion ii-d_1 at *m/z* 110 were chosen for selected ion monitoring. The ratios of the ion currents at *m/z* 109 and 110 to the sum of these ion currents provided an estimate of the extent to which deuterium was lost or retained during the metabolic oxidation of **9** and **10**. In all cases, small corrections had to be made to account for the small d_0 contaminants present in **9** and **10**, for the ^{13}C-satellite contribution of the *m/z* 109 ion to the *m/z* 110 ion, and for the stereochemical composition of the substrates. Additionally, when **9** was employed as the substrate, it was necessary to correct for the dideuteriated contaminant. Although standard curves were not run, the intensities of the principal ions observed in the spectrum of a 1:1 mixture of **7** (**7**-d_1) and **8** (**8**-d_1) were equal, suggesting that any unexpected differences in the GC–EIMS behavior of these two species, such as fragmentation differences due to isotope effects, must be minor.

The results of the human microsomal incubation experiments are summarized in Chart II. The three liver microsomal preparations were obtained from three different sources (see Acknowledgment), and therefore differences in isozyme composition due to differences in handling cannot be ruled out. Analysis of the deuterium content of the 5′-cyanonicotine isomers established that 90% of the product generated from **9** was proton enriched while 89% of the product isolated from **10** was deuteron enriched. Additionally, the deuterium/hydrogen ratio of the α-cyano amines generated from a 1:1 mixture of **9** and **10** was found to be 1:1, consistent with the absence of an intermolecular deuterium isotope effect. On the basis of these data, we have concluded that the oxidation of (*S*)-nicotine by human cytochrome P-450 enzymes proceeds in a highly stereoselective manner involving loss of the 5′-*pro-E* proton.

Chart II



9 **10**

Source of tissue	% Deuterium retention in **7** and **8**	
Human		
I	3(4)	92(4)
II	24(4)	84(4)
III	3(4)	91(4)
Mean	10	89
Dutch rabbit		
I	34(6)	87(6)
II	22(6)	81(6)
III	30(4)	89(4)
IV	28(2)	79(2)
V	27(2)	77(2)
VI	16(3)	88(3)
Mean	26	84

The stereochemical course of this oxidation also was examined with male Dutch rabbit liver microsomal preparations. Attack occurred principally at the 5′-position although the *N*-methyl group also was oxidized to a limited extent since small amounts of *N*-(cyanomethyl)nornicotine (**11**) were detected in the capillary column GC tracings. No 2′-cyano product was observed although trace amounts of (*S*)-cotinine again were detected. The results of the stereochemical studies (Chart II) demonstrate that male Dutch rabbit liver microsomal enzymes also catalyze the oxidation of the 5′-position of (*S*)-nicotine with the selective loss of the 5′-*pro-E* proton. These preparations, however, tended to be less selective, with an average loss of 74% of the label with **9** as substrate and 83% retention of the label with **10** as substrate. The results did not vary significantly with the length of the incubation time (data not shown).

The presence of (*S*)-cotinine in the incubation mixture isolates led to the possibility that the deuterium content of the α-cyano amines was not an accurate measure of the diastereoselectivity operating in the oxidation of (*S*)-nicotine since further oxidation of the iminium intermediate or of the α-cyano amines could be accompanied by a deuterium isotope effect. This possibility was eliminated by results obtained from the following experiment. A 1:1 (d_0:d_1) mixture of the diastereomeric 5′-cyanonicotines (50 μM) was incubated with rabbit liver microsomal preparations in the presence of 1 mM sodium cyanide. The concentrations of (*S*)-cotinine were determined by a capillary column GC assay using an N/P detector.[16] In the absence of NADPH, up to 4% of (*S*)-cotinine was formed after a 30-min incubation period. In the presence of NADPH, up to 20% of the mixed substrate was converted

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to (*S*)-cotinine. The deuterium content (40%) of the recovered α-cyano amines, however, was found be essentially the same as that of the starting substrate (41%).

Discussion

The results of these studies demonstrate that the cytochrome P-450 catalyzed oxidation of (*S*)-nicotine to the iminium ion **2** proceeds with the selective loss of the 5′-*pro-E* proton in human and male Dutch rabbit liver microsomal preparations. Differences in the degree of stereoselectivity (90% for the human and about 78% for the rabbit) may reflect species differences in isozyme composition. Both regio- and enantioselective variations in steroid hydroxylations have been reported for a number of cytochrome P-450 isozymes isolated from the rat.[17] More detailed studies with purified enzyme preparations will be required to address this question.

The possibility that cleavage of the carbon–hydrogen vs. carbon–deuterium bond may be accompanied by an intrinsic deuterium isotope effect in this system is difficult to assess because this transformation does not lend itself to intramolecular isotope effect measurements. No intermolecular isotope effects were detected when a 1:1 mixture of (*S*)-nicotine-d_0 and (*S*)-nicotine-d_1 served as substrate. Also, if present, deuterium isotope effects did not alter the stereoselectivity of the reaction. Finally, the limited extent to which the initial (*S*)-nicotine oxidation products were oxidized to (*S*)-cotinine did not alter the deuterium content of the α-cyano amines and, therefore, had no detectable influence on the stereochemical outcome of the initial two-electron oxidation. Consistent with these observations, literature values for both inter- and intramolecular isotope effects in the enzyme-catalyzed α-carbon oxidations of tertiary amines are small.[18]

The regio- and stereochemical profiles observed in this biooxidation suggest that the reaction is under steric constraints imposed by the enzyme. This result contrasts with those observed in related cytochrome P-450 catalyzed carbon oxidations. For example, replacement of the exo protons of norbornane with deuterons leads to a change in the exo to endo product ratio from 3.4:1 to 0.76:1.[19] Similarly, the extent of abstraction of the exo vs. endo hydrogen/deuterium atom during the 5-exo-hydroxylation of camphor by cytochrome P-450$_{cam}$ was dictated principally by deuterium isotope and not by stereochemical effects.[8] These results suggest that the species responsible for the proton abstraction step in these non-amine-containing systems has access to both the *pro-E* and *pro-Z* protons.

The regiochemistry followed in the carbon–hydrogen bond cleavage of aminium radical cations generated by singlet *trans*-stilbene has been examined with tertiary amines such as *N*-methyl-*N*,*N*-diisopropylamine.[20] The aminium radical cation formed from this amine loses hydrogen exclusively from the *N*-methyl group. The regioselectivity of this reaction appears to be governed by steric factors, which restrict overlap of the p orbital on the

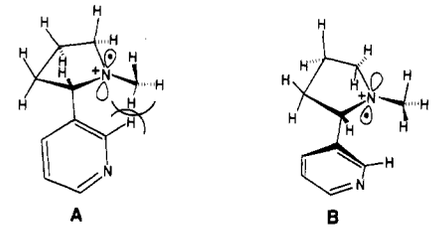


Figure 2. Drawings of the aminium radical **4** showing the steric interactions that accompany overlap of the half-filled p orbital on nitrogen with the C-5′*Z* bond (A) and the absence of such steric interactions with the corresponding overlap involving the C-5′*E* bond (B).

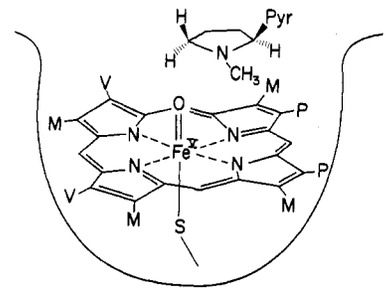


Figure 3. A depiction of (*S*)-nicotine in the active site of cytochrome P-450 oriented to explain selective loss of the 5′-*pro-E* proton.

developing methine carbon radical of the *N*-isopropyl groups with the half-empty p orbital of the planar aminium radical. A similar analysis of the radical cation **2** formed from the one-electron oxidation of (*S*)-nicotine is illustrated in Figure 2. Considerably more steric interaction between the methyl and pyridyl groups accompanies the overlap of the carbon and nitrogen p orbitals with loss of the 5′-*pro-E* proton compared with loss of the 5′-*pro-Z* proton. This model, therefore, predicts the selective loss of the 5′-*pro-Z* proton, contrary to the observed experimental results.

Studies reported by Whidby and Seeman have shown that the pyridyl and methyl substituents of (*S*)-nicotine prefer a trans orientation.[21] The energy barrier to the inversion of the *N*-methyl group, however, is likely to be relatively small, and therefore the orientation of the molecule in the active site of the enzyme may be determined by binding of the lipophilic pyridyl group to a hydrophobic pocket. In any event, we conclude that the mode of binding of (*S*)-nicotine at the active site of the enzyme is an important determinant in the stereochemical course of this oxidation. If (*S*)-nicotine binds with the bulky pyridyl group pointing away from the planar porphyrin ring of this heme protein as shown in Figure 3, the 5′-*pro-E* proton will be situated more closely to the proposed iron–oxene species than the 5′-*pro-Z* proton. According to this hypothesis, transfer of the 5′-*pro-E* proton directly to the active iron–oxene species would be facilitated by the steric constraints imposed on the substrate by the enzyme. This proposal currently is being tested with various model systems.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^{1}H and ^{2}H NMR spectra (obtained in $CDCl_3$ or H_2O) were recorded on a Varian FT-80 or a homebuilt 240-MHz instrument linked to a Nicolet 1180 computer. Chemical shifts are reported in ppm relative to Me_4Si or DSS as internal standard. Analytical GC was performed on a Hewlett-Packard 5880A series instrument with a nitro-

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gen/phosphorus detector utilizing a 25 m × 0.2 mm i.d. silica SE 54 capillary column with helium as the carrier gas. Preparative HPLC was carried out with use of a Beckman Model 100A pump with a Whatman M9 Partisil column linked to an Altex Model 153 UV detector. Analytical HPLC was performed with an Altex 110A pump connected to a Lichrosorb SI-60 silica gel column from Altex Scientific Inc. and a Hitachi 100-10 variable UV detector. GC–EIMS was performed on a J&W 30 m 0.325 mm i.d. DB5 fused silica capillary column with use of a Varian 3700 GC linked to a Kratos MS25S instrument. Elemental analyses were determined by the Microanalytical Laboratory of the University of California, Berkeley, CA. All values were within 0.4% of theory.

(*S*)-Nicotine-5′(*E*)-d_1 (9). A 70% solution of sodium dihydrobis(2-methoxyethoxy)aluminate (4.3 g, 28.8 mequiv) in 100 mL of dry ether was added dropwise to an ice-cold, stirred solution of (*S*)-cotinine[22] (6 g, 34 mmol) in 120 mL of dry ether in a flame-dried flask under N_2 over a 30-min period. After the mixture was stirred for 3 h, 50 mL of an aqueous solution of KCN (12 g, 182 mmol) was added slowly, following which the pH was adjusted to 5 with acetic acid. The reaction mixture was allowed to warm to room temperature, was made basic with a saturated solution of K_2CO_3, and was extracted with 3 × 50 mL of ether. The extracts were combined, dried over K_2CO_3, and filtered, and the solvent was removed under reduced pressure to yield a yellow oil, which was purified by column chromatography on silica gel with ether/ammonia (2000:1). Distillation [125 °C (0.8 mm)] gave 3.8 g (62%) of the diastereomeric mixture of **7** and **8** as a pale yellow oil. An ice-cold solution of this mixture (2.6 g, 13.8 mmol) in 20 mL of ethanol was treated with ethanolic perchloric acid [2.6 mL (230.2 mmol) of 70% perchloric acid dissolved in 10 mL of ethanol]. After the mixture stood for 2 days at 5 °C, the white crystalline product was collected and recrystallized from methanol containing a trace of perchloric acid to yield 2.3 g (5.9 mmol, 42%) of the bisperchlorate salt of the (*S*)-nicotine $\Delta^{1',5'}$-iminium species **2**: mp 237–241 °C dec (lit.[10] mp 230–232 °C); ^{1}H NMR (10% DCl in D_2O) δ 2.0–2.35 (m, 4 H, C3′ and C4′), 3.28 (br s, 3 H, NCH_3), 7.5–9.0 (m, 5 H, 4 aromatic pyridine H and C5′). Anal. ($C_{10}H_{15}N_2Cl_2O_6$) C, H, N.

A suspension of platinum oxide (700 mg) in 70 mL of a 2.5% DCl/D_2O solution was prereduced under 1 atm of D_2 for 30 min with vigorous stirring in a 250-mL round-bottomed flask. The iminium species **2** (7.1 g, 17.3 mmol) was added, and the flask was recharged with D_2. The reaction mixture was stirred at room temperature for 3.5 h and then was filtered through a pad of Celite. The filtrate was made basic with a saturated K_2CO_3 solution, extracted with CH_2Cl_2 (5 × 70 mL), dried (K_2CO_3), and filtered. The solvent was removed by distillation at atmospheric pressure to yield 4.34 g of (*S*)-nicotine-5′(*E*)-d_1 (**9**) as a yellow oil: 240-MHz ^{1}H NMR ($CDCl_3$) δ 1.5–2.1 (m, 4 H, C3′ and C4′), 2.2 (s, 3 H, NCH_3), 3.1 (t, 1 H, C2′), 3.3 (br d, 0.86 H, C5′-*pro*-*Z*), 7.3 (m, 1 H, C5), 7.6 (d of t, 1 H, C4), 8.3–8.5 (m, 2 H, C2 and C6); MS, *m*/*z* 163 (M^+, 40%), 134 (46%), 85 (100%). The bis-*l*-tartrate salt of **9** (5.16 g, 56.8% overall yield) was prepared in ethanol:[11] mp 138.5–139.5 °C; ^{2}H NMR (H_2O) δ 3.4. Anal. ($C_{18}H_{25}DN_2O_{12}$) C, H, N.

(*S*)-Nicotine-5′(*Z*)-d_1 (10). A solution of freshly prepared $NaAlD_4$[15] (3 mL, 20 mmol of deuteride) in 50 mL of dry THF was added dropwise to an ice-cooled, stirred solution of (*S*)-cotinine (3 g, 17 mmol) in 50 mL of dry THF under N_2. The reaction mixture was allowed to warm slowly to room temperature. After 3 h, the mixture was cooled and another aliquot of $NaAlD_4$ (1 mL) was added slowly. The system was allowed to warm to room temperature and was stirred for an additional 45 min. A solution of KCN (6 g, 17 mmol) in 50 mL of water was added, and the pH of the resulting mixture was adjusted to 5 with acetic acid. After the mixture was stirred at room temperature for 3 h, a saturated K_2CO_3 solution (20 mL) was added, the THF layer was separated, and the aqueous phase was extracted with ether (4 × 50 mL). The organic layers were combined and dried (K_2CO_3), and the solvent was removed under reduced pressure to yield 2.04 g of crude product, which was purified on preparative HPLC (silica column; mobile phase, ether/ammonia, 2000:1; flow rate, 5 mL/min) to provide 1.6 g (8.5 mmol, 50%) of a mixture of 7-d_1 and 8-d_1 as a pale yellow oil: 80-MHz ^{1}H NMR ($CDCl_3$) δ 1.9–2.6 (m, 4 H, C3′ and C4′), 2.30 and 2.32 (2 s, 3 H, NCH_3), 3.5 (m, 1 H, C2′), 7.3 (m, 1 H, C5), 7.6 (m, 1 H, C4), 8.55 (m, 2 H, C2 and C6); GC–EIMS, *m*/*z* 188 (M^+) and 110 (100%). Treatment of this mixture with ethanolic perchloric acid as described above gave, after recrystallization from methanolic perchloric acid, a 44% yield of the desired monodeuterio iminium species **2**-d_1: mp 240–243 °C dec; 80-MHz ^{1}H NMR (10% DCl in D_2O) δ 2.0–3.0 (m, 4 H, C3′ and C4′), 3.37 (br s, 3 H, NCH_3), 5.6 (t, 1 H, C2′), 8.0 (m, 1 H, C5), 8.25–8.9 (m, 3 H, C2, C4, and C6). Hydrogenation of **2**-d_1 over a platinum catalyst as described above provided an 83% yield of the crude (*S*)-nicotine-5′(*Z*)-d_1 (**10**), which was purified by silica gel chromatography using ether saturated with concentrated aqueous NH_4OH as the eluent: 240-MHz ^{1}H NMR ($CDCl_3$) δ 1.5–2.1 (m, 4 H, C3′ and C4′), 2.17 (s, 3 H, NCH_3), 2.3 (m, 1 H, $H_{5'E}$), 3.1 (t, 1 H, C2′), 7.3 (m, 1 H, C5), 7.6 (d of t, 1 H, C4), 8.3–8.5 (m, C2 and C4); GC–EIMS, *m*/*z* 163 (M^+), 134, 85 (100%). The bis-*l*-tartrate salt of **10** was obtained from ethanol:[11] mp 138.5–139 °C; ^{2}H NMR (D_2O) δ 3.8. Anal. ($C_{18}H_{25}DN_2O_{12}$) C, H, N.

2′-Cyanonicotine (12). A stirred solution of (*S*)-nicotine (2.0 g, 12.4 mmol) and mercuric acetate (15 g, 47 mmol) in 50 mL of acetic acid was heated at 80 °C for 2 h. The cooled reaction mixture was filtered, and H_2S gas was bubbled through the filtrate. The resulting black precipitate was filtered, and the process was repeated two times. The solution then was made basic with saturated K_2CO_3, and KCN (1.7 g, 26 mmol) was added. The reaction mixture was stirred for 1 h at room temperature and then was extracted with 3 × 50 mL of ether. The combined extracts were dried, filtered, and concentrated to yield 1.04 g of crude product. Chromatography on a silica gel column (30 g) gave 22.8 mg (1%) of the desired 2′-cyanonicotine and 421 mg of the 5′-cyanonicotine diastereomers. The 2′-isomer was characterized by 80-MHz ^{1}H NMR [($CDCl_3$) δ 1.9–2.4 (m, 3 H), 2.24 (s, 3 H, NCH_3), 2.4–2.9 (m, 2 H), 3.1–3.5 (m, 1 H), 7.25 (q, 1 H, C5), 7.9 (d of t, 1 H, C4), 8.6 (d, 1 H, C6), and 8.85 (m, 1 H, C2)] and by GC–EIMS [*m*/*z* 187 (M^+)]. Limited quantities precluded full characterization of this product.

Metabolic Studies. Human livers were obtained from Drs. J. Trudell (Stanford University, Stanford, CA) and L. Waskell (Veteran's Administration Hospital, San Francisco, CA). A human liver microsomal preparation also was obtained from Dr. F. Kadlubar (National Center for Toxicological Research, Jefferson, AR). Both male Dutch rabbit and human liver microsomes were prepared according to previously described methods[23] except that in the case of human tissue the initial homogenization was performed with 4 mL of KCl solution/g of liver instead of the 2 mL/g of liver used for the rabbit tissue.

The deuteriated nicotine derivatives (0.5 mM) were incubated with microsomal preparations (3 mg of protein/mL) in 0.2 M potassium phosphate buffer, pH 7.4, containing magnesium chloride (1.5 mM), sodium cyanide (1.0 mM), and NADPH (1 mg/mL) in a final volume of 5 mL. Incubations were carried out for 30 min on a metabolic shaker at 37 °C, following which the samples were cooled on ice and then extracted with an equal volume of peroxide-free ether. The aqueous phase was frozen in a dry ice bath, and the ether phase was decanted. After removal of the ether under a stream of N_2, the residue was dissolved in 50 μL of CH_2Cl_2 in preparation for GC–EIMS analysis. The sample was injected onto the column at room temperature. After the solvent had eluted (about 2 min), the column was heated rapidly to and maintained at 180 °C. The injection-port, ion-source, and inlet-line temperatures were maintained at 220, 150, and 200 °C, respectively. The ion currents at *m*/*z* 109, 110, and 111 were determined by computer-assisted selected ion monitoring techniques. A series of equations were developed to correct for the ^{13}C-satellite contributions and the enantiomeric and d_0 and d_2 contaminants present in the substrates.[24]

Incubations performed with the 1:1 mixture of the d_0 and d_1 α-cyanonicotines followed the procedures described above except

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that the total concentration of the α-cyanonicotines was 50 μM. The deuterium composition of the unmetabolized substrate was determined by the same GC-EIMS procedure. (*S*)-Cotinine analyses were performed on 1-mL aliquots of the incubation mixtures according to the procedure described by Jacob et al.[16]

Acknowledgment. We thank Drs. J. Trudell (Stanford University, Stanford, CA) and L. Waskell (Veteran's Administration Hospital, San Francisco, CA) for human liver tissue; Dr. F. Kadlubar (National Center for Toxicological Research, Jefferson, AR) for human liver microsomal preparations; Dr. N. L. Benowitz, Dr. P. Jacob, III, and C. Savanapridi (Clinical Pharmacology Unit, San Francisco General Hospital, San Francisco, CA) for cotinine analyses; and E. Roitman for technical assistance. We also acknowledge the Bio-organic, Biomedical Mass Spectrometry Resource at the University of California, San Francisco (A. L. Burlingame, Director) supported by NIH Division of Research Resources Grant RR01614. This work was supported by NIH Training Grant GMO-7175 and NCI Grant CA-35678.

Registry No. **1**, 54-11-5; **2**, 105090-00-4; **2** (bisperchlorate), 71014-67-0; **3**, 486-56-6; **7**-d_1, 105089-99-4; **8**-d_1, 105120-31-8; **9**, 105120-32-9; **10**, 105089-96-1; **10** (bis-*l*-tartrate), 105089-97-2; **12**, 105089-98-3; cytochrome P-450, 9035-51-2.

Structural Requirements for the Inhibition of 5-Lipoxygenase by 15-Hydroxyeicosa-5,8,11,13-tetraenoic Acid Analogues

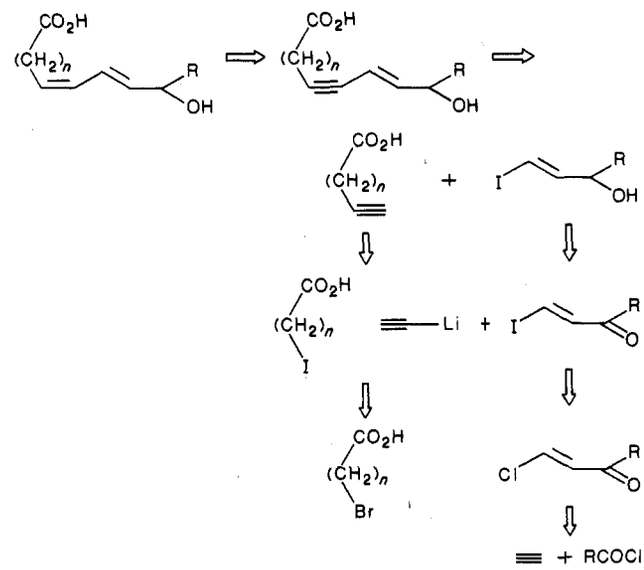
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The structural requirements for inhibition of RBL-1 (rat basophilic leukemia) 5-lipoxygenase by 15-hydroxyeicosa-5,8,11,13-tetraenoic acid (15-HETE, **1**) were studied by systematic chemical modifications of the molecule at the hydroxyl and carboxyl groups, the double bonds, and the carboxylate and ω side chains. The most potent inhibitors were analogues that contained a 5,8-*cis*,*cis*-diene system and acted as alternate substrates for the enzyme. However, several analogues in which the 5,8-diene had been reduced were also found to inhibit the enzyme. Inhibition of 5-lipoxygenase by 15-hydroxyeicosa-11,13-dienoic acid (15-HEDE) analogues was optimal in compounds that generally contained a free carboxyl group, a carboxylate side chain of nine carbons, an ω side chain of five or six carbons, a *cis*,*trans*- or *trans*,*cis*-11,13-diene or 11,13-diyne system, and a 15-hydroxyl group. Conversion of 15-HEDE to its 16-membered lactone reduced but did not eliminate 5-lipoxygenase inhibitory activity. In contrast, a 3- to 10-fold enhancement of activity occurred when 5,15-diHETE (**58**) or 5-HETE (**56**) were cyclized to their respective δ-lactones. Molecular modeling of 15-HEDE analogues, modified in the C_{11}–C_{15} region, showed that inactive analogues protrude into regions in space not occupied by active analogues. These structural studies indicate that multiple regions are important for 5-lipoxygenase inhibition by both 15-HETE and 15-HEDE analogues and that no single region plays a predominant role in inhibition.

Several laboratories have reported that 15-hydroxyeicosa-5,8,11,13-tetraenoic acid (15-HETE) is a potent inhibitor of platelet 12-lipoxygenase[1] and neutrophil 5-lipoxygenase.[2-5] Since neutrophils generate both 15-HETE and 5-HETE,[6] a possible regulatory role for 15-HETE in the control of cellular 5-lipoxygenase activity has been suggested.[1,5] Additionally, the formation of 5,15-diHETE, when 15-HPETE was incubated with human neutrophils,[4] led to the suggestion that 15-HPETE and possibly 15-HETE could serve as alternate substrates for 5-lipoxygenase. These interesting findings prompted us to investigate the structural features of 15-HETE required for 5-lipoxygenase inhibitory activity as departure point for the design of therapeutically useful 5-lipoxygenase inhibitors.

Scheme I



Synthesis

Gram quantities of 15-HETE (**1**) and analogues **7**, **8**, **10**, **12**, and **14** were prepared from the corresponding ω6 fatty

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(6) Abbreviations: 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 5,15-diHETE, 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid; 15-HEDE, 15-hydroxy-11,13-eicosadienoic acid.

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Enzymology of the metabolic pathway from nicotine to cotinine, *in vitro*

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SUMMARY

Nicotine Δ1′(5′) iminium ion was converted to cotinine by hepatic homogenates prepared from five animal species (mouse, rat, hamster, rabbit, guinea pig) and human foetus.

The pH optimum, apparent Vmax and apparent Km values were determined using animal hepatic 140,000 *g* supernatant. Use of various potential enzyme inhibitors and purified enzymes allowed further characterisation of the hepatic enzyme. Evidence presented in this paper supports the conclusion that aldehyde oxidase mediates the reaction. Human foetal hepatic enzyme appears to differ from that in adult animals. The results do not substantiate a soluble enzyme mediated metabolic pathway via γ-(3-pyridyl)-γ-methylamino butyric acid to cotinine.

INTRODUCTION

The first study on the metabolic pathway from nicotine to cotinine was carried out by Hucker *et al* (1) who proposed that cotinine arose, via a soluble enzyme, presumed to be aldehyde oxidase, from 5′ hydroxynicotine, produced by a microsomal oxidase. Murphy (2), whilst presenting evidence for the existence of the nicotine Δ1′(5′) iminium ion as a discrete entity in the pathway to cotinine, supported the proposal that 5′hydroxynicotine was the initial product of the microsomal oxidase.

The synthesis of nicotine Δ1′(5′) iminium ion diperchlorate by Brandange and Lindblom (3) allowed metabolic experiments to be conducted in order to evaluate the role of this compound in nicotine metabolism (4,5). The results showed that this ion plays an integral part in the metabolism of nicotine to cotinine, both *in vivo* and *in vitro*.

The conversion to cotinine was shown to be predominantly mediated by a soluble hepatic enzyme. This paper reports results of further work carried out to gain greater understanding of the enzymology of the pathway to cotinine.

MATERIALS

In addition to materials and animals obtained as previously described (4), human foetal livers were obtained from University College Hospital, London. The remaining compounds were obtained as follows: allopurinol and tetraethylthiuram disulphide (disulfiram) Aldrich Chemicals Co., Gillingham, Kent; ethylene-diamine-tetraacetic acid (disodium salt), Hopkin and Williams Ltd., Chadwell Heath, Essex; sodium arsenite, quinacrine dihydrochloride, l-cysteine, potassium cyanide, glutathione (oxidised (GSSG) and reduced (GSH), *p*-chloromercuribenzoate, iodoacetic acid and bovine serum albumin, B.D.H. Chemicals, Poole, Dorset; xanthine oxidase (crystallised) and alcohol dehydrogenase (hefe), Boehringer Mannheim Corporation, London; catalase, Sigma Laboratories, London. γ-(3-Pyridyl)-γ-methylamino butyric acid was kindly supplied by Prof. H. Mc Kennis Jr., Medical School of Virginia, USA; 5′-cyano-nicotine and nicotine Δ 1′(5′) iminium ion diperchlorate were prepared as described in the literature (2,3).

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METHODS

Livers from male rats, guinea pigs, hamsters and rabbits as well as those from aborted human foetuses were used to prepare various cell fractions in tris/KCl (pH 7.4) as previously described (6). Incubations consisted of tissue preparation (1 ml), substrate in distilled water (0.5 ml) and buffer to produce a final volume of 3.5 ml. In certain experiments cofactor solution consisting of NADP 2μmoles, glucose-6-phosphate 10μmoles, glucose-6-phosphate dehydrogenase 1 unit, $MgCl_2$ 20μmoles, in 0.2 mol/l phosphate buffer pH 7.4 to a final volume of 2ml, was included in the incubate (3.5 ml).

Incubations were carried out in 25 ml stoppered Ehlenmeyer flasks at 37°C in a shaking water bath, and were terminated and extracted as previously described (4). All «soluble» homogenates contained 5 to 7 mg protein per incubate unless otherwise stated. In order to determine the effect of hydrogen ion concentration on the *in vitro* conversion of the iminium ion to cotinine various buffers were prepared as follows, pH 5.0-6.8, 0.2mol/l phosphate buffers; pH 7.3-9.0, tris/KCl and glycine/NaOH for buffers (0.2 mol/l) between pH 9.4-11.0. These incubates were composed of 1 ml hamster liver 140,000 *g* supernatant, substrate 1.25 μmoles and buffer to produce a range of hydrogen ion concentrations. When a potential inhibitor was employed it was added in 0.5 ml phosphate buffer, 0.2 mol/l, pH 7.4, to give a final incubation volume of 3.5 ml.

All enzyme kinetic and inhibition experiments were carried out at pH 7.4. Experiments were carried out using rat, hamster and guinea pig liver 140,000 *g* supernatant, appropriately diluted with tris/KCl pH 7.4, to produce a range of protein concentrations from 1.8 to 9.3 mg/incubate. Protein was determined by the method of Lowry *et al.* (7) as modified by Miller (8). Incubations employing a wide range of substrate concentrations were performed using the iminium ion dissolved in 0.5 ml distilled water. Because of the rapid turnover of the iminium ion with the enzyme source, rate determinations were extremely difficult to obtain before a considerable depletion of substrate had occurred. Therefore it was found necessary to use the integrated form of the Michaelis-Menten equation (9) for obtaining apparent Km and apparent Vmax values, (app Km; app Vmax) (10). The form of equation employed was as followes :

$$\frac{t}{\ln\left(\frac{So}{So-P}\right)} = \frac{1}{Vmax}\left[\frac{P}{\ln\left(\frac{So}{So-P}\right)}\right] + \frac{Km}{Vmax}$$

(where t = time in minutes; So = Substrate concentration initially, P = product concentration at time t). The plotting of $\frac{P}{\ln\left(\frac{So}{So-P}\right)}$ against $\frac{t}{\ln\left(\frac{So}{So-P}\right)}$ produces a straight line which has a slope of $\frac{1}{Vmax}$, an intercept on the abscissa of -Km and an intercept on the ordinate of $\frac{Km}{Vmax}$ (9).

Data from incubations with fixed substrate concentration and varying time, as well as that from incubations using varying substrate concentrations and fixed time may be plotted on the same graph using the above method (11).

Bovine serum albumin (10 and 20 mg) in tris/KCl pH 7.4 (3.0 ml) was incubated with nicotine Δ1'(5') iminium ion (1.25 μmoles) in water (0.5 ml). Potential inhibitors at various concentrations were added to incubations as previously described, and the amount of cotinine formed after two minutes was compared with that formed in control experiments. Substrate concentration was in each case 1.25 μmoles per incubate. Using guinea-pig hepatic 9000 *g* supernatant, plus co-factors, the relative cotinine formation from either nicotine (1.25 μmoles) or iminium ion (1.25 μmoles) during various incubation times was compared.

The following purified enzymes were used in 1ml tris/KCl, pH 7.4: (a) xanthine oxidase (crystallised) final concentration 10^{-6} mol/l, (b) alcohol dehydrogenase (30 mg/ml) 50 μl plus NAD 4 μmoles; (c) catalase 10 μl (8750 units). In addition cows milk (1.5 mls) was used as a further source of xanthine oxidase. These were used in conjunction with nicotine Δ1'(5') iminium ion (1.25 μmoles) in water (0.5 ml) plus phosphate buffer, 0.2 mol/l, pH 7.4 to 3.5 ml. Incubations were carried out for 2,5,10 and 15 minutes at 37°C and assayed for any cotinine formed as previously described (12) but with the modification that NaCl (1g) was added prior to extraction.

The nicotine metabolite γ-(3-pyridyl)-γ-methylamino butyric acid (5 μmoles) in 0.5 ml water was

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incubated for 5 minutes at 37°C with 1 ml guinea-pig hepatic 140.000 *g* supernatant (46.2 mg protein) and phosphate buffer to 3.5 ml. Control incubates, consisting of substrate, in 1 ml tris/KCl, pH 7.4 and phosphate buffer to 3.5 ml were similarly treated.

RESULTS

The pH optimum for the conversion of the iminium ion to cotinine employing hamster hepatic 140.000 *g* supernatant was found to be 8.1 (Fig. 1).

Cotinine production in incubates containing bovine serum albumin (Table I) was insignificant compared with the enzymic reaction found in hepatic preparations (4). Human foetal liver fractions showed considerably increased enzymic activity in the 25 week specimen compared with the 20 week specimens. Maximal cotinine production required a longer incubation time with the human fetal tissue compared with that using adult animal tissue (4,5) (Figs. 2 & 3).

Cotinine formation from nicotine and the iminium ion, using guinea-pig 9000 *g* liver supernatant plus cofactors, reached approximately 8% conversion at 30 minutes from nicotine, whereas with the iminium ion maximal conversion was approximately 90% at 15 minutes (Fig. 3).

A linear relationship was established between the oxidation rate of substrate (0.5 and 1.25 µmoles/flask) and «soluble» protein concentrations up to 7 mg per incubate. The initial velocity of reaction to cotinine was linear for two minutes (Fig. 4).

Table I : Conversion of nicotine Δ1'(5') iminium ion (1.25 µmoles) to cotinine using bovine serum albumin: (a) 10 mg, and (b) 20mg per flask.

Incubation time at 37°C (mins)	Cotinine produced as percentage of substrate	
	(a)	(b)
2	0.45	0.26
5	0.76	0.32
15	0.19	0.18

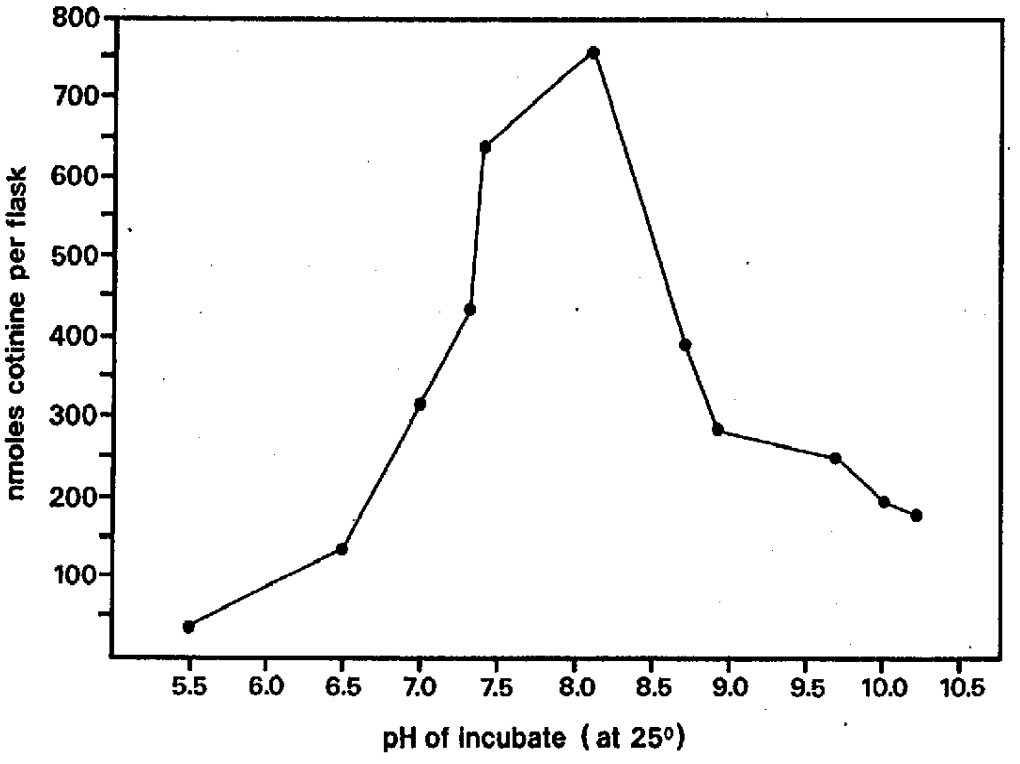


Fig. 1 : The effect of hydrogen ion concentration on the *in vitro* formation of cotinine from nicotine Δ1'(5') iminium ion by hamster 140.000 *g* supernatant.

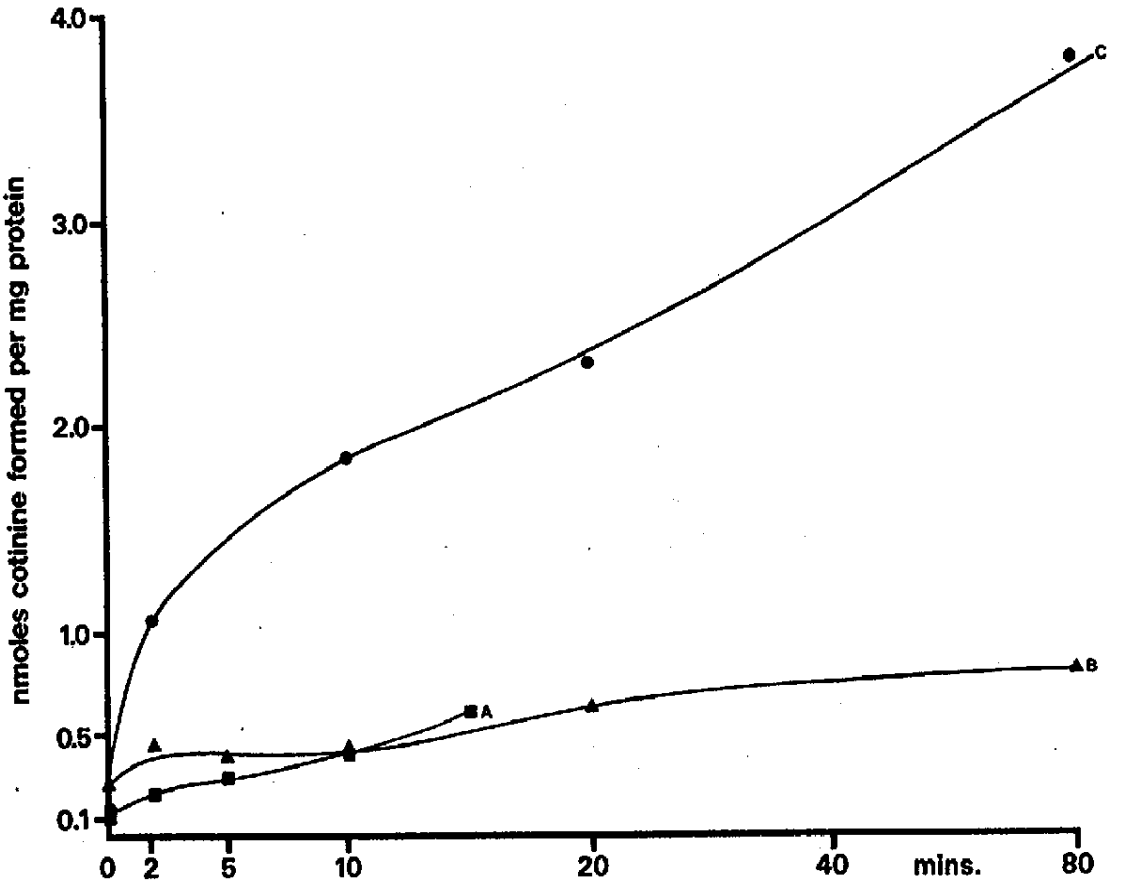
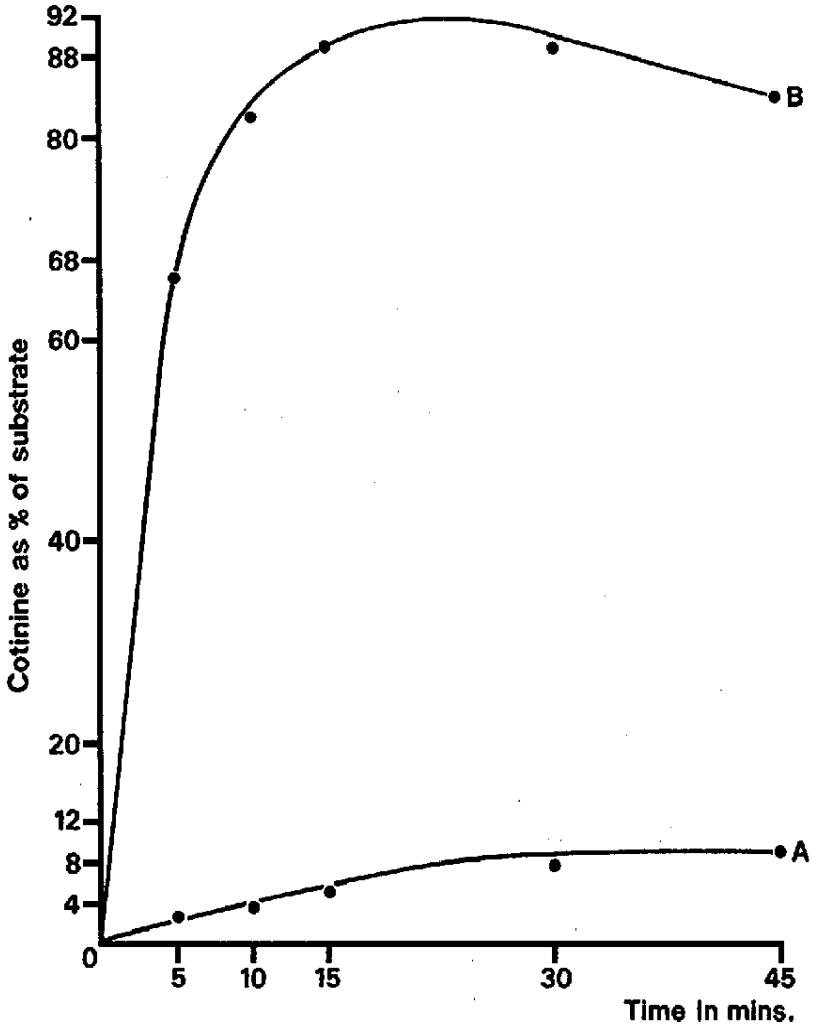


Fig. 2 : The effect of incubation time on cotinine formation from nicotine Δ1'(5') iminium ion by human foetal hepatic tissue.
A 20 week - foetal thalassemia using 140.000 × g supernatent.
B 20 week - foetal thalassemia using 140.000 × g supernatent.
C 25 week - no reported condition using 9.000 × g supernatent.

Inhibition of cotinine production occurred with increasing substrate concentration, being more noticeable at small protein concentrations (e.g., 1.0-2.0 mg/flask). However 5 to 7 mg of «soluble» protein per incubate was found to be satisfactory for all kinetic experiments, allowing a suitably wide range ($0.15\text{-}3.57 \times 10^{-4}$ mol/l) of substrate concentrations to be employed in 2 minute incubations from which data were obtained for producing plots of Vo versus S (Fig. 5). Data from these plots showed that the mouse and rat enzymes were saturated at a substrate concentration of 1.43×10^{-4} mol/l, the rabbit enzymes by approximately 2.5×10^{-4} mol/l, but that hamster and guinea-pig enzymes were still not fully saturated at a substrate concentration of 3.57×10^{-4} mol/l. Kinetic results from which app. Km and Vmax values were obtained, with the appropriate values determined for the species used, are shown in Fig. 6. Results from incubations in which various potential inhibitors were included are given in Tables II, III and IV. The percentage of substrate converted to cotinine by xanthine oxidase, cow's milk, alcohol dehydrogenase and catalase are shown in Table V. The highest conversion (0.46%) was obtained from the reaction mediated by cow's milk after 15 minutes incubation.

Results from experiments employing γ-(-3-pyridyl)-γ-methylamino butyric acid as substrate showed only a minor difference between tests and controls in that cotinine formed, as % of substrate, was 1.64 for the controls and 1.70 for the tests.

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Fig. 3 : The *in vitro* conversion of nicotine (A) and nicotine Δ1'(5') iminium ion (B) to cotinine using guinea-pig 9000 *g* hepatic supernatant preparation, plus cofactors.

DISCUSSION

The conversion of the iminium ion to cotinine in incubates containing bovine serum albumin are of the same order as that attributable to chemical reaction (4) (Table I), therefore non enzymic and non-specific macromolecular conversion is either very small or non existent (c.f., the results using other proteins Table V). The lower values of cotinine formation obtained following longer incubation times with bovine serum albumin may be attributed to the non-specific hydrolytic activity associated with the protein (13) causing hydrolysis of the cyclic amide bond. Results from human foetal liver showed a difference in reaction compared with animal tissue, which may be due to an enzymic variation. The fact that highest enzymic activity was seen with the 25 week foetal tissue may be due to increased foetal age, or also to freedom from foetal thalassemia.

The marked difference between the 25 week specimen and those of 20 weeks (Fig. 2) is unlikely to be due to the use of 9000 *g* supernatant and 140.000 *g* supernatant respectively (4).

The greatly enhanced cotinine production from the iminium ion compared with that from nicotine, using guinea pig 9000 *g* hepatic supernatant, (Fig. 3) is consistent with the ion occupying an intermediate metabolic position between nicotine and cotinine (Fig. 7). *In vivo* metabolic results also support this conclusion (4). The apparent Km values show hi-

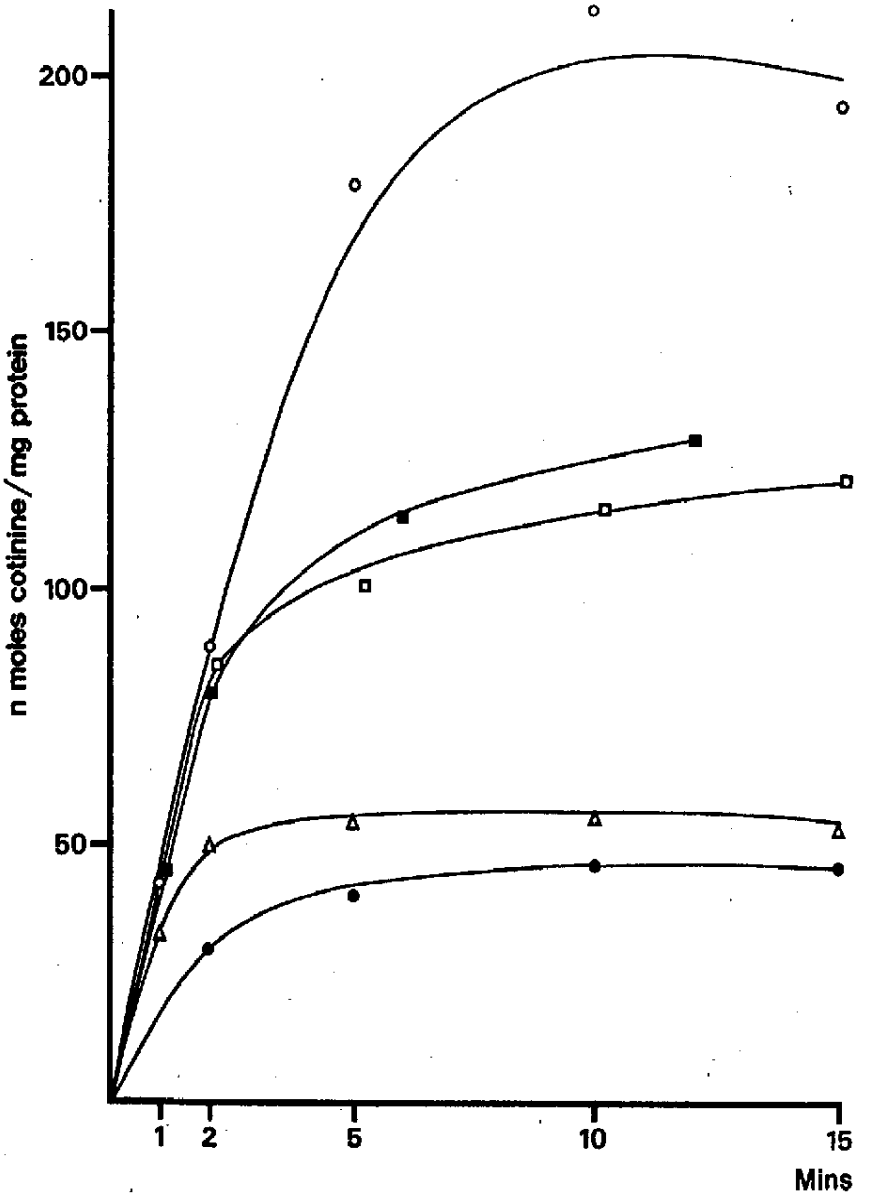


Fig. 4 : The effect of incubation time on cotinine formation from nicotine Δ1'(5') iminium ion (1.25 μmoles) using hepatic 140.000 *g* supernatant preparations from various species.
Rabbit-o-, Guinea pig -■-, Hamster -□-, Mouse -Δ-, Rat -•-

ghest enzyme substrate affinity in the mouse and rat, intermediate affinity in the rabbit and hamster, and lowest affinity in the guinea pig.

Because it has been shown that adduct formation can occur between the iminium ion and GSH (5) it was thought that both GSH and l-cysteine would be likely to act as inhibitors of cotinine production. In practice only weak inhibition of the reaction was seen using a hamster hepatic soluble preparation and concentrations of GSH and l-cysteine from 10^{-5} mol/l to 10^{-3} mol/l. However, some purified aldehyde oxidases have been shown to be slightly activated by GSH at around 10^{-3} mol/l whereas they are inhibited at higher concentrations (14). GSSG was also used as potential inhibitor because it is capable of oxidising enzymic SH groups to disulphides (15). No significant inhibition was observed in this study with up to 10^{-3} mol/l GSSG.

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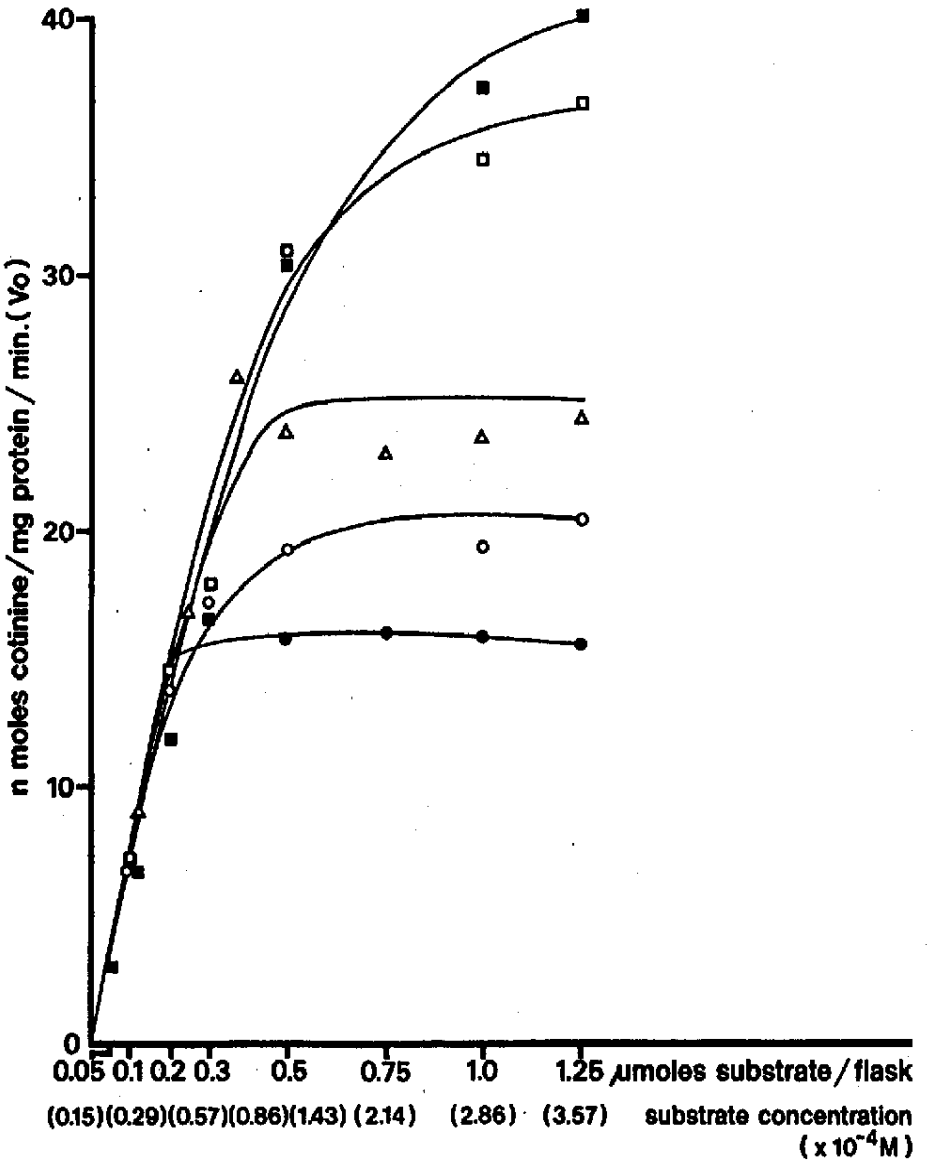


Fig. 5 : The effect of increasing nicotine Δ1'(5') iminium ion concentration on cotinine formation using hepatic 140.000 *g* supernatant from various species. Incubation time, 2 mins.
Rabbit -o-, Guinea pig -■-, Hamster -□-, Mouse -Δ-, Rat -•-

Potassium cyanide exhibited strongly increasing inhibition from 10^{-5} mol/l to 10^{-3} mol/l concentrations, in agreement with Hucker *et al.* (1), Murphy (2), Booth and Boyland (16)., Gorrod *et al.* (17) who all showed that cyanide inhibited cotinine formation from nicotine *in vitro.* Whilst cyanide, which is a metal complexing agent strongly inhibited, the inhibition with another metal complexing agent sodium azide (10^{-4} mol/l) was however weak, but this inhibition also progressively increased from 10^{-5} to 10^{-3} mol/l. Azide which is almost without effect on xanthine oxidase (11, 18, 19) does inhibit catalase and aldehyde oxidase (11), which are also cyanide sensitive (1, 11). These observations together with the 22% inhibition of cotinine formation produced by the metal chelating agent EDTA (11) (10^{-4} mol/l), suggested the activity was associated with a metalloenzyme.

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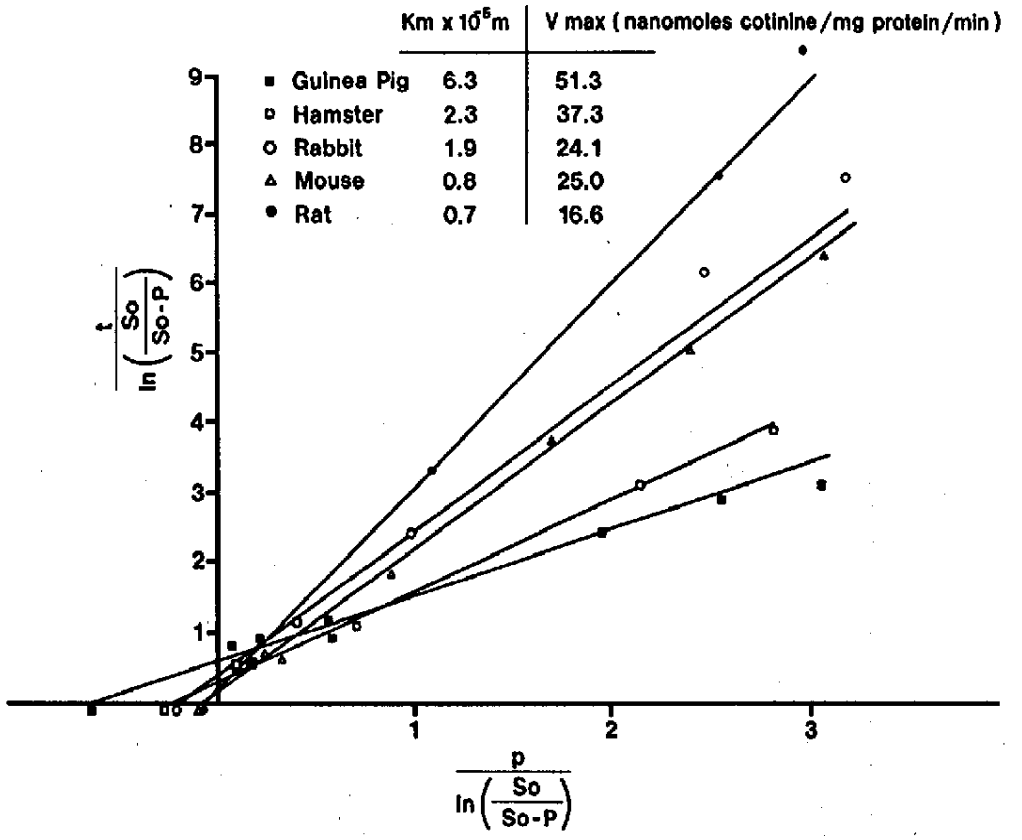


Fig. 6 : Apparent K_m and V_{max} for the hepatic cytoplasmic enzyme converting nicotine $\Delta 1'(5')$ iminium ion to cotinine.

Table II : Effect of potential inhibitors on the *in vitro* metabolism of nicotine $\Delta 1'(5')$ iminium ion to cotinine using 140.000 *g* liver supernatant from guinea-pig (G) and rat (R).

Compound added to incubate at 1×10^{-4} mol/l concentration	Percentage Cotinine formation compared with control	Species*
Nil (Control)	100	G & R
p-Chloro-mercuribenzoic acid	73	G
Iodo-acetic acid	80	G
Sodium Arsenite	54	R
EDTA (disodium salt)	78	R
5'Cyano-nicotine	27	R

* G = Guinea pig R = rat

Copper (cupric) ions exhibited strong inhibition of the enzyme probably through mercaptide formation with SH groups (15) and/or reaction with enzymic carboxyl groups (11). Other mercaptide forming agents which exhibited significant inhibition were p-chloromercuribenzoate (27%) and sodium arsenite (15) (46%). The latter reagent has long been known as an inactivator of xanthine oxidase and as a competitive inhibitor of aldehyde oxidase (18). Iodo-acetic acid which produced moderate (20%) inhibition is also known to react with SH groups (11).

Quinacrine which exhibited significantly increasing inhibition with increasing concentration ($10^{-5} - 10^{-3}$ mol/l) is commonly used as a detector of enzymic flavin moieties and its inhibition of aldehyde oxidase has been reported to be reversible by FAD (15). Ethanol was employed as an inhibitor because of the possibility that it or its metabolite, acetaldehyde (11) may competitively inhibit the enzyme responsible for the reaction to cotinine. However a slight activation was seen with the concentrations used ($10^{-5} - 10^{-3}$

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Table III : Effect of potential inhibitors on the in vitro metabolism of nicotine Δ1'(5') iminium ion to cotinine using 140.000*g* liver supernatant from various animal species.

Addition to incubate	Concentration 10^{-X} mol/l	% Inhibition (-) or Activation (+) compared with control	Species
	X =		
Glutathione (GSH)	5	- 8	Hamster
	4	- 3	»
	3	- 12	»
l-Cystine	5	- 11	»
	4	- 4	»
	3	- 7	»
Sodium Cyanide	5	- 14	»
	4	- 94	»
	3	- 99	»
Disulfiram	5	- 15	»
	4	- 29	»
	3	- 41	»
Allopurinol	5	- 24	»
	4	- 22	»
	3	- 28	»
Copper(ic)	5	- 14	Guinea Pig
	4	- 58	» »
	3	- 92	» »
Ethanol	5	+ 5	» »
	4	+ 2	» »
	3	+ 4	» »
Quinacrine	5	- 8	» »
	4	- 44	» »
	3	- 63	» »
Sodium Azide	5	- 4	Rat
	4	- 11	»
	3	- 14	»
l-Proline	5	+ 5	Rabbit
	4	- 2	»
	3	- 10	»
Glutathione (oxidased) (GSSG)	5	- 2	»
	4	- 1	»
	3	- 4	»

mol/l). Allopurinol, which produced from 22 to 27% inhibition across the concentration range 10^{-5} mol/l to 10^{-3} mol/l is a potent competitive inhibitor of xanthine oxidase (15). Accordingly, progressively stronger inhibition would have been expected with such a concentration increase, if xanthine oxidase were the mediating enzyme. Disulfiram, which exhibited a concentration dependent inhibition, up to a maximum of 43% at 10^{-3} mol/l has been reported to inhibit xanthine oxidase (19), alcohol dehydrogenases (20) and liver aldehyde oxidase (21).

l-Proline produced a small activation at 10^{-5} mol/l and only 11% inhibition at 10^{-3} mol/l. This amino acid was employed as a potential inhibitor on

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the basis of its ability to inhibit the oxidation of Δ^1-pyrroline-5-carboxylate to glutamic acid, by glutamic semialdehyde dehydrogenase (22). There is some similarity between the above reaction and one possible mechanism for cotinine formation from the iminium ion and aldehyde in equilibrium (Fig. 7) thence via γ-(3-pyridyl)-γ-methylamino butyric acid (23, 24). It is known that γ-(3-pyridyl)-γ-methylamino butyraldehyde exists in aqueous solution of the iminium ion pH 7.4 (3). It has also been shown that γ-(3-pyridyl)-γ-methylamino butyric acid can cyclise chemically to cotinine (23). However, the lack of inhibition observed suggests that glutamic semialdehyde dehydrogenase does not play a significant role in the conversion of the iminium ion to cotinine, *in vitro*.

The use of γ-(3-pyridyl)-γ-methylamino butyric acid as substrate, designed to test the possibility of metabolic cyclisation of this compound to cotinine, showed only a minor difference between test incubates and controls. Cotinine does not therefore appear to arise metabolically *in vitro* by this route.

Cotinine inhibited its own formation by almost 20% when 1.0 μmole per incubate was used and by 9% at 0.5 μmoles per incubate (Table IV). Control (uninhibited) incubations produces 0.53 μmoles cotinine from the iminium ion substrate (1.25μmoles). The incubates which included cotinine at t = 0 also

Table IV : The influence of cotinine on the conversion of nicotine Δ 1′(5′) iminium ion to cotinine by hamster supernatant preparation.

Cotinine added (μmole/incubate)	% Inhibition of cotinine formation with compared control
0.50	9
0.75	18
1.00	20

(a) nicotine
(b) nicotine -Δ1'(5')-iminium ion
(c) 5'hydroxy-nicotine
(d) γ-(3-pyridyl)-γ-methylamino butyrlaldehyde
(e) cotinine

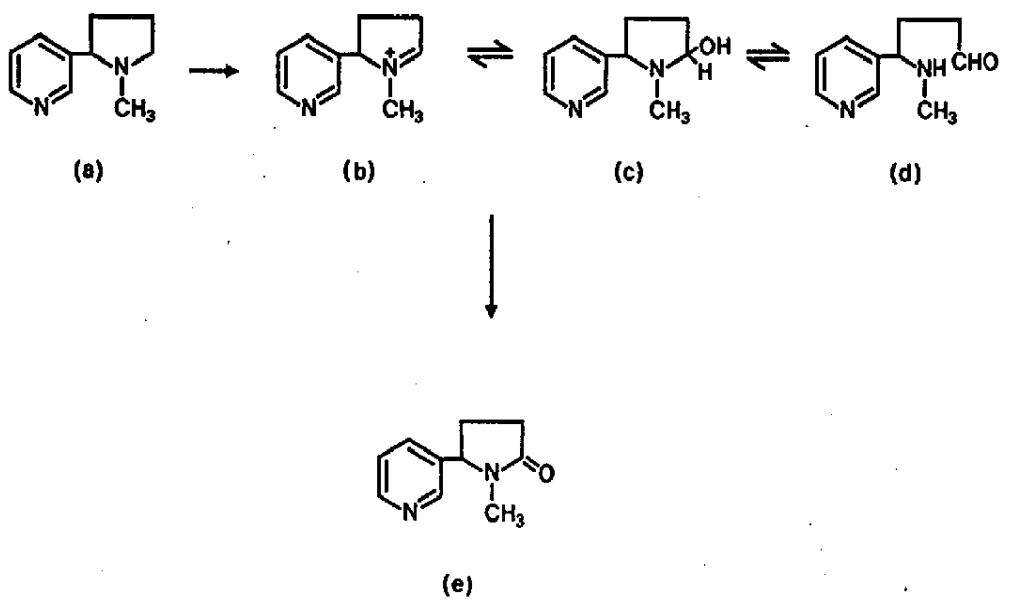


Fig. 7 : Proposed metabolic pathway from nicotine to cotinine.
a = Nicotine, b = Nicotine Δ1′(5′) iminium ion, c = 5′-Hydroxynicotine, d = γ-(3-Pyridyl)-γ-methylaminobutyraldehyde, e = Cotinine.

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Table V : Effect of incubation of nicotine Δ1′(5′) iminium ion with (A) crystallised milk xanthine oxidase, (B) fresh cow's milk (as a source of xanthine oxidase), (C) animal alcohol dehydrogenase and (D) catalase.

Incubation time (mins) at 37°C	Cotinine formation, expressed as % of substrate, for each potential enzymic mediator.			
	A	B	C	D
0	0.06	0.21	Trace	Trace
2	0.06	0.22	—	—
5	0.07	0.36	—	—
10	0.06	0.18	Trace	Trace
15	0.06	0.46	—	—

had metabolically formed cotinine in addition to the original amount, throughout the incubation period. Therefore 9% inhibition arises from a final cotinine concentration of almost 1.0 μmole per flask. In controls, however the figure is 0.53 μmoles. It may therefore be reasonably assumed that product inhibition over the initial rate of oxidation (two minutes) would be, at most 5%. Accordingly, product inhibition was not considered to be a factor which introduced unacceptable error into the above kinetic studies.

5′Cyano-nicotine was employed as a potential inhibitor on the assumption that because of its structural similarity it may have been capable of occupying the same enzymic active site as the possible ultimate precursors of cotinine, viz 5′-hydroxy-nicotine (1,2) (Fig. 7). Powerful inhibition of cotinine formation, 73% at 10^{-4} mol/l 5′cyano-nicotine was observed.

The above evidence points to the activity being associated with a metallo, thiol containing enzyme, which possibly implicates either alcohol dehydrogenase, aldehyde oxidase, xanthine oxidase, xanthine dehydrogenase or catalase (11, 15, 18, 19, 20). However results from the use of purified xanthine oxidase, cow's milk (as a natural source of xanthine oxidase), alcohol dehydrogenase and catalase showed that none of these played a significant role in cotinine formation from the iminium ion. The excess substrate inhibition observed during the course of this work suggested that the formation of cotinine may be due to another enzyme(s). Inhibition due to excess substrate is a phenomenon common to both xanthine oxidase (18, 19, 25) and aldehyde oxidase (18, 25) and the results are consistent with the iminium oxidase being a metalloflavoprotein; amongst this group are aldehyde oxidase, xanthine oxidase and xanthine dehydrogenase, which catalyse reactions of the form, $RH \xrightarrow[+OH^-]{-2e,\ -H^+} > ROH$ where RH is the reducing substrate. The oxygen introduced into RH being derived from water (18).

A well supported proposal for the mechanism of aldehyde oxidase mediated oxidation of *N′*-methyl-nicotinamide to 2- and 4-pyridone (14) is applicable to the reaction to cotinine from the iminium ion, proposed here (Fig. 7). Previous evidence (2,4) has strongly indicated that the oxygen in cotinine is derived from water.

K_m values for the oxidation of *N′*methylnicotinamide by purified rabbit and hog liver aldehyde oxidase have been reported to be 6.6×10^{-4} mol/l and 3.6×10^{-4} mol/l respectively (14). Recently it has been shown that purified aldehyde oxidase has a considerably higher affinity for the nicotine Δ1′(5′) iminium ion than for N′methylnicotinamide (26). This adds support to the app Km values reported in this paper (Fig. 6).

The results from this study support the idea that aldehyde oxidase is the hepatic soluble enzyme which mediates the conversion of the iminium ion to cotinine (26). Differences in this enzyme in animal species and man have previously been noted (14) and these observations are consistent with the differences observed between animal and human fetal tissue used in these studies.

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THE METABOLISM OF NICOTINE INTO TWO OPTICALLY-ACTIVE STEREOISOMERS OF NICOTINE-1′-OXIDE BY ANIMAL TISSUES *IN VITRO* AND BY CIGARETTE SMOKERS

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Abstract—Nicotine-1′-oxide, prepared by the oxidation of (−)-nicotine with hydrogen peroxide, has been resolved into two optically active stereoisomers by fractional precipitation with ammonium reineckate. (−)-Nicotine is enzymically oxidized into both isomers of nicotine-1′-oxide *in vitro*. These enzyme systems are stereospecific since guinea-pig and rabbit liver form similar amounts of each stereoisomer while BALB/c mouse and hamster liver and guinea-pig lung synthesize chiefly *laevo*-rotatory nicotine-1′-oxide. Both stereoisomers of nicotine-1′-oxide were identified in the urine of cigarette smokers.

THE ABSORPTION and metabolism of nicotine after the smoking of tobacco were demonstrated by the identification of cotinine in the urine of smokers.[1] The distribution and fate of nicotine has been reviewed by McKennis[2] and cotinine was found to be a major metabolite in numerous species. Another metabolic oxidation product of nicotine is nicotine-1′-oxide which was identified in the liver, kidney and urine of the rabbit after administration of nicotine.[3] In *in vitro* experiments nicotine is converted into cotinine by a combination of the microsomal and soluble fraction of rabbit liver in the presence of NADPH and O_2,[4] and into nicotine-1′-oxide by a rabbit liver supernatant preparation.[5]

The present paper shows that nicotine-1′-oxide can be resolved into two isomers with different optical activities and demonstrates the *in vitro* formation of both stereoisomers by animal tissues and their presence in the urine of cigarette smokers. The numbering of the nicotine molecule used in this paper is shown in Fig. 1.

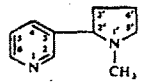


FIG. 1. Numbering of nicotine molecule.

MATERIALS

(−)-Nicotine, purchased from British Drug Houses Ltd., Poole, Dorset, England, was distilled under reduced pressure and stored in ampoules, a fresh ampoule being used for each experiment.

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(−)-Cotinine was prepared by an oxidation of nicotine.[6] Nicotine-1′-oxide was prepared by the oxidation of nicotine with hydrogen peroxide and characterized as the dipicrate, m.p. 156° (lit.[7] m.p. 154–158°).

METHODS AND RESULTS

Chromatography. Paper chromatograms were developed on Whatman No. 1 or 3 MM paper with *n*-butanol:*n*-propanol: 2N NH_4OH (2:1:1) by the descending technique. Thin-layer chromatography (TLC) plates were coated with 0·25 mm of Silica gel $HF_{254+366}$ or aluminium oxide GF_{254} (type E) (E. Merck A.G., Darmstadt, Germany) and activated at 100° for 1 hr. Ascending chromatograms were developed with one of the solvent systems described in the legends to the tables.

Compounds on chromatograms were located as: (1) dark spots when viewed under ultraviolet light; (2) black spots on a pink background after spraying with iodoplatinate reagent;[8] (3) orange spots after spraying with potassium bismuth iodide.[9]

Semi-quantitative estimations of spot intensity depended on the precipitation of a black complex when nicotine-1′-oxide was treated with iodoplatinate. Compounds were located on paper or thin-layer chromatograms under ultraviolet light. The appropriate areas were removed from the chromatograms and treated with 4 ml dilute iodoplatinate solution (spray reagent[8] diluted 1 in 13). This solution has E_{max} at 494 mμ and formation of the black complex, which was removed by centrifugation, resulted in a decrease in absorption at this wavelength. The decrease produced by biologically formed nicotine-1′-oxide was compared with values obtained by adding known amounts of synthetic nicotine-1′-oxide.2 HCl to reaction mixtures or non-smoker's urine.

Resolution of nicotine-1′-oxide into two isomers

When either nicotine-1′-oxide or its dipicrate was examined by chromatography on Whatman No. 1 paper with *n*-butanol:*n*-propanol:2N NH_4OH (2:1:1) as the solvent system they were resolved into two compounds with R_f values of 0·51 and 0·41. Semi-quantitative estimations of spot intensity indicated that, in freshly prepared samples, the ratio of the amount of the faster running compound (R_f 0·51) to that of the slower running one (R_f 0·41) was approximately 2:1. These compounds, which were stereoisomers of nicotine-1′-oxide with different optical activities, were separated by fractional precipitation of the reineckates. After the addition of ammonium reineckate (14 m-moles in 50 ml methanol) to nicotine-1′-oxide (28 m-moles in 500 ml water) and stirring for 0·5 hr, the pink precipitate was removed by filtration. A further addition of ammonium reineckate (14 m-moles in 50 ml methanol) to the filtrate produced a second precipitate which was again removed after stirring for 0·5 hr. This procedure was repeated until a total of 56 m-moles of ammonium reineckate had been added; the final filtrate was kept overnight and the crystalline material that separated removed by filtration. Chromatography of the nicotine-1′-oxide reineckates as described previously caused the complex to split into the red reinecke acid (R_f 0·77) and nicotine-1′-oxide (R_f 0·51 or 0·41). Chromatograms of the various fractions showed that the first precipitate was the reineckate of nicotine-1′-oxide with R_f 0·52 and the final precipitate the reineckate of nicotine-1′-oxide with R_f 0·41. Intermediate fractions contained both isomers. The reineckates were dissolved in methanol containing 3% ammonia and the solutions filtered to remove an insoluble brown contaminant. The reineckates were precipitated from the filtrates by addition of acetic acid and crystallized from aqueous methanol.

The absorption spectra of reineckates of colourless bases resemble that of ammonium reineckate between 360 and 600 mμ because the absorption is exclusively due to the reineckate moiety of the molecule. Spectrophotometric analysis of reineckates can therefore be used for molecular weight determinations.[10] The nicotine-1′-oxide reineckates and ammonium reineckate dissolved in methanol containing 3% (v/v) ammonia have an absorption maximum at 520 mμ and molecular weights were calculated by comparing $E^{1\%}_{1cm}$ values at this wavelength. Table 1 shows that after purification both isomers of nicotine-1′-oxide existed as monoreineckates.

TABLE 1. PROPERTIES OF NICOTINE-1′-OXIDE REINECKATES

Compound	$E^{1\%}_{1cm}$(520 mμ)	Mol. wt. Found	Mol. wt. Required	m.p.
Ammonium reineckate ($NH_4[Cr(NH_3)_2(SCN)_4]H_2O$)[a] Mol. wt. 354,4	2·97	—	—	—
Nicotine-1′-oxide (R_f 0·41) reineckate	2·07	509	—	136–140° (decomp.)
Nicotine-1′-oxide (R_f 0·51) reineckate	2·11	499	—	184–188° (decomp.)
Nicotine-1′-oxide monoreineckate ($C_{14}H_{31}CrN_8OS_4$)	—	—	497	—
Nicotine-1′-oxide direineckate ($C_{18}H_{32}Cr_2N_{14}OS_8$)	—	—	816	—

The nicotine-1′-oxides were recovered from the monoreineckates by elution from a column of aluminium oxide. The monoreineckates were dissolved in a small volume of methanol containing 3% (v/v) ammonia and applied to the column. On elution with chloroform:methanol (5:2) the nicotine-1′-oxides were eluted and the red reinecke acid remained at the top of the column.

The nicotine-1′-oxides were also recovered and the isomers separated by applying a solution of the reineckates in methanol containing 3% (v/v) ammonia as a strip to Whatman 3 MM chromatography paper. After development in *n*-butanol:*n*-propanol:2N NH_4OH (2:1:1) the nicotine-1′-oxide isomers which appeared as dark bands under ultraviolet light were eluted with methanol. After evaporating the eluates to dryness under reduced pressure at 30° and drying, the residues were washed with a small volume of acetone and crystallized from ethyl acetate.

Optical activities were determined in a Perkin–Elmer Model 141 polarimeter using micro cells (1 ml) with 10 cm light path. After crystallization from ethyl acetate, the solvent was decanted, the crystals dried with a stream of dry N_2 and immediately dissolved in methanol for determination of the optical activity. The concentration was determined by transferring 1 ml of the methanolic solution into a weighed flask fitted with an outlet and inlet. The solution was evaporated to dryness with a stream of dry N_2 and the flask closed and re-weighed. The faster running isomer (R_f 0·51) crystallized as colourless deliquescent prisms, m.p. 168° decomp., $[\alpha]_D^{22} + 65{\cdot}1$ (c 4·38 in methanol). The other isomer (R_f 0·41) also crystallized in colourless prisms, $[\alpha]_D^{22} - 76{\cdot}0$

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(c 3·05 in methanol) but was considerably more deliquescent and the m.p. was not determined. For nicotine-1′-oxide lit. m.p. 172–173°;[11] 172–173°;[12] 154–155·2° and $[\alpha]_D^{21.5}$ + 65·3 (c 5·13 in methanol);[13] and $[\alpha]_D^{21}$ + 66 (c 5·0 in methanol).[5]

Identification of nicotine-1′-oxide isomers as metabolites of nicotine

(a) *By guinea-pig liver and lung* in vitro. Fresh tissue from male animals was homogenized in 3 vol. ice-cold KCl (1·15% w/v) using an Ultra-Turrax homogenizer. The homogenates were centrifuged at 5° in an Angle 50 centrifuge (Measuring and Scientific Equipment Ltd., London) for 20 min at 2650 g_{max} and the supernatant phase used for incubations with nicotine. Incubation mixtures contained Na_2HPO_4–NaH_2PO_4 buffer pH 7·5 (1·2 m-moles); nicotinamide (480 μmoles); glucose 6-phosphate (200 μmoles); NADP (4 μmoles); glucose 6-phosphate dehydrogenase (20 μg); nicotine (144 μmoles); and 4 ml homogenate supernatant in a total volume of 24 ml. Mixtures, with nicotine omitted, were pre-incubated with shaking at 37° for 10 min, the reaction was started by addition of the nicotine and the mixtures incubated for 2 hr. A similar mixture without incubation after the addition of nicotine or complete reaction mixtures in which the tissue had been boiled before incubation were used as controls. The reactions were stopped with 36% (w/v) trichloroacetic acid (2 ml) and the protein removed by centrifugation. The supernatant phase was made alkaline with 5 N NaOH (1·5 ml) and extracted with three portions of dichloromethane (10 ml). The aqueous phase was treated with glacial acetic acid (1·5 ml) and decolorising charcoal (800 mg) (May and Baker Ltd., Dagenham, England). The charcoal was removed by filtration, washed with water (40 ml) and the metabolites eluted with 3% (v/v) ammonia in methanol (20 ml). The eluate was evaporated at 35° under reduced pressure to approximately 0·2 ml and applied as a band to a silica gel HF_{254} + $_{366}$ TLC plate which had also a sample of nicotine-1′-oxide applied as a marker. After development in *n*-butanol:ethanol:2N NH_4OH (4:1:1) the strip of silica gel corresponding to the position of nicotine-1′-oxide (R_f 0·15) was removed from the plate and shaken with methanol (14 ml). The mixture was filtered, the filtrate evaporated to dryness and the residue dissolved in methanol (0·2 ml) for chromatography. Table 2 shows that the

TABLE 2. CHROMATOGRAPHIC PROPERTIES OF NICOTINE-1′-OXIDE ISOMERS AND NICOTINE METABOLITES

Compound	R_f Chromatographic system				
	1	2	3	4	5
Nicotine-1′-oxide (*dextro*-rotatory)	0·33	0·59	0·34	0·51	0·15
Nicotine-1′-oxide (*laevo*-rotatory)	0·25	0·66	0·25	0·41	0·15
Guinea-pig liver metabolites	0·26, 0·35	0·59, 0·67	0·27, 0·34	0·42, 0·51	0·15
Guinea-pig lung metabolites	0·24, 0·32	0·58, 0·66	0·25, 0·33	0·42, 0·51	0·15
Metabolites in smoker's urine	0·26, 0·33	0·58, 0·66	0·24, 0·34	0·42, 0·51	0·15

Chromatographic systems: 1, 2 and 3 on TLC plates coated with $Al_2O_3GF_{254}$; 4 on Whatman No. 1 chromatography paper; 5 on TLC plates coated with silica gel HF_{254} + $_{366}$. Solvent systems: 1, benzene:methanol (9:1); 2, chloroform:ethyl acetate-acetic acid (7:2:1); 3, *iso*-amyl alcohol: 5% (w/v) citric acid (9:1); 4, *n*-butanol:*n*-propanol:2N NH_4OH (2:1:1); 5, *n*-butanol:ethanol:2N NH_4OH (4:1:1).

Compounds were located as dark spots when chromatograms were viewed under ultraviolet light, as grey spots against a pink background when sprayed with iodoplatinate reagent, or as orange spots when sprayed with potassium bismuth iodide.

chromatographic properties of the nicotine metabolites synthesised by guinea-pig liver and lung were identical with those of the two isomers of nicotine-1′-oxide in chromatographic systems. Nicotine-1′-oxides formed either chemically or by guinea-pig tissues also had the same R_f values in system 5 although in this case the isomers were not separated. The relative intensity of the spots of each isomer formed by the two tissues showed that the liver synthesised similar amounts of each (Table 3) but that

TABLE 3. COMPARISON OF NICOTINE-1′-OXIDE ISOMERS FORMED BY LIVER HOMOGENATES FROM DIFFERENT SPECIES

Species	Nicotine-1′-oxide isomers	
	Laevo-rotatory	*Dextro*-rotatory
Albino guinea-pig	*	*
Albino rabbit	†	†
BALB/c mouse	‡	‡
CB Cream Syrian hamster	†	‡

Incubation mixtures were as described in the text. Results represent the mean values of at least three experiments and indicate the approximate amounts of each isomer of nicotine-1′-oxide formed by 300 mg liver in 30 min. * = 100–150 μg; † = 50–100 μg; ‡ = 0–50 μg.

lung formed almost entirely the *laevo*-rotatory isomer with only trace amounts of the *dextro*-rotatory form. Under the same conditions liver formed approximately 10 times as much *laevo*-rotatory isomer and 50 times as much *dextro*-rotatory isomer as lung. No metabolites were seen if complete reaction mixtures were boiled before incubation. The identity of the metabolites was confirmed by two colour reactions. All substances listed in Table 2 appeared as black spots on a pink background when sprayed with iodoplatinate reagent or as orange spots when sprayed with potassium bismuth iodide.

(b) *By livers of other species* in vitro. The ability of livers from male animals of other species to form the two isomers of nicotine-1′-oxide was investigated by the same method used for guinea-pig tissues employing chromatographic system 4 (Table 2). Both isomers of nicotine-1′-oxide were identified in reaction mixtures after incubating *nicotine* with homogenates of liver from BALB/c mice, rabbits or hamsters. Considerable differences in the relative size of the spots of the two isomers and the intensity of colours after spraying chromatograms with colour reagents (as above) indicated that the amounts of each isomer formed varied in different species. For comparisons of spot intensities, 6 ml reaction mixtures, with concentrations of constituents as for guinea-pig liver, were used and incubations were for 30 min. After treatment with charcoal as before the eluates were applied directly to 3 MM paper and chromatograms developed in system 4 (Table 2). The results are shown in Table 3 and although the values are presented as semi-quantitative results they indicate that whereas guinea-pig and rabbit formed comparable amounts of each isomer, mouse and hamster synthesised chiefly *laevo*-rotatory nicotine-1′-oxide. The stereospecificity of the enzymic oxidation of nicotine to nicotine-1′-oxide by different sepecies and tissues is being studied by quantitative methods.

(c) *By cigarette smokers*. Specimens of urine were collected from male non-smokers or cigarette smokers between 16·00 and 19·00 hr. Several specimens from each group

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(total volume 300–600 ml) were pooled, cooled to 5° and centrifuged at 2000 g_{max} for 15 min. The supernatant phases were adjusted to pH 3·0 with 40% (w/v) trichloroacetic acid and treated with decolorising charcoal (1 g/100 ml urine). The charcoal was removed by filtration and washed with water (300 ml) followed by ethanol (20 ml). The metabolites were eluted with 300 ml methanol containing 3% (v/v) ammonia which was then concentrated at 35° under reduced pressure to approximately 100 ml. This elutate was shaken with aluminium oxide (60 g) which adsorbed most of the coloured impurities present at this stage. The aluminium oxide was removed by filtration and washed with methanol (80 ml). The filtrate and washing were combined and concentrated to 0·6 ml at 35°. The concentrate was transferred to a 500 × 60 mm tube, small amounts of finely divided aluminium oxide removed by centrifugation and the supernatant phase applied as bands to silica gel $HF_{254+366}$ TLC plates. A sample of nicotine-1′-oxide was also applied as a marker and the chromatograms developed in *n*-butanol:ethanol:2N NH_4OH (4:1:1). The marker (R_f 0·15) was located as a dark spot under ultraviolet light or after spraying with iodoplatinate reagent. The areas of silica gel corresponding to this location were scraped from the plates and shaken with methanol (20 ml) which was filtered. The filtrates were evaporated to dryness at 35°, the residues dissolved in methanol (0·2 ml) and applied as bands to aluminium oxide HF_{254} TLC plates which also had marker spots of both isomers of nicotine-1′-oxide. After development of chromatograms in chloroform:ethyl acetate:acetic acid (7:2:1), the aluminium oxide corresponding to the nicotine-1′-oxide position was scraped off and the metabolites eluted with methanol and concentrated as before for chromatography.

Table 2 shows that the urine of cigarette smokers contains two compounds which are chromatographically identical with the isomers of nicotine-1′-oxide in five chromatographic systems. In all experiments the amount of *dextro*-rotatory nicotine-1′-oxide (as judged by the relative size of the spots) exceeded that of the *laevo*-rotatory isomer. For semi-quantitative experiments, known amounts of nicotine-1′-oxide (mixed isomers) were added to non-smoker's urine, which was then subjected to the same procedure, and the spot intensities compared with those of smoker's urine. In a typical experiment the combined urine samples (340 ml) from four smokers, who had collectively smoked 60 cigarettes on the day of urine collection, contained approximately 80 μg of *laevo*-rotatory nicotine-1′-oxide and approximately 800 μg of the *dextro*-rotatory isomer.

Reaction of nicotine-1′-oxide with potassium dichromate and isatin

The treatment of amine oxides with chromate or dichromate may cause oxidation, demethylation, isomerization in which the oxygen atom is transferred from the nitrogen to the α-carbon (or 5′ position) atom or a combination of these reactions.[14] Treatment of nicotine-1′-oxide with potassium dichromate produced several reaction products which could be separated by TLC but their chromatographic properties varied, presumably because of their instability. The inclusion of isatin in the reaction mixture, however, resulted in the formation of a stable crimson complex which could be extracted into organic solvents. Isatin has been used to detect certain pyrrolidine derivatives after chromatography.[15]

Reaction mixtures contained nicotine-1′-oxide in 1 ml water, 2% (w/v) potassium dichromate (0·1 ml) and isatin reagent[8] (0·2 ml) and were heated at 100° for 15 min.

After cooling, the mixtures were shaken with ethyl acetate (6 ml) which was removed then shaken with N NaOH (0·2 ml). The ethyl acetate extracts contained a crimson and complex with absorption max. at 505 mμ. Similar reactions were carried out using nicotine-1′-oxide isolated from mixtures in which nicotine had been incubated with guinea-pig liver, or lung, or from the urine of cigarette smokers as described above. The ethyl acetate extracts were evaporated to dryness and the residues dissolved in methanol (0·1 ml) and examined by TLC. Table 4 shows that synthetic nicotine-1′-oxide formed a product with chromatographic properties identical with those of

Table 4. Chromatographic properties of complex formed by isatin and potassium dichromate oxidation product of nicotine-1′-oxide

Source of nicotine-1′-oxide	R_f Chromatographic systems 1	2	3
Synthetic	0·66	0·31	0·57
Nicotine + guinea-pig liver incubation	0·66	0·31	0·57
Nicotine + guinea-pig lung incubation	0·66	0·31	0·57
Cigarette smoker's urine	0·64	0·31	0·56

Complex was visible as a crimson spot.
Chromatographic system: 1 and 2 on TLC plates coated with silica gel $HF_{254+366}$; 3, with $Al_2O_3GF_{254}$. Solvent systems: 1, acetone:benzene:ethanol:ammonia (40:50:5:3); 2 and 3, benzene:methanol (9:1).

products from nicotine-1′-oxide synthesised by guinea-pig tissues or isolated from the urine of smokers. Reaction mixtures containing 25 μg nicotine-1′-oxide produced a clearly visible crimson spot. This was absent if either nicotine-1′-oxide, potassium dichromate or isatin was omitted or if complete mixtures were not heated. By analogy with other amine-*N*-oxides[14] possible oxidation products are nornicotine, cotinine, desmethylcotinine, 3-(5′-hydroxy-1′-methylpyrrolid-2′-yl)pyridine and 3-(5′-hydroxypyrrolid-2′-yl)pyridine. However, reaction mixtures in which nornicotine, cotinine or desmethylcotinine were substituted for nicotine-1′-oxide did not form the crimson complex. Therefore, it seems probable that the complex is due to a reaction between isatin and 3-(5′-hydroxypyrrolid-2′-yl)pyridine. On the other hand, McCaldin[15] considers that a ring containing the —NH—CH_2—CH_2— sequence is required for the production of a coloured complex. The structure of the crimson complex formed between isatin and a potassium dichromate oxidation product of nicotine-1′-oxide has not been determined.

DISCUSSION

The factor or factors responsible for the carcinogenic effect of the smoking of tobacco on the human lung[16] has not been identified, but it is possible that nicotine or some of its metabolites play a part in this process. One of the metabolites of nicotine, nicotine-1′-oxide, might be considered as a possible carcinogen by analogy with the purine *N*-oxides which induce tumours when injected into rats.[17, 18] The rapid absorption, excretion and metabolism of nicotine have been demonstrated by Beckett and

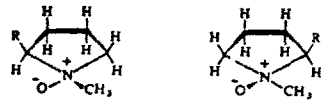
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Triggs,[19] who found that the maximum rate of nicotine excretion in urine occurred during the first 15 min after smoking two cigarettes and the maximum rate of cotinine excretion about 2 hr after smoking.

Nicotine is converted into cotinine *in vitro* by lung tissue of numerous species including man[20, 21] and to both isomers of nicotine-1′-oxide by guinea-pig lung. Hence these metabolites may be formed in the lungs of smokers by enzymic oxidation of absorbed nicotine. Alternatively, they could be transported to the lungs after formation in the liver, which is the principal site of nicotine metabolism.[20] The enzymic oxidation of nicotine to cotinine is catalyzed by a combination of the microsomal drug metabolizing system of rabbit liver, which requires NADPH and O_2, and the soluble liver fraction.[4] Since rabbit liver preparations oxidize nicotine to nicotine-1′-oxide in the presence of NADPH[5] this reaction is probably also catalyzed by the microsomal enzymes. The presence of nicotine in the urine of smokers indicates that it circulates in the body and would be available as a substrate for these enzyme systems. The formation of both cotinine and nicotine-1′-oxide are detoxication mechanisms since, when tested by subcutaneous injections into mice, nicotine is 50 times as toxic as cotinine[22] and 20 times as toxic as nicotine-1′-oxide.[23]

The oxidation of nicotine to nicotine-1′-oxide with hydrogen peroxide[7] produced two isomers which could be separated by chromatography. Since (—)-nicotine was used in all experiments the configuration at the asymmetric C atom of the pyrrolidine ring is fixed. However, the existence of two isomers due to different arrangements of groups at the quaternary nitrogen atom is possible, one isomer having the pyridyl and methyl groups *cis* to each other and the other having *N*-oxide oxygen *cis* to the pyridine ring. When the methyl and pyridyl groups are not *cis* to each other they offer the minimum of mutual steric hindrance; this suggests that nicotine-1′-oxide is likely to exist predominantly in this form.[12] In the present work, chromatograms of nicotine



R = PYRID—3—YL

Fig. 2. Isomers of nicotine-1′-oxide.

oxidation products indicated, by reaction with iodoplatinate, that freshly prepared samples contained approximately twice as much *dextro*-rotatory as *laevo*-rotatory isomer, although quantitative experiments are needed to confirm this observation. Although the stereoisomers did not have equal and opposite optical activities, as would be predicted from the fact that they are not enantiomorphs, one was *dextro*-rotatory and one *laevo*-rotatory. Both isomers are fairly stable in solution but form deliquescent solids. The *laevo*-rotatory isomer is the more unstable and is probably the isomer with the methyl and pyridyl groups in *cis* positions.

When nicotine was oxidized enzymically both isomers of nicotine-1′-oxide were synthesised but the relative amounts of each varied with the species and with the tissue.

For example, whereas guinea-pig liver synthesised similar amounts of the two isomers, liver from BALB/c mice and guinea-pig lung formed almost exclusively the *laevo*-rotatory isomer. Although the *dextro*-rotatory isomer predominated in smokers' urine this may not reflect the amounts synthesized by various organs, since *in vivo* the isomers may show stereospecificity during further metabolism or reactions with cell constituents before excretion. Stereospecificity is also demonstrated in the oxidation of ethylbenzene into optically active isomers of methylphenyl-carbinol by rat-liver microsomes in which 80·9 per cent of the product was the *dextro* isomer. The stereospecificity was reduced when the microsomal enzymes were induced by pretreatment of the rats with phenobarbital.[24] This suggests that many conditions, such as species, strain, age, sex, hormone regulation, nutritional status and diet, which are known to affect these enzymes[25] may also change their stereospecificity.

Other drugs which are converted to *N*-oxides by liver microsomes are chlorpromazine,[26, 27] imipramine[28] and dimethylaniline.[29] In these compounds the N atom is in the side-chain of the molecule and diphenhydramine is also metabolized by this route by the Rhesus monkey.[30] However, the formation of *N*-oxides of tremorine by rabbit liver slices,[31] chlorcyclizine by men and rats,[32] guanethidine by rats,[33] perazine by men[34] and nicotine by men and animal tissues suggests that *N*-oxidation of ring *N*-atoms is a general route for the conversion of nitrogenous foreign compounds into water-soluble metabolites.

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SOME METABOLITES OF BROMOCYCLOPENTANE, BROMOCYCLOHEXANE AND BROMOCYCLOHEPTANE IN THE RABBIT

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Abstract—Rabbits have been dosed with bromocyclopentane, bromocyclohexane and bromocycloheptane and the excretion of bromine (estimated as bromide), glucosiduronic acid and ethereal sulphate measured. With all three compounds there is a relatively rapid excretion of bromide accounted for by the formation of a bromine-containing glucosiduronic acid. The glucosiduronic acids isolated as their tri-acetyl methyl esters are conjugates of bromocycloalkanols. Evidence is presented that hydroxylation of the bromocycloalkane occurs at position 2. The extent of hydroxylation increases with increasing ring size.

POLYHALOGENATED derivatives of alicyclic compounds are used widely as insecticides and herbicides but few detailed studies of their metabolic fate in mammals have been reported. Grover and Sims[1] found that hexachlorocyclohexane (Gammexane) was dehydrochlorinated and hydroxylated by the rat with the formation of trichlorophenols. Few accounts of the metabolism of partly halogenated derivatives of alicyclic compounds have been given although the monohalogenoparaffins have been extensively studied. This paper describes an investigation of those hydroxylated derivatives which are formed from bromocyclopentane, bromocyclohexane and bromocycloheptane and excreted conjugated with glucuronic and sulphuric acids. A preliminary account[2] of the excretion of a bromine-containing glucosiduronic acid by rabbits dosed with bromocyclohexane has been given. The compounds studied also give rise to sulphur-containing metabolites which will be reported separately.

MATERIALS AND METHODS

Bromocyclohexane was purchased from B.D.H.; bromocyclopentane, bromocycloheptane, cyclopentene, cycloheptene, the cyclohexane diols and β-glucuronidase from Koch-Light Laboratories Ltd.

Cyclopentene epoxide was prepared either by the method of Durbetaki[3] or that of Goodman *et al.*[4] The product had b.p. 99–101°, n_D^{20} 1·4380 in close agreement with the properties recorded in the literature[5] (b.p. 99–102°, n_D^{16} 1·4370). Cycloheptene epoxide was prepared by the same methods and had b.p. 80–81° at 50 mm, n_D^{20} 1·4617 (lit.[6] gives b.p. 77–78° at 50 mm, n_D^{25} 1·4620).

cis-Cyclopentane-1,2-diol prepared by the method of Owen and Smith[6] had b.p. 105–106° at 16 mm (lit.[6] b.p. 104° at 15 mm).

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[Federal Register: May 23, 1994]

Part II

Department of Health and Human Services

Food and Drug Administration

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21 CFR Parts 173 and 573

Secondary Direct Food Additives Permitted in Food for Human Consumption; Food Additives Permitted in Feed and Drinking Water of Animals; **Aminoglycoside** 3'-Phosphotransferase II; Final Rule
DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

21 CFR Parts 173 and 573

[Docket No. 93F-0232]

Secondary Direct Food Additives Permitted in Food for Human Consumption; Food Additives Permitted in Feed and Drinking Water of Animals; **Aminoglycoside** 3'-Phosphotransferase II

AGENCY: Food and Drug Administration, HHS.

ACTION: Final rule.

SUMMARY: The Food and Drug Administration (FDA) is amending the food additive regulations to provide for the safe use of **aminoglycoside** 3'-phosphotransferase II (APH(3')II) as a processing aid in the development of new varieties of tomato, oilseed rape, and cotton. APH(3')II is a protein encoded by the kanamycin resistance (kan<SUP>r) gene. This action is in response to a petition filed by Calgene, Inc.

DATES: Effective May 23, 1994; written objections and requests for a hearing by June 22, 1994.

ADDRESSES: Submit written objections to the Dockets Management Branch (HFA-305), Food and Drug Administration, rm. 1-23, 12420 Parklawn Dr., Rockville, MD 20857.

FOR FURTHER INFORMATION CONTACT: Nega Beru, Center for Food Safety and Applied Nutrition (HFS-206), Food and Drug Administration, 200 C St., SW., Washington, DC 20204, 202-254-9523.

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I. Introduction

A. Regulatory History

In accordance with 21 CFR 10.85, Calgene, Inc., submitted to FDA on November 26, 1990, a request for advisory opinion regarding whether the kan<SUP>r gene, a selectable marker, may be used in the production of genetically engineered tomato, cotton, and oilseed rape plants intended for human food and animal feed uses (kan<SUP>r Gene: Safety and use in the production of genetically engineered plants, Docket Number 90A-0416). In the Federal Register of May 1, 1991 (56 FR 20004), FDA announced that the request had been received and solicited comments from interested persons. The data submitted to the agency with the request for advisory opinion and the comments received were made available to the public at the Dockets Management Branch.

Subsequent to the submission of the request for advisory opinion, FDA published its ``Statement of Policy: Foods Derived From New Plant Varieties'' (the 1992 policy statement) in the Federal Register of May 29, 1992 (57 FR 22984). This policy statement clarified FDA's interpretation of the Federal Food, Drug, and Cosmetic Act (the act) with respect to human foods and animal feeds derived from new plant varieties, including plants developed by new methods of genetic modification such as recombinant deoxyribonucleic acid (DNA) techniques.

In the 1992 policy statement, FDA stated that the postmarket authority under section 402(a)(1) of the act (21 U.S.C. 342(a)(1)) would continue to be the primary legal tool for ensuring the safety of whole foods derived from genetically modified plants. FDA also noted that under the statutory definition of ``food additive'' in section 201(s) of the act (21 U.S.C. 321(s)), the transferred genetic material and the intended expression products could be subject to regulation as food additives, if such material or expression products were not generally recognized as safe (GRAS) (57 FR 22984 at 22990). FDA further stated that the agency would use its food additive authority to the extent necessary to ensure public health protection (such as when an intended expression product in a food differs significantly in structure, function, or composition from substances found currently in food) (57 FR 22984 at 22990).

The 1992 policy statement specifically discussed selectable markers that provide antibiotic resistance in product selection and development. With such markers, both the antibiotic resistance gene and the gene product, unless removed, are expected to be present in foods derived from such plants. FDA stated:

Selectable marker genes that produce enzymes that inactivate clinically useful antibiotics theoretically may reduce the therapeutic efficacy of the antibiotic when taken orally if the enzyme in the food inactivates the antibiotic. FDA believes that it will be important to evaluate such concerns with respect to commercial use of antibiotic resistance marker genes in food, especially those that will be widely used.

(See 57 FR 22984 at 22988.)

Subsequently, in January 1993, Calgene requested that FDA convert

its request for advisory opinion to a food additive petition under section 409 of the act. FDA then announced in the Federal Register of July 16, 1993 (58 FR 38429), that a food additive petition (FAP 3A4364) had been filed by Calgene, Inc., 1920 Fifth St., Davis, CA 95616, proposing that the food additive regulations be amended to provide for the safe use of APH(3')II as a processing aid in the development of new varieties of tomato, oilseed rape, and cotton.

After completing its review of the data submitted by Calgene, FDA convened a public meeting of its Food Advisory Committee on April 6 through 8, 1994, to undertake a scientific discussion of the agency's approach to evaluating the safety of whole foods produced by new biotechnologies; a genetically modified tomato developed by Calgene containing the kan<SUP>r gene served as an example and focus of the discussion. The membership of the standing committee was supplemented with temporary members and consultants to the committee, representing scientific disciplines appropriate to the evaluation of foods derived from new plant varieties developed using recombinant DNA techniques.

At the meeting, Calgene presented a summary of the data they considered adequate to show safety of the tomato, and FDA presented its evaluation of the data. The committee was asked to comment on the approach used by FDA to evaluate whole foods and specifically, on the approach used for the Calgene tomato (Ref. 1). During committee discussion of the Calgene and FDA presentations, the committee members generally expressed the view that the approach used by FDA to evaluate the safety of the tomato, including the safety of the kan<SUP>r gene, was appropriate and that all relevant scientific questions had been adequately addressed.

In regard to the use of the kan<SUP>r gene, Calgene and the agency presented, and the committee discussed, such issues as the potential allergenicity of APH(3')II and the potential for ingested APH(3')II to inactivate orally administered antibiotics. Most of the discussion concerning the kan<SUP>r gene focused on the potential transfer of the gene to microorganisms in the gastrointestinal (GI) tract or in the environment. In evaluating Calgene's food additive petition for the use of the kan<SUP>r gene product, APH(3')II, in the development of new varieties of tomato, oilseed rape, and cotton, FDA has considered the committee's discussions and recommendations on this subject, which are summarized in section III.B.3. of this document.

B. Scope of the Regulation

Having completed its evaluation and having considered the deliberations of the Food Advisory Committee, the agency is amending the food additive regulations to permit the use of APH(3')II in the development of genetically modified tomatoes, oilseed rape, and cotton intended for food use. Only the translation product of the kan<SUP>r gene, APH(3')II, and not the gene itself, is being regulated as a food additive. As the 1992 policy statement indicated, FDA does not anticipate that transferred genetic material (deoxyribonucleic acid (DNA)) would itself be regulated as a food additive (57 FR 22984 at 22990). DNA is present in the cells of all living organisms, including every plant and animal used for food by humans or animals, and is efficiently digested (Ref. 2). In this respect, the DNA that makes up the kan<SUP>r gene does not differ from any other DNA and does not itself pose a safety concern as a component of food.

This final rule is being promulgated after consideration of the issues relating to the safety of the use of APH(3')II in the selection

of transgenic plants. In addition, as noted above, because of the property of the kan<SUP>r gene to confer antibiotic resistance, the agency has considered the possibility that the gene might be transferred to other organisms (discussed in section III.B. of this document).

Potential safety issues specific to particular food products that contain the kan<SUP>r gene are not addressed by the agency in this document because such issues are beyond the scope of this rulemaking. For example, issues associated with other co-transferred DNA sequences, including other genes intended to impart specific traits, and issues related to potential genetic instability are not addressed because such issues will vary with specific products.

Developers of new plant varieties are responsible for addressing potential safety issues associated with specific food products resulting from the transfer of genetic materials and for ensuring the safety of the food products that they market. The policy statement contains a ``Guidance to Industry'' section (57 FR 22984 at 22991) that outlines an approach for the safety evaluation of foods derived from transgenic plants and suggests that the agency be consulted, as needed, to resolve critical issues.

As noted, issues related to genetic instability are not addressed because such issues are not unique to the kan<SUP>r gene but apply to any transferred genetic material irrespective of the transfer techniques used. Genetic instability could arise as a result of insertion of multiple copies of a given construct, especially if insertion occurs at multiple loci. Recombinations of the transferred DNA could cause deletions, duplications, or rearrangements within the plant genome (Ref. 3). Hence, in the 1992 policy statement, the agency noted that the genetic stability of a new plant variety is an important safety consideration and further stated that, ``Factors that favor stability include a minimum number of copies of the introduced genetic material, and insertion at a single site.'' (57 FR 22984 at 23004).

In developing new plant varieties, developers are therefore responsible for following good manufacturing and good agricultural practices to ensure that they have developed a genetically stable transgenic plant. As a practical matter, this would ordinarily include using such techniques as segregation and Southern blot analysis to ensure that new plant varieties chosen for development have the new genetic material inserted into a single locus and that the number of copies of inserted DNA at a given site is limited to the minimum sufficient to achieve the intended effect.

C. Determination of Safety

Under section 409(c)(3)(A) of the act, a food additive cannot be approved for a particular use unless a fair evaluation of the data available to FDA establishes that the additive is safe for that use. The concept of safety embodied in the Food Additives Amendment of 1958 is explained in the legislative history of the provision: ``Safety requires proof of a reasonable certainty that no harm will result from the proposed use of an additive. It does not--and cannot--require proof beyond any possible doubt that no harm will result under any conceivable circumstance.'' (H. Rept. 2284, 85th Cong., 2d sess. (1958)). FDA has incorporated this concept of safety into its food additive regulations. Under 21 CFR 170.3(i), a food additive is ``safe'' if ``there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended

conditions of use.''

The agency has reviewed the data and studies submitted in the request for advisory opinion, material that was submitted subsequent to the conversion of the request for advisory opinion to a food additive petition, the deliberations of the Food Advisory Committee that took place at the April 1994 meeting, as well as other information in its files. In addition, the agency has considered the comments that were received in response to the Federal Register notice announcing receipt of the request for advisory opinion. The comments are addressed in section IV. of this document. As discussed below, FDA has concluded, based upon its review, that the use of **aminoglycoside** 3'-phosphotransferase II is safe for use as a processing aid in the development of new varieties of tomato, oilseed rape, and cotton intended for food use.

II. Use of the kan<SUP>r Gene As a Selectable Marker in Transgenic Plants

A. Background

Developers have for many years used plant breeding techniques to introduce desirable genetic traits into new varieties that can be used in agriculture. Traditionally, breeders have relied on selection of mutants and on hybridization between different varieties of the same species to achieve this goal. More recently, recombinant DNA techniques (commonly referred to as ``genetic engineering'' techniques) have come into use to generate new plant varieties with desirable characteristics. Recombinant DNA techniques involve the isolation, and subsequent introduction into a host plant, of discrete DNA segments containing the gene(s) of interest. This introduction of exogenous DNA into a cell, resulting in its acquisition of a new phenotype, is commonly referred to as ``transformation,'' and transformed plants that contain genetic material derived from sources other than the host plant itself are called transgenic.

The desired gene(s) may be introduced into a host plant by one of several methods, including: (1) Direct DNA uptake by the plant cells mediated by chemical or electrical treatments; (2) microinjection of DNA directly into plant cells; (3) biolistics, or firing tiny particles coated with the DNA of interest into plant cells; and (4) the use of a bacterium, such as the soil bacterium Agrobacterium tumefaciens, as a vehicle to carry the DNA into plant cells. (For a discussion of these processes, see Ref. 4).

B. Need for a Selectable Marker

Transformation of plant cells by introducing exogenous DNA is an inefficient process and, in general, only a small proportion of cells will successfully take up, integrate, and express the new genetic material (Ref. 5). Further, the few cells that do so are not readily distinguishable from the vast majority of cells that do not. Therefore, developers of transgenic plants need a means to distinguish cells that are successfully transformed from those that are not. Selectable markers, such as the kan<SUP>r gene, perform this function.

The kan<SUP>r gene is linked to the gene (or genes) of interest and then this genetic material is inserted into plant cells. Because plant cells are sensitive to the antibiotic kanamycin, incorporation of the kan<SUP>r gene into cells and subsequent expression of APH(3')II

provides a convenient method for selecting successfully transformed cells. Kan<SUP>r works as a marker because only successfully transformed cells (which contain both the kan<SUP>r and the desired genetic material) survive when grown in a kanamycin-containing medium. These cells are subsequently regenerated into transgenic plants.

C. Identity of the Additive

APH(3')II<SUP>1 (CAS Reg. No. 58943-39-8) is encoded by the kan<SUP>r gene, which was originally isolated as a component of transposon Tn5<SUP>2 from the bacterium Escherichia coli (Refs. 6 and 7). APH(3')II is an enzyme with an apparent molecular weight of 25,000 that catalyzes the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of **aminoglycoside** antibiotics (see below), thereby inactivating the antibiotics.

\1\Other names for this enzyme include neomycin phosphotransferase II (NPT II), neomycin phosphotransferase, and kanamycin phosphotransferase II.

\2\A transposon is a segment of DNA that is mobile and has the capacity to move from one site in the genome to another. Transposons vary in size and frequently contain, as does Tn5, antibiotic resistance genes in addition to genes coding for functions concerned with movement of the transposon.

APH(3')II inactivates the **aminoglycoside** antibiotics neomycin, kanamycin, paromomycin, ribostamycin, gentamicins A and B, as well as butirosins (Refs. 8 and 9). Of the antibiotics that are inactivated by APH(3')II, only neomycin and kanamycin are currently approved for use in humans or animals in the United States (Refs. 10 and 11).<SUP>3

\3\Gentamicin, which is used therapeutically, is composed of a complex mixture of the antibiotic substances produced by Micromonospora purpurea that contain primarily gentamicin C<INF>1 (25 to 50 percent), gentamicin C<INF>1a (10 to 35 percent), and gentamicins C<INF>2a and C<INF>2 (25 to 55 percent) (Ref. 10). Gentamicins A and B are at most minor components of the commercial drug. Thus, APH(3')II does not confer resistance to gentamicin that is used therapeutically (Ref. 12).

The APH(3')II evaluated in this document is the enzyme whose synthesis is directed by the kan<SUP>r gene derived from transposon Tn5. This enzyme is not to be confused with enzymes that may be similarly named (e.g., a type I **aminoglycoside** phosphotransferase encoded by a gene isolated from transposon Tn601) or other bacterial enzymes (including acetyltransferases, nucleotidyltransferases, and phosphotransferases) that inactivate kanamycin and neomycin (Refs. 8 and 12).

D. Use and Intended Technical Effects

Aminoglycoside antibiotics exert their effect on bacteria by binding to bacterial ribosomes and inhibiting protein synthesis. Phosphorylation of the antibiotics by APH(3')II interferes with this binding and thus prevents the antibiotics from inhibiting protein synthesis (Ref. 13). In this way, cells that contain the kan<SUP>r gene and that express APH(3')II are rendered resistant to the action of the antibiotics. In plant cells, the antibiotics exert their effect on mitochondria and chloroplasts where protein synthesis takes place on ribosomes that resemble bacterial ribosomes (Ref. 14).

The proposed use of the kan<SUP>r gene and gene product APH(3')II is as a processing aid in the development of new varieties of tomato, cotton, and oilseed rape intended for food use. As discussed above, because transformation of plant cells is an inefficient process, the presence of APH(3')II and the consequent ability of the plant cells to grow in the presence of antibiotics is used to distinguish between transformed and nontransformed cells. Therefore, the intended technical effect of APH(3')II is to permit, in the early phases of development of genetically modified plants, the selection of transformants carrying the kan<SUP>r gene along with the genetic material of interest. However, APH(3')II has no intended technical effect in the final plant or final crop product.

III. Safety Evaluation

A. APH(3')II

Safety issues associated with APH(3')II can be divided into two areas: (1) Those associated with the direct effects of ingestion of the protein, including the possibility of allergenicity; and (2) those associated with the biological activity of APH(3')II (i.e., the effect of the enzyme on the therapeutic efficacy of orally administered antibiotics).

1. Direct Effects of Ingestion

Calgene provided evidence that APH(3')II is rapidly inactivated by stomach acid, is degraded by digestive enzymes, and is not modified by glycosylation (i.e., does not contain sugar molecules attached to the protein) when produced in the transgenic plants under consideration. In addition, Calgene noted that enzymes such as APH(3')II are heat labile. Thus, Calgene concluded that APH(3')II does not possess any of the characteristics associated with allergenic proteins such as proteolytic stability, glycosylation, or heat stability (Ref. 15). In April 1992, Calgene also conducted protein and DNA sequence comparisons using sequences in four separate databases (GenBank, EMBL, PIR 29, and Swiss-Prot) and established that APH(3')II does not have significant homology to any proteins listed as food allergens or toxins in these databases.

FDA agrees with Calgene that the characteristics of APH(3')II do not raise a safety concern. First, each whole food, on average, contains several thousands of different proteins (Ref. 16). As a class, proteins are rarely toxic (Ref. 17) and APH(3')II is not known to be toxic. Second, APH(3')II is a phosphorylating enzyme, and all plants and animals that are part of the food supply contain such phosphorylating enzymes without adverse consequences. Third, APH(3')II has been shown to be rapidly degraded under simulated gastric conditions (Refs. 18 through 21). Finally, the estimated dietary exposure to APH(3')II is very low (480 <greek-m>g APH(3')II per person

per day,<SUP>4 or 0.16 part per million in the diet, based on a 100-percent market share for tomatoes containing APH(3')II (Ref. 18)).

\4\Because oils produced from transgenic cottonseed and rapeseed would not contribute APH(3')II to the human diet (see also section 2 below), the exposure estimate was derived exclusively for tomatoes. The agency made several conservative assumptions in arriving at the probable per capita exposure to APH(3')II of 480 <greek-m>g/person/day. For example, FDA assumed that all tomatoes contain APH(3')II at a level of 0.1 percent of total protein although, of the two lines intended for commercialization by Calgene, one contains less than 0.01 percent and the other less than 0.002 percent of APH(3')II (as a percentage of total protein). Second, FDA included APH(3')II in processed products in its estimate although high temperature treatment used in the production of processed products would be expected to result in loss of enzymatic activity of APH(3')II. In summary, the exposure estimate represents a theoretical maximum rather than a realistic estimate of exposure to APH(3')II.

Based upon the available evidence, the agency believes that this protein does not possess any properties that would distinguish it toxicologically from other phosphorylating enzymes in the food supply. Further, because of the low exposure levels and normal digestibility of APH(3')II, the agency concludes that no limits other than good manufacturing practice are needed to ensure the safety of the petitioned use of APH(3')II (Ref. 20).<SUP>5

\5\A recently published study (Ref. 22) also showed that APH(3')II is rapidly degraded under simulated mammalian digestive conditions. In addition, in an acute mouse feeding study, the investigations showed that feeding highly exaggerated doses of purified APH(3')II caused no deleterious effects.

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2. Effects on the Therapeutic Efficacy of Orally Administered Antibiotics

a. APH(3')II in human foods. i. Relevant source of APH(3')II. Calgene considered whether APH(3')II could affect the therapeutic efficacy of orally administered **aminoglycoside** antibiotics. In doing so, Calgene stated that only APH(3')II from fresh tomatoes is relevant because it is the only form that is enzymatically active. Processed tomato products (such as processed whole tomatoes, chili, juice, pulp, paste, catsup, and soup) are subjected to temperatures in the range of 82 to 100 deg.C; these temperatures would be expected to inactivate the APH(3')II enzyme. For edible oils extracted from cottonseed and rapeseed, high temperature treatment, solvent extraction, and subsequent purification steps generally included in the processing of such oils would also be expected to inactivate APH(3')II.

FDA agrees that high temperature treatment denatures proteins and inactivates enzymes and therefore, processed products that contain

tomatoes with the kan<SUP>r gene are unlikely to contain any enzymatically active APH(3')II. In addition, purified oils essentially do not contain protein; therefore, oils derived from transgenic cottonseed and rapeseed modified using the kan<SUP>r gene would not be expected to contain active or inactive APH(3')II (Refs. 18 and 23). Thus, FDA agrees that fresh tomatoes from plants developed using the kan<SUP>r gene are the only source of active APH(3')II.

ii. Effect of APH(3')II in fresh tomatoes on the therapeutic efficacy of orally administered antibiotics. Calgene performed several experiments intended to address whether APH(3')II consumed as a component of fresh tomatoes could render orally-administered kanamycin ineffective. These experiments were performed under simulated gastric and intestinal conditions (i.e., appropriate pH, reagent concentrations, temperature, and reaction times) chosen to reflect conditions expected in vivo. In some studies both tomato extract and nonfat milk were added to determine whether the presence of additional food-source proteins in the simulated gastric and intestinal fluids might slow the proteolytic degradation of APH(3')II by competition. After evaluating the loss of immunologically detectable APH(3')II, Calgene concluded that, under normal gastric and intestinal conditions, APH(3')II would be effectively degraded before the enzyme could inactivate kanamycin or neomycin and therefore, APH(3')II would not interfere with orally administered kanamycin or neomycin therapy. The results of Calgene's experiments were the same whether done in the presence or the absence of tomato extract and nonfat milk.

In addition, Calgene presented the results of in vitro degradation studies performed under simulated abnormal gastric conditions, such as may exist in patients treated with drugs that reduce stomach acidity. Calgene stated that these studies demonstrated that APH(3')II is not degraded in neutralized (pH 7.0) simulated gastric fluid and thus, APH(3')II may remain active in such abnormal gastric conditions. However, Calgene pointed out that, even under those conditions, APH(3')II would not be expected to inactivate orally administered kanamycin or neomycin because the concentration of ATP, which the enzyme requires to inactivate kanamycin and neomycin, would be limiting. In support of this contention, Calgene presented data from the published literature on ATP levels in fresh fruits and vegetables. Calgene then estimated ATP intake and calculated the fraction of neomycin that would be phosphorylated assuming that all of the available ATP reacted with the antibiotic. Under the worst-case situation (high intake of ATP-containing food, low dose of antibiotic) Calgene's calculations showed that only a small fraction (no more than 1.5 percent) of the antibiotic would be inactivated. Moreover, Calgene presented data that showed that no significant inactivation of kanamycin was observed during in vitro studies conducted with tomato extract containing APH(3')II and kanamycin over a 4-hour incubation period.

iii. Agency conclusions. The agency has evaluated the data and other information presented by Calgene (Refs. 18 through 21 and 24). FDA agrees that Calgene's in vitro digestion studies show that, as is the case for dietary protein in general, the biological activity of APH(3')II is destroyed during gastric and intestinal phases of digestion. Further, the agency has determined that any active APH(3')II that might remain would not significantly inactivate kanamycin or neomycin in the gut because the small amount of ATP in fruits and vegetables would limit the amount of antibiotic that could be phosphorylated. ATP is an extremely labile molecule that is susceptible

to inactivation both by heat (e.g., cooking) and by enzymes, such as alkaline phosphatases (Ref. 25), that are found in the intestine. Because the ATP in meat, poultry, fish, and cooked vegetables would be broken down by cooking, the primary source of ATP in the gastrointestinal (GI) tract of patients would be uncooked fruits and vegetables. However, the amount of ATP in a variety of fruits and vegetables would provide enough ATP to inactivate only a small percentage of kanamycin or neomycin, even if one makes the conservative assumption that all of the ATP in these fruits and vegetables would survive the alkaline phosphatases in the intestines and would be available for catalytic phosphorylation of kanamycin or neomycin.

In addition, the agency has considered the patient population likely to be exposed to **aminoglycoside** antibiotics. Oral aminoglycosides are most commonly administered to either pre-operative patients (prior to bowel surgery) or patients with hepatic encephalopathy. Neither patient population would be expected to be ingesting tomatoes or any other fresh fruits and vegetables; therefore there is little or no risk of inactivating the oral antibiotic in these patients (Refs. 24 and 26). For these reasons, FDA concludes that the presence of APH(3')II in food will not compromise the therapeutic use of orally administered kanamycin or neomycin.

b. APH(3')II in animal feed. Calgene also considered the potential inactivation of neomycin that is used in animal feeds manufactured using cottonseed meal and rapeseed meal obtained from transgenic plants. The transgenic tomato was not considered because only small amounts of tomato and tomato byproducts are used in the animal feed industry. Further, neomycin is primarily used to treat calves and swine whereas tomato byproducts, to the extent that they are used in animal feed, are primarily used as ingredients in cattle diets (Ref. 27).

Calgene analyzed neomycin levels both in nontransgenic medicated cottonseed and rapeseed meals and in transgenic medicated cottonseed and rapeseed meals over a storage period of 56 days (considered a worst-case situation) and concluded that there was no significant inactivation of neomycin.

FDA reviewed the data submitted by Calgene and concludes that there was no significant difference with respect to neomycin stability between medicated cottonseed and rapeseed meals prepared from transgenic cottonseed and rapeseed containing APH(3')II, and appropriate controls (Ref. 28). Therefore, the agency concludes that transgenic strains of cottonseed and rapeseed containing APH(3')II have no apparent untoward effect regarding the stability of neomycin and that the therapeutic efficacy of neomycin in animal feed will not be affected. The agency also considers this conclusion applicable to other **aminoglycoside** antibiotics, e.g., gentamicin, when orally administered.

B. The Kan<SUP>r Gene

The agency also evaluated issues relevant specifically to the safety of the use of the kan<SUP>r gene in tomato, oilseed rape, and cotton. In particular, FDA evaluated the potential for horizontal transfer of the gene and subsequent expansion of the population of antibiotic-resistant pathogens. The agency evaluated whether efficacy of oral antibiotic treatment of humans or animals could be compromised by consumption of food containing the kan<SUP>r gene either because of the development of resistant intestinal microflora in humans and animals or because the cells lining the intestinal lumen might become transformed. In addition, the agency considered the possible transfer

of the kan<SUP>r gene from transgenic plants to soil microorganisms and expansion of the antibiotic-resistant bacterial population.

1. Potential Transfer of the kan<SUP>r Gene to Intestinal Microorganisms and Cells Lining the Intestinal Lumen

Calgene presented theoretical and experimental evidence to demonstrate that the potential for compromise of antibiotic therapy by horizontal transfer of the kan<SUP>r gene to gut microorganisms or intestinal epithelial cells is not of significant concern. Calgene considered the sources of the kan<SUP>r gene, the role digestion plays in degrading DNA, and possible DNA transfer mechanisms.

a. Relevant source of the kan<SUP>r gene available for transformation. Calgene considered potential transfer of the kan<SUP>r gene only from fresh tomatoes because processing is expected to inactivate the kan<SUP>r gene in processed tomato products and in food products derived from cotton and oilseed rape. The kan<SUP>r gene is not expected to survive procedures used to process tomatoes because heating processes, such as those used in commercial processing, can directly degrade DNA or can damage DNA by releasing cellular DNA-degrading enzymes.

The kan<SUP>r gene is also not expected to survive the process of oil production from cottonseed and rapeseed. Mechanical grinding or flaking of oilseeds during the production of oils and meals from oilseeds is expected to liberate degradative enzymes normally present within the cell that would degrade the kan<SUP>r gene. In addition, oil processing also includes high temperatures and solvent extractions, both of which would be expected to inactivate the kan<SUP>r gene. Moreover, because DNA is hydrophilic, it is unlikely to fractionate into oil, which is hydrophobic, during the extraction of oil from cottonseed and rapeseed. Therefore, intact DNA, including the kan<SUP>r gene, is not expected to survive the production of oils and animal feeds from cottonseed and rapeseed.

b. Effect of digestion on the availability of the kan<SUP>r gene for possible transformation. Calgene demonstrated that most if not all of the DNA comprising the kan<SUP>r gene ingested by humans will be degraded in the stomach and upper small intestine before it reaches the lower small intestine, cecum, and colon, and would be unavailable for potential transformation of gut microorganisms. Calgene estimated that 99.9 percent of fresh tomato DNA would be digested to fragments smaller than 1,000 base pairs. This estimate was based on in vitro studies that found that only 0.1 percent of DNA could be detected as fragments of 1,000 base pairs or longer after exposure to stomach-simulating fluids for 10 minutes and to intestinal-simulating fluids for another 10 minutes. Thus most of the DNA remaining after digestion would be smaller than the kan<SUP>r gene which is about 1,000 base pairs long.

Regarding animal feed, food-producing animals consume primarily processed forms of cottonseed and rapeseed, in which, as discussed above, the kan<SUP>r gene is not expected to remain intact. In addition, researchers have shown that nucleic acids introduced into the rumens of calves, or incubated with calf, sheep, or cow rumen contents in vitro, were rapidly and completely degraded to nucleotides and nucleosides (Ref. 29).

c. Calculation of worst-case transformation frequencies. In its submission, Calgene addressed the potential for horizontal transfer of the kan<SUP>r gene. Natural transformation, i.e., the uptake and incorporation into the genome of free DNA, is known to occur in some bacterial species. This is the only possible mechanism by which intestinal microflora could take up free DNA (Ref. 30). However, none

of the species known to be present in the GI tract has been found capable of acquiring exogenous DNA by natural transformation. Nonetheless, to consider the worst-case scenario, Calgene assumed that all microbes in the intestine would be able to take up and incorporate exogenous DNA at a frequency found for certain species of the genus Streptococcus. Calgene noted that although the firm developed its transformation model for certain Streptococcus species, they are not aware of any information indicating that Streptococcus species found in the GI tract can be naturally transformed.

To undergo natural transformation, the recipient bacterium must be transformation-competent, i.e., ready to take up DNA. As noted, none of the bacterial species that occur in the GI tract is known to be capable of becoming transformation-competent. In addition, the genome of a recipient bacterium should contain DNA homologous to the incoming DNA (Refs. 31 and 32). Because the genomes of intestinal Streptococci or other intestinal bacteria are not expected to exhibit homology to the DNA constructs containing the kan<SUP>r gene<SUP>6, Calgene assumed that the kan<SUP>r gene could only undergo ``illegitimate'' recombination, a process that does not require significant DNA homology. Calgene noted that illegitimate recombination occurs in microorganisms at a much lower rate than homologous recombination.

\\6One population that does contain DNA segments homologous with part of the kan<SUP>r construct is E. coli, because the kan<SUP>r construct contains part of an E. coli gene. Although E. coli constitutes one of the predominant species of aerobic GI tract bacteria, E. coli is not transformation-competent under conditions that prevail in the GI tract (Ref. 33). Thus, transformation of E. coli due to homologous recombination is not an issue.

Under the foregoing worst-case assumptions, Calgene estimated that if a person consumes fresh tomatoes at the 90th percentile level (i.e., eats more tomatoes than 89 percent of the individuals in the population), the transformation frequency of the intestinal microorganisms with the kan<SUP>r gene will be approximately 3 x 10<SUP>-15 transformants per day. This transformation frequency is more than 5 orders of magnitude less than the frequency of mutation to kanamycin resistance per bacterial replication, i.e., 10<SUP>-9 (Ref. 12). Thus, Calgene showed that for every 300,000 bacteria that mutate to kanamycin resistance per replication (generally a matter of hours), there would be, at most, under worst-case conditions, one kanamycin-resistant bacterium per day added to that number due to transformation.

Calgene stated that the potential for food-producing animals to experience decreased efficacy of antibiotic therapy as a result of pathogenic intestinal microflora incorporating and expressing the kan<SUP>r gene would be similar to that described for humans, i.e., equally improbable. In reaching this conclusion, Calgene relied on the finding that DNA is rapidly and completely digested in the gut of food animals (Ref. 29) and on the contention that the worst-case transformation scenario described above for human gut microorganisms also applies to microorganisms found in the gut of food-producing animals.

With respect to epithelial cells lining the intestinal lumen,

Calgene provided information that no transformation of human epithelial cells has been demonstrated in vivo (Ref. 2). In addition, even if transformed, intestinal epithelial cells are terminally differentiated (i.e., do not divide) and have a relatively short life span (Ref. 34), and thus would continually be shed and replaced by nontransformed cells.

2. Potential Transfer of the kan<SUP>r Gene to Soil Microorganisms

Calgene also considered the possibility that the kan<SUP>r gene might be transferred to soil microorganisms, thereby increasing the level of antibiotic-resistant organisms in the environment. Calgene pointed out that the only plausible mechanism by which gene transfer could occur between plants and bacteria is through natural transformation. Taking this mechanism into consideration and using worst-case assumptions similar to those discussed above for intestinal microorganisms, Calgene calculated that, at worst, kanamycin-resistant transformants resulting from plant DNA left in the fields would represent not more than one in 10 million of the existing kanamycin-resistant soil population.

3. Food Advisory Committee Discussions Regarding Potential Horizontal Transfer of the Kan<SUP>r Gene

As part of its discussion of the scientific issues related to the evaluation of Calgene's genetically engineered tomato, the Food Advisory Committee discussed the possibility that the kan<SUP>r gene might be transferred to microorganisms in the GI tract and in the environment (Ref. 1).

The committee members concluded that transfer of the kan<SUP>r gene consumed as a component of tomatoes to microorganisms in the GI tract was highly unlikely based on published data in the scientific literature. Similarly, the committee members judged that the potential for transfer of the kan<SUP>r gene from plants to microorganisms in the environment is highly unlikely based on the members' knowledge of mechanisms of gene transfer. In addition, members of the committee pointed out that the rate at which such transfer could take place, if at all, was of so small a magnitude that, coupled with the high prevalence of kanamycin resistant organisms already present in the environment, it would not cause a significant environmental impact.

Some members of the committee, while convinced by the information presented at the meeting that the transfer of the kan<SUP>r gene from tomato plants to microorganisms in the soil was improbable, expressed concern regarding the use of the kan<SUP>r gene in other crops that may be grown on a wide scale. In addition, some committee members were concerned that a determination of safety with regard to the use of kan<SUP>r gene in Calgene's tomato might signal to producers that it is now permissible to use the kan<SUP>r gene in other crops. In light of such concerns, these committee members advised that use of the kan<SUP>r gene in other crops should be evaluated on a case-by-case basis.

4. Agency Conclusions

The agency has considered the recommendations of the members of the Food Advisory Committee. The agency agrees that the potential transfer of the kan<SUP>r gene, as well as other antibiotic resistance marker genes, from crops to microorganisms should be evaluated on a case-by-case basis. As noted, Calgene petitioned for the use of the kan<SUP>r gene product, APH(3')II, in the development of genetically engineered cotton and oilseed rape in addition to tomato. As discussed below, the agency has evaluated data and information concerning horizontal transfer of the kan<SUP>r gene from its use in all three crops. This is

consistent with the committee's advice that safety of the use of the kan<SUP>r gene be evaluated on a case-by-case basis. In addition, Calgene's petition seeks to amend the food additive regulations to permit the use of APH(3')II only in tomato, cotton, and oilseed rape; approval of Calgene's petition would not mean that developers could use the kan<SUP>r gene in crops other than those identified in the petition.

FDA has also evaluated the information submitted by Calgene and has determined that the probability of transfer of the kan<SUP>r gene to gut microflora is remote and that even under worst-case conditions, the number of microorganisms that would be converted to kanamycin resistance is negligible when compared to the reported prevalence of gut microflora that are already resistant to kanamycin (Ref. 35). This conclusion applies to both humans and animals. The agency has determined that exposure to foods that contain the kan<SUP>r gene will not compromise the efficacy of antibiotic treatment because the likelihood of increasing the number of antibiotic resistant microorganisms is extremely low. Further, the agency has determined that there is no evidence that free DNA containing the kan<SUP>r gene, even if present, can transform cells lining the GI tract (Ref. 2).

FDA has also evaluated the information submitted by Calgene concerning soil microorganisms and agrees with Calgene that there would be no increase in kanamycin-resistant soil microorganisms because it is highly unlikely that the kan<SUP>r gene could move from the plant genome into soil microorganisms via horizontal gene transfer. Further, the agency has determined that, even if such transfer could occur, the rate at which it could occur is such that it would not result in a detectable increase over the existing background population of kanamycin-resistant bacteria (Ref. 36). Based on the foregoing, FDA has concluded that the use of the kan<SUP>r gene does not pose safety concerns in terms of increase in the population of antibiotic-resistant pathogens due to the potential for horizontal transfer of the gene.

IV. Response to Comments

FDA received 47 comments on Calgene's request for an advisory opinion on the use of the kan<SUP>r gene in the development of new varieties of tomato, oilseed rape, and cotton plants. Comments were received from members of academia, industry and industry-related organizations, State and Federal agencies, environmental groups and other nonprofit organizations, and individual consumers. Additionally, several comments on the agency's 1992 policy statement addressed the use of the kan<SUP>r gene.

Most of the comments supported the use of the kan<SUP>r gene in crop development, stating that there were no health or environmental issues precluding its use. Several comments expressed opinions on a wide range of issues including regulatory approaches for genetically engineered foods, concerns relating to human and animal food safety, and to the environmental effects of the kan<SUP>r gene, and whether foods containing the kan<SUP>r gene and APH(3')II should be specially labeled.

A. Regulatory Issues

Some comments stated that it was not appropriate for FDA to evaluate the safety of the kan<SUP>r gene and APH(3')II under an advisory opinion and that the kan<SUP>r gene and APH(3')II should be

treated as food additives by FDA. FDA has discussed above the basis for its decision not to regulate the DNA that makes up the kan<SUP>r gene itself as a food additive. Further, in light of Calgene's conversion of its request for advisory opinion on the use of the kan<SUP>r gene to a food additive petition, the comment concerning the regulation of APH(3')II as a food additive no longer requires a response.

B. Food Safety

Several comments stated that the presence in food of APH(3')II raised no food safety concerns whatsoever. Others questioned whether Calgene had supplied adequate data to ensure the safety of the kan<SUP>r gene and gene product, APH(3')II, when present in food. The substantive questions raised are discussed in sections IV.B.1 through 5 of this document.

1. Glycosylation

Two comments stated that APH(3')II might be glycosylated (i.e., might contain sugar molecules attached to the protein via the amino acid asparagine (N-linked) or via the amino acids serine, threonine, or hydroxyproline (O-linked)) when produced in tomatoes or other plants and, therefore, might become a food allergen. One of the comments asserted that for this reason, Calgene should be required to test whether APH(3')II is glycosylated. The comments, however, did not provide any information showing that glycosylated APH(3')II is likely to be, or is, allergenic.

At this time, FDA is unaware of any practical method to predict or assess the potential for new proteins in food to induce allergenicity. Although many food allergens that have been characterized at a structural level are glycosylated (Ref. 37), the agency is not aware of any information on structural or other properties of glycosylated proteins that would be predictive of their allergenicity. As noted, the comments did not provide such information. Moreover, glycosylated proteins are widespread in food. For these reasons, glycosylation is not a useful positive predictor of a potential allergenic effect. Accordingly, FDA did not request that Calgene determine whether APH(3')II is glycosylated.

Nevertheless, in a submission dated October 24, 1991, entitled ``Response to Public Comments,'' Calgene addressed whether APH(3')II is likely to be glycosylated and concluded that it is not. Calgene noted that APH(3')II lacks the amino terminal sequence of amino acids (commonly referred to as a ``signal peptide'') that is necessary to direct the protein into the cellular compartments where glycosylation occurs. Calgene also asserted that the unchanged molecular weight of APH(3')II in plants (relative to the molecular weight of bacterial APH(3')II, which is not glycosylated) supports the conclusion that APH(3')II is not glycosylated in plants. Finally, Calgene stated that the amino acid sequence (asparagine-X-serine/threonine) that is required to direct N-linked glycosylation to specific asparagine moieties is not present in APH(3')II. (Calgene noted that a corresponding argument for the lack of the appropriate amino acid sequence to direct O-linked glycosylation cannot be made because the sequences that direct O-linked glycosylation have not been defined.)

FDA has considered the information and arguments submitted in the comments and Calgene's response and has concluded that the available evidence indicates that APH(3')II is not glycosylated in plants. However, even if glycosylation had been demonstrated, FDA emphasizes that glycosylation alone does not necessarily establish that APH(3')II

is likely to produce an allergenic response because the positive predictive value of glycosylation with respect to the potential for inducing allergenicity has not been demonstrated.

2. In Vitro Digestibility Studies

In its original submission, Calgene presented the results of in vitro digestibility studies that demonstrated that APH(3')II enzymatic activity is rapidly decreased in simulated gastric fluid and in simulated intestinal fluid.

One comment asserted that Calgene should provide a more thorough study of degradation of APH(3')II in the digestive tract because the conditions of the in vitro digestibility study submitted by Calgene did not fully mimic the complex environments of the human gut. The comment further asserted that it was not clear whether the digestibility data also apply to neonates and to people with coeliac disorders or ulcers who can absorb peptides and intact proteins through their intestines. The comment noted that the applicability of the data to neonates would be of special importance should kan<SUP>r be used in soybeans because soy protein is a major component of some infant formulas. Importantly, however, the comment presented no information to provide a basis for concluding that the absorption of APH(3')II occurs, or that if it does, such absorption presents a health concern greater than that posed by the absorption of any other protein in the diet.

As discussed above, FDA has evaluated the studies presented by Calgene to demonstrate the normal digestibility of the enzyme and concurs with Calgene's conclusion that APH(3')II is rapidly degraded under normal conditions in the GI tract. Therefore, FDA believes that the intestinal transfer of intact or large fragments of APH(3')II is not likely to occur in individuals with normal GI tracts.

In regard to the possibility of increased intestinal absorption of proteins in neonates and individuals with special conditions (e.g., ulcers), FDA has concluded that there is no reason to expect that absorption of the intact or partially digested APH(3')II protein would present a safety problem different from absorption of any other protein in the diet. As discussed above, proteins, as a class, are rarely toxic. Furthermore, APH(3')II is a phosphorylating enzyme and does not contain any properties that would distinguish it toxicologically from any other phosphorylating enzymes that historically have been part of the food supply without adverse consequences. Finally, because Calgene did not petition FDA for the use of APH(3')II in soybeans, it is not necessary to address the comment concerning the applicability of Calgene's digestibility data to neonates fed soybean-derived formulas.

3. Copy Number of the kan<SUP>r Gene and Expression Level of APH(3')II

In its submission of November 26, 1990, Calgene stated that it did not intend to commercialize lines that contained more than 10 copies of the kan<SUP>r gene. In addition, Calgene also declared that, in tomatoes, the APH(3')II level would be no more than 0.1 percent of the total protein of the tomato and that processing procedures would destroy APH(3')II in processed tomatoes and edible oils extracted from cottonseed and rapeseed.

One comment asserted that Calgene inadequately described the methods by which it would ensure that no lines with greater than 10 copies of the kan<SUP>r gene would be marketed. The comment further asserted that many of the analyses offered by Calgene to prove the safety of the kan<SUP>r gene depend on estimates of the number of genes per cell and that, if the company cannot ensure this relatively low level of gene incorporation, many of its safety arguments are undermined. The comment, however, did not identify which of Calgene's

safety analyses depended on estimates of the numbers of genes per cell.

The comment may have been referring to Calgene's assumption that each plant cell would contain 10 copies of the gene when it calculated a worst-case frequency of transformation of microorganisms with the kan<SUP>r gene that would result from use of the gene in transgenic plants. However, the agency notes that the outcome of those calculations, i.e., Calgene's conclusion that the transformation frequency of microorganisms with the kan<SUP>r gene is insignificant, would not change had Calgene assumed much higher gene copy numbers in its calculations. Therefore, FDA's safety assessment does not depend on precise estimates of gene copy number. Nor does the comment provide a basis for concluding that it is necessary to have precise methods for ensuring that no plants with more than 10 copies of the gene will be marketed.

A second comment maintained that Calgene provided an inadequate description of the quality control and assurance procedures the company would use to ensure that APH(3')II would be kept to no more than 0.1 percent of total protein of the tomato, and that a number of the company's safety analyses rely on the amount of APH(3')II in the food. The comment, however, did not identify which of Calgene's safety analyses relied on estimates of the concentration of APH(3')II in the food.

FDA has determined that there is no need to set a tolerance for the amount of APH(3')II that will be consumed because the agency knows of no reason why this protein would have any properties that would distinguish it toxicologically from any other phosphorylating enzymes in the food supply. Also, as discussed above, APH(3')II will not affect efficacy of orally administered antibiotics because APH(3')II is rapidly digested under normal conditions in the GI tract, and even in abnormal gastric conditions where APH(3')II may not be rapidly digested, the amount of ATP available in food would allow only a small proportion of kanamycin and neomycin to be inactivated. Therefore, the agency concludes that there is no need to require quality control and assurance procedures to ensure that the APH(3')II level will be no more than 0.1 percent of the total protein in commercial tomato varieties.

A third comment argued that Calgene did not provide data to establish that APH(3')II would not be present after tomato processing and after extraction of edible oils.

The agency's exposure estimates included an assumption that APH(3')II would be present in both processed tomatoes and fresh tomatoes even though the high temperatures involved in processing inactivate enzymes and therefore, processed tomato products are unlikely to contain enzymatically active APH(3')II (Ref. 18). In addition, well-established processing procedures used to extract edible oils from oilseed crops do not extract significant amounts of protein (Ref. 23). Therefore, exposure to APH(3')II obtained from rapeseed oil and cottonseed oil would be negligible (Ref. 18). The comment did not present any information to contradict FDA's analysis and conclusion on this point.

4. The Potential for Side Effects From Consumption of Genetically Engineered Foods

One comment asked whether there might be side effects from consumption of genetically engineered foods, and if so, whether these side effects would be short term or long term. Another comment noted that food plants and humans exhibit complex and unpredictable behavior and that therefore, the safety of a food substance should be based on thoughtfully gathered empirical evidence.

The comments did not point to any specific side effects of genetically engineered foods. FDA has evaluated the safety of APH(3')II and has determined that it is safe for its proposed use. This safety assessment is in fact based on empirical evidence, such as the structure and function of APH(3')II, the low level at which APH(3')II occurs in foods, the digestibility of APH(3')II, and the inability of APH(3')II to interfere with clinically useful antibiotics under usual conditions of use for the antibiotics.

5. Relevance of Clinical Studies

Several comments noted that a National Institutes of Health (NIH) gene therapy trial in which cancer patients were infused with cells containing the kan<SUP>r gene, and which was cited by Calgene as strong evidence for the safety of the kan<SUP>r gene, provides little information concerning the safety of the kan<SUP>r gene and APH(3')II in food. One comment also noted that the combination of data from the in vitro studies and the gene therapy study was an inadequate basis for a safety determination of the kan<SUP>r gene and APH(3')II in food that millions of people might eat.

In determining that APH(3')II is safe for its proposed food additive use, FDA did not rely on the NIH gene therapy trial. However, FDA does believe that the in vitro degradation data provide important information that should be and was considered by the agency as part of its overall safety assessment of the kan<SUP>r gene and APH(3')II, as discussed earlier in this document.

C. Possible Effect on Clinical Efficacy of Orally Administered Kanamycin or Neomycin

Several comments questioned whether the presence of APH(3')II in tomatoes or other foods might compromise the clinical efficacy of orally administered kanamycin or neomycin. One comment noted that Calgene claimed that at most only 76,800 people annually were administered kanamycin or neomycin orally, and argued that those people deserved not to be put at risk. The comment further requested that Calgene be required to perform animal studies on the effects of ingestion of APH(3')II on the efficacy of orally administered kanamycin and neomycin. The comment asserted that if APH(3')II were shown to compromise clinical efficacy of kanamycin or neomycin, food containing APH(3')II should be appropriately labeled.

Other comments observed that ingested APH(3')II would not impair the efficacy of orally administered kanamycin and neomycin, that these antibiotics are rarely administered orally, and that the kan<SUP>r gene is therefore a good choice as a selectable marker gene.

FDA agrees with Calgene that kanamycin and neomycin are rarely administered orally. The primary clinical role for orally administered neomycin, and to a lesser extent kanamycin, is cleansing the bowel of microbes prior to bowel surgery. This use is relatively minor because of severe side effects (auditory nerve damage and kidney damage) that may result from the antibiotic that is absorbed from the GI tract (Ref. 38).

As discussed above, for most individuals receiving oral kanamycin or neomycin, APH(3')II will be inactivated by the acidic environment of the stomach and degraded by the digestive enzymes present in the GI tract. More important, even for patients receiving simultaneous treatment to reduce stomach acidity, the amount of ATP available from food would allow, at most, only a small fraction of kanamycin or neomycin to be inactivated. The comment advocating animal studies did

not contradict directly or indirectly FDA's analysis concerning the inactivation and degradation of APH(3')II or the information concerning ATP levels. FDA has therefore determined that the presence of APH(3')II in food will not compromise therapy with orally administered kanamycin or neomycin. On this basis, FDA has concluded that neither animal studies on the effects of ingestion of APH(3')II on the efficacy of the antibiotics, nor special labeling of foods containing APH(3')II for patients receiving orally administered kanamycin or neomycin, are necessary.

D. Fate of the kan<SUP>r Gene in the Environment

1. Potential Transfer of the kan<SUP>r Gene From Crops to Microorganisms

One comment posited a connection between ``the prophylactic use of antibiotics [resulting] in antibiotic-resistant bacteria reaching the human population'' with a health risk from the possible addition of up to ``10 antibiotic genes [sic] in most of the cells of major crops.'' The comment agreed with Calgene's documentation that the widespread use of antibiotics has led to an increase in antibiotic-resistant bacteria in the environment, but went on to postulate that this was evidence that introducing antibiotic-resistance genes into plants has human health implications.

The comment further asserted that the ``scientific question is whether the resistance genes in the crops can be transferred by any mechanism [to] organisms that might be human pathogens,'' and that the company should be required experimentally to ``determine the rates of gene transfer to soil bacteria from plant debris, the persistence or selection of organisms containing such genes in soil ecosystems, and other important factors in the assessment of the likelihood of releases compromising the use of antibiotics.'' The comment noted that Calgene analyzed these issues ``in some detail,'' but with ``arm chair calculations, most based on extrapolations from experiments done with other organisms under other circumstances.''

A second comment noted that Calgene had supplied information that three kinds of bacteria, with and without plasmids<SUP>7 carrying antibiotic resistance genes, had little effect on several measures of soil ecosystems, but wrote that the ``relevance of experiments on bacteria to releases of plants is marginal, at best.'' A third comment asserted, without any supporting evidence, that ``genetic resistance to antibiotics in these plants could be transferred by plasmids to microorganisms in the soil and elsewhere in the food chain.''

\7\Plasmids are self-replicating units of DNA commonly found in bacteria and are responsible for transfer of antibiotic resistance between bacteria.

FDA agrees that increasing the number and prevalence of antibiotic-resistant microbes may have serious human health implications if those microbes are themselves pathogens of humans or domesticated animals, or share the same microenvironment as such pathogens. FDA considers the relevant scientific question to be whether there would be a meaningful increase in antibiotic-resistant pathogenic microbes in the human

environment due to transfer of the kan<SUP>r gene from plants to microbes. This issue was also the subject of considerable discussion at the April 1994 Food Advisory Committee meeting. As discussed in detail above, FDA has determined, based on the body of evidence presented by Calgene and based on the discussions of the Food Advisory Committee (Ref. 1), that the transfer of the kan<SUP>r gene from plants to microbes will not occur at a detectable frequency and overall will result in no significant increase in the numbers of antibiotic-resistant microbes. Regarding whether Calgene should be required to determine experimentally the rate of transfer, the agency notes that Calgene's calculations represent worst-case scenarios, and the agency believes it would not be useful to do experiments to attempt to measure that which is too small to measure.

Regarding the relevance of experiments on bacterial releases to the environment, FDA finds that information concerning the lack of an environmental effect from the release of microbes with and without antibiotic resistance genes is of limited direct relevance to the environmental effects of plants with antibiotic resistance genes. The agency did not rely on this information in reaching its determination that there will be no significant increase in the antibiotic-resistant microorganism population of the soil.

Finally the claim that the kan<SUP>r gene could be transferred from plants to bacteria by plasmids is without basis because there is no evidence that plasmids exist in plants.

2. Potential Transfer of the kan<SUP>r Gene to Other Crops and to Wild Relatives

Comments were also received on the potential transfer of the kan<SUP>r gene to other crops and wild relatives. These comments address environmental issues and do not bear on the safety of APH(3')II for its proposed food additive use and are therefore addressed in section VII. of this document.

E. Possible Effects of Consumption of Animal Feeds Containing APH(3')II on Animals and Their Gut Microflora

One comment argued that empirical evidence should be gathered to assess the potential effects of modified foods on animals and their gut microflora.

The agency is aware of no information that APH(3')II would affect animals or their gut microflora any differently than any other protein in the diet, nor did the comment provide such information. The comment may have been referring to the theoretical potential for APH(3')II in animal feed to affect efficacy of neomycin administered to animals, and the theoretical potential for the gut microflora to take up the kan<SUP>r gene and become resistant to neomycin. As discussed above, the likelihood of transfer of the kan<SUP>r gene to gut microflora of food animals is extremely remote. Also, as discussed above, FDA has evaluated the study presented by Calgene addressing the possibility of inactivation of neomycin by APH(3')II in animal feed and has concluded that the therapeutic efficacy of neomycin in animals would not be affected by consumption of feed containing transgenic cottonseed and rapeseed modified through the use of the kan<SUP>r gene.

F. Labeling of Foods Containing the Kan<SUP>r Gene and APH(3')II

One comment asserted that APH(3')II should be labeled as an ingredient. The comment further stated that, if FDA exempted APH(3')II

from ingredient labeling requirements (based on its classification as a processing aid that is present at insignificant levels in a finished food and has no technical or functional effect in that food), FDA should require special labeling if the ingestion of food containing APH(3')II could compromise the clinical efficacy of orally administered kanamycin or neomycin.

FDA's authority over food labeling is based on section 403 of the act (21 U.S.C. 343). Section 403(i) of the act requires that, in the case of foods fabricated from two or more ingredients, a food product bear on the label the common or usual name of each ingredient, unless compliance with the requirement for labeling is impracticable or results in deception or unfair competition. FDA considers an ``ingredient'' to be a substance used to fabricate (i.e., manufacture or produce) a food. FDA does not consider those substances that are inherent components of food to be ingredients that must be disclosed in the food's label.

A genetic substance introduced into a plant by breeding becomes an inherent part of the plant as well as of all foods derived from the plant. Consistent with FDA's general approach on ingredient labeling, the agency has not treated as an ingredient a new constituent of a plant introduced by breeding, regardless of the method used to develop the new plant variety. The comment provides no basis for FDA to deviate from its current practice in the case of APH(3')II.<SUP>8 Accordingly, FDA has determined that neither the kan<SUP>r gene nor APH(3')II is an ingredient that, under section 403(i) of the act, must be individually identified in labels of foods containing them.

\8\Furthermore, APH(3')II satisfies the definition of ``processing aid'' in Sec. 101.100(a)(3)(ii)(c) (21 CFR 101.100(a)(3)(ii)(c)) and will be regulated as such by this final rule. As the comment acknowledges, FDA's labeling regulations exempt processing aids like APH(3')II from the labeling requirements of section 403(i)(2) of the act. Thus, even if APH(3')II were properly considered an ingredient, its presence in a food would not be required to be disclosed in the food's labeling.

FDA has also determined that the presence of APH(3')II is not a material fact that must be disclosed in the labeling of foods that contain the enzyme. Under section 403(a)(1) of the act (21 U.S.C. 343(a)(1)), a food is misbranded if its labeling is false or misleading. Under section 201(n) of the act (21 U.S.C. 321(n)), labeling is misleading if it fails to reveal all facts that are ``* * * material with respect to consequences which may result from the use of the article * * *.'' As discussed at length above, FDA has determined that the ingestion of food containing APH(3')II will not compromise the clinical efficacy of orally administered kanamycin or neomycin. Because the consequences alleged in the comment--compromise of clinical efficacy--will not occur, the presence of APH(3')II is not a material fact requiring disclosure.

V. Conclusions

FDA has evaluated data in the petition and other relevant material

and concludes that the proposed use of APH(3')II as a processing aid in the development of new varieties of tomato, oilseed rape, and cotton is safe, and that 21 CFR parts 173 and 573 should be amended as set forth below.

VI. Inspection of Documents

In accordance with Secs. 171.1(h) and 571.1(h) (21 CFR 171.1(h) and 571.1(h)), the petition and the documents that FDA considered and relied upon in reaching its decision to approve the petition are available for inspection at the Center for Food Safety and Applied Nutrition by appointment with the information contact person listed above. As provided in 21 CFR 171.1(h) and 571.1(h), the agency will delete from the documents any materials that are not available for public disclosure before making the documents available for inspection.

VII. Environmental Impact

Calgene's initial submission requesting an advisory opinion regarding whether the kan<SUP>r gene may be used in the production of genetically engineered tomato, cotton, and oilseed rape plants included an environmental assessment (EA). The agency received comments on this EA. As noted earlier, the request for advisory opinion was later converted to a food additive petition at Calgene's request at which time Calgene submitted an updated EA. At the time the notice of filing was published in the Federal Register, FDA announced that the petitioner's EA was being made available to the public at the Dockets Management Branch (address above) and expressly solicited comments on the EA. No additional comments were received in response to this request for comments. The comments received on the original EA are discussed below.

One comment asserted that the kan<SUP>r gene could spread from tomato, cotton, and oilseed rape plants to other crops and related weeds by pollen flow when the kan<SUP>r gene-containing crops are grown near nontransgenic crops, and in locations where the kan<SUP>r-gene containing crops have wild relatives. The comment noted that transfer of the kan<SUP>r gene would create a problem if it were to make wild and weedy relatives more difficult to control.

The comment also criticized the Calgene submission for not addressing whether it is ``wise to contribute foreign genes to the gene pools of wild plants even where the plants do not become weeds or manifest other obviously harmful traits'' and stated that Calgene's submission ``too easily dismissed the problem of outcrossing from the engineered oilseed rape.'' The comment noted that oilseed rape has wild and weedy relatives with which it can breed, and that ``it is not sufficient to rely on traditional commercial control practices to control gene flow,'' but that the rate of gene flow must be experimentally determined and then ``controlled by procedures that are demonstrated, not assumed, to work.''

The agency has considered the potential for adverse environmental effects from the commercial use of cotton, tomato, and oilseed rape plants modified to contain the kan<SUP>r gene. The agency notes that it is possible for cotton and tomato plants to transfer the kan<SUP>r gene to neighboring plants of the same species via cross-pollination, although commercially grown cotton and tomatoes are primarily self-pollinating. Oilseed rape plants are also capable of pollinating sexually compatible wild relatives, although not all crosses with wild

relatives prove fertile. Importantly, however, introduction of the kan<SUP>r gene will not confer a competitive advantage upon a plant receiving it. That is, the gene will not enhance the plant's capacity to compete with other plants for available resources. In particular, there will be no selective pressure on plants containing the kan<SUP>r gene because kanamycin will not be present in the environment in sufficient concentrations to create such pressure. First, there are no specific therapeutic uses of kanamycin that would result in its widespread application to agricultural crops. Also, kanamycin does not accumulate in the environment from production by soil microbes or by land application of animal wastes (Ref. 36). Accordingly, FDA has concluded that transfer of the kan<SUP>r gene to other crops or related weeds will have no significant adverse environmental effects.

With regard to the comment about outcrossing from engineered oilseed rape, the comment provided no information to show that the transfer of the kan<SUP>r gene to wild or weedy relatives of oilseed rape will be any more frequent or have any greater significance than the transfer of other genes from cultivated oilseed rape. FDA is aware of no human health or environmental concern associated with such transfer. Therefore, the agency does not agree that the cultivation of kan<SUP>r-containing oilseed rape should be subject to control practices any different from those used traditionally.

The agency has carefully considered the potential environmental effects of this action, including those described in the comments discussed in this document. FDA has concluded that the action will not have a significant impact on the human environment and that an environmental impact statement is not required. The agency's finding of no significant impact and the evidence supporting that finding, contained in an environmental assessment, may be seen in the Dockets Management Branch between 9 a.m. and 4 p.m., Monday through Friday.

VIII. Objections

Any person who will be adversely affected by this regulation may at any time on or before June 22, 1994, file with the Dockets Management Branch (address above) written objections thereto. Each objection shall be separately numbered, and each numbered objection shall specify with particularity the provisions of the regulation to which objection is made and the grounds for the objection. Each numbered objection on which a hearing is requested shall specifically so state. Failure to request a hearing for any particular objection shall constitute a waiver of the right to a hearing on that objection. Each numbered objection for which a hearing is requested shall include a detailed description and analysis of the specific factual information intended to be presented in support of the objection in the event that a hearing is held. Failure to include such a description and analysis for any particular objection shall constitute a waiver of the right to a hearing on the objection. Three copies of all documents shall be submitted and shall be identified with the docket number found in brackets in the heading of this document. Any objections received in response to the regulation may be seen in the Dockets Management Branch between 9 a.m. and 4 p.m., Monday through Friday.

IX. References

The following references have been placed on display in the Dockets Management Branch (address above) and may be seen by interested persons

between 9 a.m. and 4 p.m., Monday through Friday.

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19. Memorandum from Z. Olempska-Beer, FDA, to J. Maryanski, FDA, July 14, 1992.
20. Memorandum from C.B. Johnson, FDA, to V. Zenger, FDA, September 7, 1993.
21. Memorandum from C.B. Johnson, FDA, to J. Maryanski, FDA, July 14, 1992.

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List of Subjects

21 CFR Part 173

Food additives.

21 CFR Part 573

Animal feeds, Food additives.

Therefore, under the Federal Food, Drug, and Cosmetic Act and under authority delegated to the Commissioner of Food and Drugs, 21 CFR parts 173 and 573 are amended as follows:

PART 173--SECONDARY DIRECT FOOD ADDITIVES PERMITTED IN FOOD FOR

HUMAN CONSUMPTION

1. The authority citation for 21 CFR part 173 continues to read as follows:

Authority: Secs. 201, 402, 409 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 321, 342, 348).

2. New Sec. 173.170 is added to subpart B to read as follows:

Sec. 173.170 **Aminoglycoside** 3'-phosphotransferase II.

The food additive **aminoglycoside** 3'-phosphotransferase II may be safely used in the development of genetically modified cotton, oilseed rape, and tomatoes in accordance with the following prescribed conditions:

(a) The food additive is the enzyme **aminoglycoside** 3'-phosphotransferase II (CAS Reg. No. 58943-39-8) which catalyzes the phosphorylation of certain **aminoglycoside** antibiotics, including kanamycin, neomycin, and gentamicin.

(b) **Aminoglycoside** 3'-phosphotransferase II is encoded by the kan<SUP>r gene originally isolated from transposon Tn<SUP>5 of the bacterium Escherichia coli.

(c) The level of the additive does not exceed the amount reasonably required for selection of plant cells carrying the kan<SUP>r gene along with the genetic material of interest.

PART 573--FOOD ADDITIVES PERMITTED IN FEED AND DRINKING WATER OF ANIMALS

3. The authority citation for 21 CFR part 573 continues to read as follows:

Authority: Secs. 201, 402, 409 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 321, 342, 348).

4. New Sec. 573.130 is added to subpart B to read as follows:

Sec. 573.130 **Aminoglycoside** 3'-phosphotransferase II.

The food additive **aminoglycoside** 3'-phosphotransferase II may be safely used in the development of genetically modified cotton, oilseed rape, and tomatoes in accordance with the following prescribed conditions:

(a) The food additive is the enzyme **aminoglycoside** 3'-phosphotransferase II (CAS Reg. No. 58943-39-8) which catalyzes the phosphorylation of certain **aminoglycoside** antibiotics, including kanamycin, neomycin, and gentamicin.

(b) **Aminoglycoside** 3'-phosphotransferase II is encoded by the kan<SUP>r gene originally isolated from transposon Tn5 of the bacterium Escherichia coli.

(c) The level of the additive does not exceed the amount reasonably required for selection of plant cells carrying the kan<SUP>r gene along with the genetic material of interest.

Dated: May 17, 1994.
Fred R. Shank,
Director, Center for Food Safety and Applied Nutrition.
Linda A. Suydam,
Interim Deputy Commissioner for Operations.
David A. Kessler,
Commissioner of Food and Drugs.
[FR Doc. 94-12492 Filed 5-18-94; 12:39 pm]
BILLING CODE 4160-01-P

Transgenic Research 1, 239–249 (1992)

REVIEW

Biosafety of kanamycin-resistant transgenic plants

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Kanamycin resistance is one of the most frequently used selection markers for obtaining transgenic plants. The introduction of these transgenic plants into agricultural practice will cause the kanamycin resistance gene and the gene product to be present on a large scale. The desirability of this situation is analysed. The nature, properties and applications of the antibiotic kanamycin are briefly reviewed, as are the mechanisms of kanamycin resistance. It is argued that the gene used for resistance is an excellent choice because of the high substrate specificity of the enzyme encoded. Human or veterinary antibiotic therapies will not be compromised. Also, the physico-chemical characteristics of the antibiotic exclude the existence of selective conditions in the environment. Therefore, a transgenic plant or any other organism that might have acquired the gene will not get any selective advantage because of this gene. Evidence further suggests there is no toxicity or predictable harm of both gene or gene product for human or animal consumption. Full legislative clearance of this transgenic trait is therefore acceptable.

Keywords: antibiotics; kanamycin resistance; neomycin; risk assessment; nutrition; veterinary efficacy

Introduction

The number of agronomically important crops that have become amenable to genetic modification by means of recombinant DNA techniques is increasing rapidly (Fraley, 1989; van Wordragen and Dons, 1992). Not hampered by classical crossing barriers and focused on individual genes, R-DNA techniques have an enormous potential to improve the crop of interest in a relatively precise way. Commercialization and, consequently, large scale application of the first transgenic crops seem nearby. However, there is general concern with respect to the relative safety of the transgenic plants involved. In the Netherlands, several field trials with transgenic plants have been ruined by environmental pressure groups, demonstrating that the apparent fear may even turn into violence. Due to the novelty of the transgenic approach, and because of the apparent concern, careful evaluation of the acquired characteristics of any transgenic plant is required. The methods to introduce foreign DNA in a plant cell, either by *Agrobacterium*, particle gun, microinjection or protoplast transformation, are relatively inefficient (Fraley, 1989). To enhance the efficiency of the procedures for obtaining transgenic plants, a selectable marker gene is cointroduced with the gene of interest. The selection gene allows the transgenic cell to proliferate in the presence of an antibiotic or a herbicide, thus ensuring convenient and easy selection of the few transformed cells in large populations of untransformed cells.

In 1990, Calgene submitted a 600 page document to the American Food and Drug Administration (FDA) to establish the biosafety of such a marker gene, the gene conferring resistance to the antibiotic kanamycin (Calgene, 1990). This report received favourable comments recently (Flavell *et al.*, 1992). The gene involved, *aphA2*, codes for the enzyme aminoglycoside-3'-phosphotransferase II (APH(3')II). The APH(3')II activity allows growth in medium containing the antibiotics kanamycin, neomycin and geneticin. As a consequence of the laboratory procedures, *aphA2* DNA and APH(3')II protein are present in most, if not all, cells of the resulting transgenic plant throughout its full life cycle.

In this article, the main points of the Calgene report are briefly reviewed with special reference to the situation in the Netherlands, and new data are added to further contribute to the safety assessment of the kanamycin resistance gene.

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Kanamycin: properties and applications

Kanamycin was first isolated in Japan in 1957 (Otten *et al.*, 1988). As a member of the sugar-containing antibiotics, the aminoglycosides, it is a tri-saccharide composed of a deoxystreptamine and two glucosamine units, chemically designated 3-D-glucosamine-2-deoxystreptamine-6-D-glucosamine (Fig. 1). The antibiotic is produced by the soil-borne actinomycete *Streptomyces kanamyceticus*. In industrial production, the culture filtrate of this organism contains several water-soluble oligosaccharides, among which are several forms of the antibiotic kanamycin. Fermentation has been adapted to produce mainly kanamycin A. Commercial preparations are 98% pure. Kanamycin sulphate is a colourless to yellowish powder that is not soluble in organic solvents, whereas its solubility in water depends on pH. Both the substance and aqueous solutions are very stable: the activity of a solution at pH 7 will not deteriorate more than 2% in 6 months, while autoclaving will decrease the biological activity by only 5%. These percentages, however, are significantly higher at other pH values. The related aminoglycoside antibiotic neomycin, that is also inactivated by APH(3')II, is a tetrasaccharide with chemical properties much like kanamycin (Kors, 1991). It is produced by the actinomycete *S. fradiae*.

Kanamycin is bactericidal for a broad spectrum of both Gram-positive and Gram-negative bacteria, while very low concentrations can have a bacteriostatic effect. The antibiotic interacts with the 30S and 50S subunits of ribosomes (Mouton, 1985; Davis, 1988), eliminating protein synthesis. Organisms that are (facultative) anaerobes tend to be less or not sensitive, probably because the electron transport required for the antibiotic to enter the cell is absent or less efficient (Bryan and Kwan, 1981).

In eukaryotic cells, the action of kanamycin is based upon the effect it exerts on mitochondria and chloroplasts (Kors, 1991), in which protein synthesis occurs on ribosomes that resemble bacterial ribosomes. In plant cells, impaired chloroplast protein synthesis results in chlorosis (Weide *et al.*, 1989). Little is known about the transport of kanamycin into the plastids of the eukaryotic cell. It is supposed that the eukaryotic membranes have a lower permeability for kanamycin than the prokaryote membrane. In plants both the cell membrane and the organelle membranes have to be passed, eukaryotes require 15–60 fold higher kanamycin concentrations for intracellular biological activity.

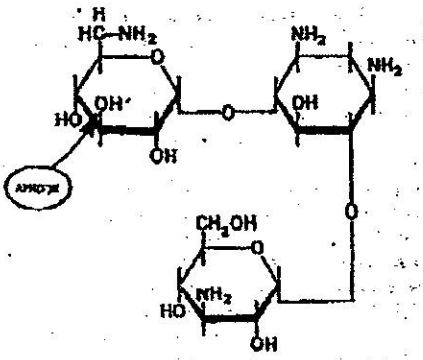


Fig. 1. Structure of the aminoglycoside antibiotic kanamycin A. The 3'-OH that is phosphorylated by APH(3')II is indicated by an arrow.

This difference in susceptibility is the basis for the clinical use of kanamycin and neomycin. Both chronic and acute toxicity is, however, considerable. The LD_{50} values (in mice) for intravenal application of kanamycin are 1450 mg kg^{-1} and for oral application 20.7 g kg^{-1} (Bodvari *et al.*, 1989). Neomycin is approximately ten times more toxic. The daily dose of kanamycin allowed in human prescription is 8 g per day, when administered over a period of 4 h (Reynolds, 1989). Both kanamycin and neomycin can cause deafness, and/or disorders of the kidneys or the nervous system. Although kanamycin has been used successfully against *Mycobacterium tuberculosis*, it has largely been replaced by third generation antibiotics (Siegenthaler *et al.*, 1986). Widespread bacterial resistance has limited the efficacy and use of this aminoglycoside antibiotic in human medicine (Pratt and Fekety, 1986). In the Netherlands, approximately 100 kg is used annually in human medicine, most of it as a constituent of liniments.

In veterinary medicine, however, kanamycin and especially neomycin are used in considerable amounts. These antibiotics are used to prevent and treat gastrointestinal infections and, in the case of cattle, mastitis. The addition of subtherapeutical amounts in animal food to stimulate growth is another vetedinary application of these antibiotics, which, however, is under debate. In the Netherlands, this application of kanamycin and neomycin is forbidden by law. Although production and sales figures are kept confidential, it is estimated that in the Netherlands annually approximately 20000 kg neomycin and 10000 kg kanamycin are used in veterinary medicine.

In the last decade, kanamycin resistance has become an indispensible tool in laboratories studying and exploiting the molecular genetics of bacteria, plants or animals (Sambrook *et al.*, 1989). In the Netherlands, approximately 1 kg kanamycin is used annually for these scientific applications.

Kanamycin resistance

Bacterial resistance to kanamycin can be caused by decreased permeability for and/or decreased accumulation of the antibiotic, so that the required intracellular

concentration is not reached (Vliegenthart, 1991). For the related aminoglycoside antibiotic streptomycin ribosomal resistance is known (Shannon and Phillips, 1982), but because kanamycin has several binding sites on the ribosomal subunits (Vliegenthart, 1991), resistance would only occur as result of a series of mutations in ribosomal proteins and has not been described.

The most important mechanism of bacterial resistance to kanamycin is the activity of aminoglycoside-modifying enzymes. In 1965, Okamoto and Suzuki (1965) demonstrated kanamycin inactivation by cell-free extracts of *Escherichia coli* strains carrying an R plasmid. Kanamycin can be inactivated by acetylation, phosphorylation and adenylation. Dependent upon the type of reaction, aminoglycoside-modifying enzymes are classified as aminoglycoside-*N*-acetyltransferase (AAC), aminoglycoside-*O*-phosphoryltransferase (APH) or aminoglycoside-*O*-nucleotidyltransferase (ANT). At present, over 27 different enzymes have been characterized, at least 18 of which inactivate kanamycin (Table 1). The relative occurrence of these enzymes shows local and temporal variations in populations. In Europe, AAC(3)-types are the enzymes observed most frequently (Vliegenthart, 1991).

The aminoglycoside-modifying enzymes are found in a variety of bacterial species and also in the antibiotics-producing actinomycetes (Trieu-Cuot *et al.*, 1987; Benviste and Davies, 1973), possibly to protect the producer against its own product (Demain *et al.*, 1974). In several cases, the genes coding for these

Table 1. Activities of aminoglycoside-modifying enzymes

Enzyme activity	*Protein*	*Gene*	*Resistance against*				
			kana	*neo*	*gen*	*ami*	*net*
phosphorylation							
	APH(3')I	*aphA1*	+	+	−	−	−
	APH(3')II	*aphA2*	+	+	−	−	−
	APH(3')III	*aphA3*	+	+	−	+/−	−
	APH(3')IV	*aphA4*	+	+	−	+/−	−
	APH(3')V	*aphA5*	+	+	−	−	−
	APH(3')VI	*aphA6*	+	+	+	+	−
	APH(3')		−	−	−	−	−
	APH(4)		−	−	−	−	−
	APH(5")		−	+	−	−	−
	APH(6)		−	−	−	−	−
	APH(2")/AAC(6')	*aphD*	+	−	+	+/−	+
adenylation							
	ANT(2")I		+	−	+	−	+/−
	ANT(2")II	*aadB*	+	−	+	−	+
	ANT(3",9)		−	−	−	−	−
	ANT(4',4")	*aadC*	+	+	−	+	−
	ANT(6)	*aadE*	−	−	−	−	−
	ANT(9)		−	−	−	−	−
acetylation							
	AAC(2')		−	+	+	−	+
	AAC(3)I	*aacC1*	−	−	+	−	+/−
	AAC(3)II	*aacC2*	+/−	−	+	−	+/−
	AAC(3)III	*aacC3*	+	+	+	−	+/−
	AAC(3)IV	*aacC4*	+/−	+	+	−	+
	AAC(6')I	*aacA1*	+	+/−	+/−	+	+
	AAC(6')II	*aacA2*	+	+	+/−	+	+
	AAC(6')IV	*aacA4*	+	+	+	−	+
	AAC(6')V	*aacA5*	+	+	+	+	+
	AAC(6')VI	*aacA6*	+	+/−	+/−	+	+
	AAC(6')/APH(2")	*aacA3*	+	−	+	+/−	+

Abbreviations: kana: kanamycin A, neo: neomycin B, gen: gentamycin C1a, ami: kanikacin, net: netilmycin. Data from Mouton (1985) and Vliegenthart (1991).

aminoglycoside-modifying enzymes have been isolated and characterized (Table 1). They can now be distinguished using PCR (Vliegenthart *et al.*, 1990). Most genes are located on plasmids and plasmid-encoded resistances contribute especially to their spread in bacterial populations (Stewart, 1989; Olson *et al.*, 1991).

The *aphA2* gene is most commonly used as a selectable marker in plants (Flavell *et al.*, 1992). This gene originates from transposon Tn5 from *E. coli* (Beck *et al.*, 1982) and codes for the enzyme APH(3')II, that catalyses the ATP-dependent phosphorylation of the 3'-hydroxy moiety of the aminohexose ring. Phosphorylated kanamycin is no longer able to interact with the 30S ribosomal subunit (Dickie *et al.*, 1978). In bacteria, the protein is present in the cytoplasm, probably associated with the cytoplasmic membrane (Matsuhashi *et al.*, 1976). The enzyme consists of 264 amino acids and has a high substrate specificity: only the aminoglycoside antibiotics kanamycin, neomycin and the 3'-OH-containing gentamycin-derivative geneticin (G-418) are inactivated, but not the newer aminoglycoside antibiotics, such as, for example, amikacin or netilmycin (Table 1). Although APH(3')II has been reported to modify the modern semi-synthetic kanamycin derivative amikacin (Courvalin and Davies, 1977), catalysis is sufficiently slow to prevent bacterial resistance.

Almost 10 years ago, the *aphA2* gene was shown to confer kanamycin resistance to plants, if supplied with promoter sequences active in plant cells (Bevan *et al.*, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983). Since then, the use of *aphA2* as dominant selectable marker gene in transgenic plants has boomed (Reynaerts *et al.*, 1988). In the remainder of this article, the *aphA2* gene fused to regulatory sequences active in eukaryote cells will be designated *eu-aphA2*. The biosafety of the presence of this *eu-aphA2* gene and its APH(3')II gene product in transgenic plants will be evaluated in more detail below.

Concerns about kanamycin resistance

In general, concern about release and use of transgenic plants focuses on the effects such plants may have on ecosystems (Jones and Maryanski, 1991, Keeler and Turner, 1991). The introduction of genes beyond the barrier of species with recombinant DNA techniques is a novel phenomenon with consequences not fully known. The specific concerns with respect to kanamycin and kanamycin resistance can be summarized as follows: (1) kanamycin resistance might transform a crop into an uncontrollable weed or might disturb the ecological relations of the plant in another unknown way; (2) in addition, or alternatively, the kanamycin resistance gene may spread from the crop plant to wild relatives or to other organisms, which as a result become uncontrollable. Agricultural application may thus result in an undesirable increase in the occurrence of the kanamycin resistance gene in nature; (3) also, the presence of kanamycin resistance in crops may result in increased use of the antibiotic as herbicide; (4) furthermore, the presence of the kanamycin resistance gene or its gene product may render the plant unsuitable for consumption or industrial processing.

The chemotherapeutical and veterinary applications of kanamycin and neomycin imply additional, specific concerns. The development of antibiotic resistance in pathogenic micro-organisms as result of high antibiotic selection pressures has caused severe problems in human medicine (Pratt and Fekety, 1986; Olson *et al.*, 1991). Therefore, the use of kanamycin and the *eu-aphA2* gene in modern plant breeding (5) could contribute to an unacceptable increase in resistant, potentially pathogenic microorganisms and (6) could thus impair the human and veterinary applications of these antibiotics. A hypothetical flow diagram containing most of the various possibilities is presented in Fig. 2. The concerns will be addressed in more detail below.

Kanamycin resistance and weediness

The weediness of a plant is supposed to depend on intrinsic characters of the plant, such as those proposed in Keeler (1989), in combination with the specific habitat the plant lives in (Williamson *et al.*, 1990). In the latter view, the better the biological and ecological properties of a plant are adapted to a given habitat, the less additional knowledge is required to be able to predict its potential weediness (Sharples, 1991). Laboratory experience has shown that the genetically modified plant is generally identical to its untransformed parent plant with the exclusive exception of the added property. In fact, the highly specific and relatively little genetic change achieved by molecular breeding can be considered to be one of the major advantages in comparison with classical cross breeding. As major agronomical projects aim at cultivation of the transgenic crop in ecosystems in which the parent plant was already in use for many years, a drastic change in habitat is not likely.

Kanamycin resistance is introduced to give transformed cells an absolute selective advantage in the presence of the antibiotic. The only situation in which kanamycin resistance is a feasible contribution to a selective advantage of the transgenic plant is when selective antibiotic concentrations are found in nature. An evaluation of the probability that such a situation arises will give insight into the potential weediness of kanamycin resistant plants. Selective antibiotic concentrations would develop as result of (1) antibiotic production in soil or (2) antibiotic addition to soil.

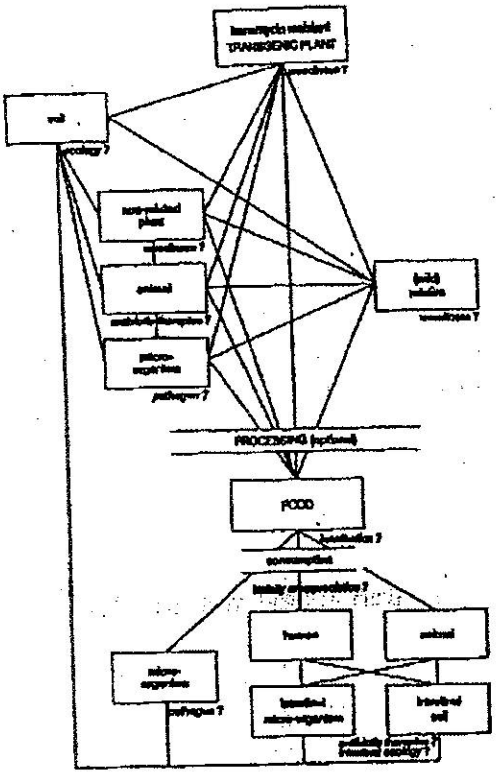


Fig. 2. Hypothetical spread of the kanamycin resistance gene and/or gene product into the environment. For the sake of clarity, not all possible relationships are shown in the flow diagram. The main concern for each possibility is given in italics.

Kanamycin production in soil

Kanamycin and neomycin are produced by soil micro-organisms, but it is unclear whether they are only produced under laboratory and industrial conditions, or also in the natural environment (van Elsas, 1992). Despite a lot of research efforts, the presence and biological activity of antibiotics in natural soil could not be demonstrated (Williams and Vickers, 1982), except for phenazine-1-carboxylic acid in the rhizosphere of wheat (Thomashow *et al.*, 1990) and a possibly ecological role of streptomycin (Li and Alexander, 1990). Whereas the presence and biological activity of soil-produced antibiotics in microniches cannot be excluded (van Elsas, 1992), the amount of soil-produced kanamycin will be small and, also in view of the physico-chemical characteristics of the compound (see below), will not result in conditions that can give kanamycin resistant plants any selective advantage over the non-resistant parent plants.

Kanamycin addition to soil

Veterinary use of kanamycin and neomycin is a potential source of antibiotic addition to soil. Both antibiotics are poorly resorbed in the gastro-intestinal tract and an estimated 97% leaves the body unchanged in the faeces (Reynolds, 1989). Intensive animal factory farming as in the Netherlands, could establish selective conditions for kanamycin resistant plants through the application of manure. It can be calculated that if the annually consumed 30000 kg kanamycin and neomycin is ploughed into the top 5 cm of three quarters of the Dutch agricultural area (1.5 million ha (CBS, 1991) soil density 1.5 g cm^{-3}, relative humidity 20%, no biodegradation) the resulting concentration would be 0.13 μg ml^{-1} ground water. The lower limit for kanamycin sensitivity of microorganisms (MIC value) is 0.12–8 μg ml^{-1} (Otten *et al.*, 1988). Therefore, only the most sensitive microorganisms would experience a limited selection pressure. However, this estimate of the effective concentration of kanamycin and neomycin in soil proves to be a gross overestimation when the physico-chemical properties of these aminoglycoside antibiotics are taken into account. Most substances (glucose, lipids, proteins, nucleic acids, clay, phosphates, various ions) can form complexes with aminoglycoside antibiotics and thus reduce the biological activity. Especially, loss of biological activity through adsorption to clay particles has been well documented for kanamycin and neomycin (Soulides, 1962). Clay is an important constituent of all agriculturally relevant soil types. The type of clay determines the adsorption capacity and whether adsorption is reversible or irreversible. The clay mineral illite, comprising 35% of the Dutch clay grounds, adsorbs irreversibly 42 g neomycin or 26 g kanamycin per kg, while the clay mineral kaolinite adsorbs reversibly 10 g neomycin or 8 g kanamycin per kg (Pinck *et al.*, 1961). The other two important soil constituents, humus and organic matter, are also able to adsorb and inactivate substantial amounts of antibiotics (Gottlich, 1976). Most, if not all, soils are thus able to inactivate substantial amounts of kanamycin and neomycin. The precise mechanisms underlying the adsorption or desorption of antibiotics by soil are, however, unknown. It can be calculated that if the Dutch agricultural area (see above) would contain 5% illite that is able to bind irreversibly 30 g kanamycin and/or neomycin per kg, with an annual application of 30000 kg in the top 5 cm of the area, it would take 56250 years for the area to be saturated with antibiotic, in the absence of biodegradation and with only illite adsorption taken into account.

Although kanamycin and neomycin are relatively stable compounds, it is likely that the effective concentration is

not only limited by adsorption to soil constituents, but also by biodegradation (Misato *et al.*, 1977). For the related aminoglycoside streptomycin, it has been demonstrated that recurrent application to soil increases the rate of its breakdown and the ease by which streptomycin-degrading organisms can be isolated from that soil (Gottlieb, 1976).

Taken together, it can be excluded that any soil will be able to accumulate kanamycin or neomycin in concentrations that are selective for transgenic plants. Kanamycin resistance will therefore not contribute to enhanced weediness of a plant.

Conversely, it has been argued that, especially in the absence of selection, the burden of an extra but useless gene would lower the fitness of the organism (Tiedje *et al.*, 1989), suggesting that the *eu-aphA2* gene may decrease weediness. Data from laboratory studies (Hilder and Gatehouse, 1991) and field trials now performed (Kaniewski *et al.*, 1990) do not support this view. Another aspect of the presence of the *eu-aphA2* gene is the possibility that the gene product could alter metabolism in another way than inactivating kanamycin, by interfering with existing metabolic pathways. However, the substrate specificity of the APH(3')II enzyme is sufficiently high and available data show that the enzyme does not have any interference with the basic functions of growth and development of a plant. It could also be argued that if the *eu-aphA2* gene would mutate in a number of steps into a gene encoding another enzyme with other substrate specificities, undesirable effects could occur. However, the likelihood for (a series of) mutations resulting in a new and functional enzyme with substrate specificity altered in such a way that plant metabolism is disturbed and to give selective advantage to the plant seems negligible.

Increased use of kanamycin in agriculture

The presence of kanamycin resistance in crops opens the possibility to use the antibiotic as herbicide and could thus contribute to an (increase in the) use of kanamycin in agriculture. Although aminoglycoside antibiotics have been used as weed killer in the sixties (Misato *et al.*, 1977), this application has diminished. Very occasionally, streptomycin is used against fire blight caused by *Erwinia amylovora*. For some basic research purposes and breeding programmes spraying seedlings with kanamycin may be advantageous (Weide *et al.*, 1989), but its contribution to the total consumption of this antibiotic is negligible. Apart from the relatively high costs of production, kanamycin and neomycin are not likely to be used anymore for such purposes, because of their limited efficacy and the intrinsic resistance of various plants. Hence, there is no basis for the concern of any increased use of these antibiotics in agriculture. Even if large amounts of these antibiotics were used, their physico-chemical characteristics will prevent the establishment of selective conditions.

Spread of kanamycin resistance

The presence of the *eu-aphA2* gene in transgenic plants might result in a transfer of the gene to the related wild flora by cross pollination or to unrelated organisms by direct DNA transfer. As a result of this transfer, the newly transformed organism may become a plague, a pathogen, or both.

Cross pollination

The probability of the spread of a transgene in populations of wild relatives by outcrossing depends on a myriad of ecological, genetic and statistical factors (Keeler and Turner, 1991). Several factors concerning the crop itself and the ecological relations with other species of that crop at the location of interest need therefore to be taken into account to be able to estimate the likelihood of cross pollination (Keeler and Turner, 1991). For example, potato (*Solanum tuberosum*) is not likely to outbreed in the Netherlands, because it is a vegetatively propagated crop that has only a limited number of wild relatives in the Dutch flora, with which it cannot, or only with extreme difficulty, be crossed (Eijlander and Stiekema, 1990). In Peru, however, transgenic potato species are rather likely to exhibit outbreeding (Cohen and Chambers, 1991). In view of the global interests of current agriculture, it is prudent to assume that a transgene may spread by cross pollination in some conditions and at some locations with a certain probability, that – at considerable costs – can be estimated. More relevant, therefore, is the foreseeable effect of the spread of, in this case, the *eu-aphA2* gene. Kanamycin resistance would only be able to contribute to the weed characteristics of a crop when the environment poses a selection pressure. Likewise, wild relatives that receive the gene would only become less controllable than the parent plant in a situation of selective antibiotic conditions. As argued above, this situation will not occur.

In addition, it should be noted that large plant families, notably the monocotyledonous plants, are already highly tolerant to the antibiotic, probably because the antibiotic can not enter the cell in sufficient amounts. In tissue culture and plant regeneration, enhanced kanamycin tolerance is occasionally observed, demonstrating the variability of the plant genome in this respect (Evans, 1989). Kanamycin resistance may thus develop as result of natural mutations and does not seem to require the transgene. Therefore, (phenotypical) kanamycin resistance should not be considered a novel characteristic for any ecosystem and will not contribute to enhanced weediness of any crop or wild relative.

Horizontal gene transfer

'Horizontal' gene transfer to another organism requires direct transfer of DNA. It is conceivable that during growth, decay or herbivory, the transgene is made available and is transferred to other (micro)organisms. As

plant cells do contain large amounts of DNAses, most of the DNA will be degraded before it is available for such transfer event. High concentrations of microorganisms are found in the rhizosphere (Bakken, 1990) and near decaying plant material (Rämheld, 1990), but the probability of natural transformation in such environments is unknown. DNA transfer between microorganisms is supposed to occur frequently in soil, but the available data are related to conjugal transfer of plasmids. Plant DNA will not be made available as plasmids, so conjugal DNA transfer can be excluded.

The transformation frequency of microorganisms in soils can be estimated for any given case. Detailed considerations of the various assumptions have been published by Calgene (1990). For example, when all tomato plants including all fruits annually produced in the Netherlands (5×10^8 kg fruit; fruit:plant ratio 2:1; density and volume plant cell 1 g cm^{-3}, and 1.25×10^{-7} cm^3 respectively; genome size 7×10^5 kb; assuming 10 copies of 1 kb *eu-aphA2*) are ploughed into the first 30 cm of the area in which tomato plants are cultivated (1600 ha, relative humidity 20%), assuming 10% of the DNA to be released and 10% of the released DNA to be intact, the amount of DNA per hectare would be 5.6 kg per year resulting in a concentration of 6.2×10^{-2} µg ml^{-1} ground water, of which 8.7×10^{-7} µg ml^{-1} is *eu-aphA2* DNA. This concentration of DNA, assuming 10% of the soil organisms transformable, 5% of which is competent, and 0.01% natural transformation frequency (Calgene, 1990), would give a transformation frequency of 8.7×10^{-12}. The 10^7 viable microorganisms supposed to be present per g soil (Stotzky, 1989) would thus result in 8.7×10^{-5} transformants per g soil. Henschke and Schmidt (1990) found 1600 kanamycin resistant microorganisms per g agricultural soil, while van Elsas and Pereira (1986) demonstrated 3000 - 30000 kanamycin resistant bacilli in various Brazillian soils. This shows that kanamycin resistance is not a novel characteristic for a soil ecosystem. When transfer of the *eu-aphA2* transgene would result in 8.7×10^{-5} transformants per g soil containing 10^7 microorganisms of which 1600 were already kanamycin resistant, the contribution to the natural kanamycin resistance would be 5.4×10^{-9}%. In addition, it should be noted that a given transfer of the *eu-aphA2* gene to a (soil) microorganism will not immediately result in kanamycin resistance, because the gene carries regulatory sequences that will generally not work in microorganisms. Upon transfer, recombination should therefore occur to make the gene functional, the microorganism should be present in a natural environment in which a selective advantage of kanamycin resistance occurs and should be able to outcompete the kanamycin resistant organisms already present. The likelihood for a subsequent occurrence of all these events is negligible. Therefore, horizontal transfer of *eu-aphA2* DNA will not alter or disturb a soil ecosystem.

Consumption of kanamycin-resistant plant material

The uptake of transgenic plant material by insects, cattle or man introduces both the transgene *eu-aphA2* and the transgene product APH(3')II in the digestive tract of the consumer. Dependent upon the intactness of either gene or product, undesirable effects may occur. Consumption may prove toxic, or give rise to unacceptable side effects. As most food processing generally results in irreversible inactivation and degradation of DNA and proteins, processed food will not be taken into consideration here. Food that potentially carries the largest risk is the food consumed fresh, such as a tomatoes. The safety of consumption of the *eu-aphA2* gene and the APH(3')II protein need to be evaluated in relation to their properties and the 'natural' consumption of these compounds (IFBC, 1990).

Consumption of the eu-aphA2 gene

The large amount of DNA that passes the digestive system daily indicates that DNA in itself is not intrinsically toxic to human beings. Most DNA is efficiently degraded in the intestinal tract and no functional genes are assumed to remain present (Berkowitz, 1990). In this respect, *eu-aphA2* DNA will not differ from any other DNA and will not pose any (additional) threats.

Another concern should be the natural transformation of intestinal cells or of intestinal bacteria. Although most of the DNA will be degraded and diluted several orders of magnitude, natural transformation can not *a priori* be excluded. The cells that may come into direct contact with the transgene are the epithelium cells of the mucus membrane that covers the surfaces of the digestive system. No data exist to indicate any transformability of these cells. As they have a limited life span of only 7 days (Johnson *et al.*, 1986), any putatively transformed cell will disappear quickly and pose no threats. Natural transformation of microorganisms that are present in the digestive tract should also be considered. The transfer of antibiotic resistance to clinically important bacteria has been repeatedly observed among indigenous bacteria, thereby reducing the choices of antibiotics available to treat infections (Olson *et al.*, 1991). In the digestive tract, approximately 10^{14} bacteria are present (Savage, 1977), 10^{12} of which are not intrinsically kanamycin resistant and may be target for noticeable transformation. No data are available concerning the natural transformation of bacteria in the human gastro-intestinal tract. The transit time of food in mouth, pharynx, oesophagus, stomach and duodenum is rapid, and conditions in the stomach will prevent any natural transformation. The most relevant location for possible DNA transfer to the intestinal flora therefore is the lower part of the small intestine (ileum) and the colon, in which consumed food stays relatively long.

Calgene (1990) has presented a detailed hypothetical

scenario for *Streptococcus* transformation in this part of the body, concluding that the contribution is negligible. Alternatively, it is illustrative to estimate the probability of DNA transfer in the intestine using data from plasmid rescue. A transgene insertion could be rescued as a plasmid from the mouse genome with reasonable efficiency when special *E. coli* methylation restriction mutants (*mcrABC*$^-$, *mrr*$^-$) were used for transformation (Grant *et al.*, 1990). The amount of 50 ng digested and ligated genomic mouse DNA in a volume of 100 µl yielded 20 colonies per transgene if the transformation efficiency was calibrated to 10^9, depending upon, among other things, the methylation status of the DNA (Grant *et al.*, 1990). The tomato genome (7×10^5 kb) is half the mouse genome, implying twice the amount of transgene. Further assuming that all transformable bacteria in the intestinal tract are *E. coli*, that the transformation efficiency of plasmid DNA in this tract is 10^{-6}, as is easily attainable in laboratory conditions (Sambrook *et al.*, 1989), that linear DNA transforms at 0.1% the frequency of circular plasmid DNA (Sambrook *et al.*, 1989), that 10 copies *eu-aphA2* are present per tomato genome and that there is no breakdown of DNA in the intestinal tract, the number of colonies that would arise from 50 ng transgenic tomato DNA in 100 µl is 4×10^{-4}. The number of bacteria present in 100 µl competent cells is approximately 10^8, so also 4×10^{-4} colonies would be expected if 0.5 mg DNA is added to 10^{12} bacteria in 1000 ml. Consumption of 250 g tomatoes (density and volume plant cell 1 g cm^{-3} and $1.25\ 10^{-7}$ cm^3 per cell; 10 copies *eu-aphA2* DNA per genome; 7×10^5 kb genome size) implies the uptake of 3 mg tomato DNA. The volume of the fluid in the small intestine is estimated to be 1500 ml (Johnson *et al.*, 1989; Calgene, 1990). In the above model, 3 mg genomic DNA in 1500 ml would generate 0.0024 transformants, an increase of 2.4×10^{-12}% of the transformable intestinal flora or 2.4×10^{-15}% of the total intestinal flora. Also now it should be noted that transfer of the *eu-aphA2* gene to an intestinal microorganism will not immediately result in kanamycin resistance, because recombination and subsequent selection should occur to make the gene functional and effective. The actual contribution of the *eu-aphA2* gene would therefore be considerably smaller. The number of kanamycin resistant microorganism that would thus result from potential DNA transfer in the human digestive tract thus is negligible with respect to the numbers of intrinsically resistant microorganisms already present.

The biochemistry of food digestion of cattle is largely comparable to that of humans, so in cattle the probability of generating a kanamycin resistant organism as result of DNA transfer from transgenic food is equally negligible. The high 'natural' background of kanamycin resistant microorganisms in the digestive tract indicates that there is no reason for concern that the in itself highly unlikely spread of the *eu-aphA2* gene by consumption will have undesirable side effects.

Consumption of the APH(3')II enzyme

The presence of the APH(3')II enzyme in the digestive tract may interfere with the oral therapeutic usage of aminoglycoside antibiotics. In the Netherlands, kanamycin and neomycin are no longer administered orally, and the high substrate specificity of the enzyme (Table 1) ensures that modern generations of aminoglycoside antibiotics such as amikacin or netilmycin are not inactivated. The high substrate specificity of the enzyme also guarantees that no novel secondary metabolites will be formed. Moreover, APH(3')II requires ATP as a cofactor. ATP is unstable at low pH and the amounts of ATP supposed to be present in the digestive tract will not result in any catalytic activity. The protein is not likely to be active in the digestive tract of cattle, either. Therefore, any impairment of the large scale veterinary applications of kanamycin and neomycin will not go beyond the already existing problems due to antibiotic resistant microorganisms.

In the absence of catalytic activity, the APH(3')II protein itself, or its degradation products could be toxic. However, in general, proteins are nontoxic (Jones and Maryanski, 1991). As protein, APH(3')II will essentially be fully degraded in the gastro-intestinal tract. In simulated gastric conditions the protein was totally degraded in 20 min (Calgene, 1990). Feeding rodents with transgenic tomatoes had no deleterious effects (Calgene, 1991; Flavell *et al.*, 1992). Empirical data evaluating human toxicity are not available, but indirect evidence suggests no or very low toxicity. Analyses of the microbial populations in food (Corpet, 1988), drinking-water (Kelch and Lee, 1978) and faeces (Levy, 1978) show widespread occurrence of resistance to antibiotics (Levy *et al.*, 1988). In purified water, the relative number of antibiotic resistant organisms is higher than in unpurified water (Armstrong *et al.*, 1981), and can be up to 50% of the isolated strains, possibly due to the method of water purification (Calomires *et al.*, 1981). More than half of the tested samples of human faeces, from individuals that had not been treated with antibiotics for a longer period, contained over 10% organisms resistant to at least one antibiotic out of a panel of seven, among which was kanamycin (Levy *et al.*, 1988). Plasmids have been shown to be maintained and transferred in natural populations even in the absence of selection (Stotzky and Babich, 1984). Apparently, a natural resistance pool exists independent from the actual use of antibiotics (Hughes and Datta, 1983). As result of this 'natural' background of kanamycin resistant organisms, part of which is resistant because of APH(3')II, the digestive tract will be challenged by the protein. A continuous exposure to the APH(3')II protein can therefore be assumed. Consequently, there is no reason for concern with respect to the toxicity of APH(3')II.

The presence of APH(3')II could interfere with the appreciation of consumption by either humans or other feeding organisms. There are no clues to the potential influence of APH(3')II on the overall taste of a plant. This type of subtle change will be very difficult to approach experimentally, but attention in controlled field trials and consumer panels seems worthwhile.

Concluding remarks

The phenotype of a plant to be able to grow on the aminoglycoside antibiotic kanamycin as result of transformation with the *eu-aphA2* selectable marker will not give the plant any selective advantage outside the laboratory as compared to the untransformed parent plant. The phenotype is already quite ubiquitous in nature: species are already kanamycin resistant, or are likely to develop resistance by natural mutations. In case of vertical or even horizontal transfer of the gene, any resulting kanamycin resistant organism will either not have any selective advantage over the parent organism or will have to compete with the natural background of resistant organisms. The physico-chemical characteristics of the antibiotic itself seem to prevent a situation of significantly selective conditions in the environment.

From the health point of view, the *eu-aphA2* gene proves to be a very good choice of marker gene. For human therapy, both kanamycin and neomycin have largely been replaced by more modern antibiotics. The high substrate specificity of the enzyme guarantees the absence of development of resistance against these newer aminoglycoside antibiotics. At no point will there be any interference with modern antibiotic therapies. Even the probability that the *eu-apha2* gene would interfere with the efficacy of the veterinary applications of kanamycin and neomycin is negligible. The acceptance and permissibility of (human) consumption of *eu-aphA2* containing crops will require toxicological evaluation, but in view of the data available it is unlikely that gene and/or gene product will turn out to be unacceptable for consumption. Therefore, kanamycin-resistant transgenic plants can generally be recognized as safe. No restrictions are required for the introduction of kanamycin resistant plants in the field or on the market place. Even if methods for removal of a (selection) gene are available, such as for example the Cre/*lox* system (Dale and Ow, 1991), and these prove generally applicable, gene removal should not be required in case of the kanamycin resistance gene. Full legislative clearance of kanamycin resistance in transgenic plants will hopefully partly contribute to the social acceptance of products from agricultural biotechnology (Scholten *et al.*, 1991). For every additional transgene considered for agricultural application, however, specific case studies should contribute to insight in the biosafety and admissibility of the gene in question.

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AMINOGLYCOSIDE 3'-PHOSPHOTRANSFERASE II (APH(3')II): REVIEW OF ITS SAFETY AND USE IN THE PRODUCTION OF GENETICALLY ENGINEERED PLANTS

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ABSTRACT

Calgene conducted a thorough review and analysis of the use of the *kan*^{r} gene and gene product, aminoglycoside 3'-phosphotransferase II (APH(3')II), for use as a selectable marker in FLAVR SAVR™ tomatoes, in BXN™ cotton and oil-modified rapeseed. The data generated support the conclusions that APH(3')II is not a toxin or allergen, that the *kan*^{r} gene is highly unlikely to move from the plant genome into microorganisms via horizontal gene transfer, that if such transfer could occur the impact would be minimal, and that APH(3')II in transgenic plants will not compromise antibiotic use in humans or animals.

At FDA's Food Advisory Committee meeting on April 6-8, 1994, the FDA Center for Food Safety and Applied Nutrition (CFSAN) concluded that the FLAVR SAVR tomato was as safe and nutritious as other commonly consumed tomatoes and "that the use of the *kan*^{r} gene and APH(3')II will not have a significant impact on the environment and that an environmental

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impact statement is not required." On May 18, 1994, the FDA finished its safety review of the FLAVR SAVR tomato and APH(3')II. The FDA concluded that "FLAVR SAVR™ tomatoes have not been significantly altered when compared to varieties of tomatoes with a history of safe use" (Federal Register 59:26646) and "that the use of aminoglycoside 3'-phosphotransferase II is safe for use as a processing aid in the development of new varieties of tomato, oilseed rape, and cotton intended for food use" (Federal Register 59:26700).

INTRODUCTION

Calgene began consultations with the U.S. Food and Drug Administration (FDA) in 1989 to determine the type of data necessary for assessing the safety of the transgenic foods. Since then, many consultations have occurred and a number of documents have been submitted to the FDA, beginning with "*kan*^{r} Gene: Safety and Use in the Production of Genetically Engineered Plants" (Request for Advisory Opinion, U.S. Food and Drug Administration, Docket #90A-0416, November 26, 1990). This document was submitted in a Food Additive Petition (FAP) format because there was a potential that Calgene might choose an FAP for APH(3')II after subsequent consultations with the FDA. Calgene has continued to consult with the FDA and submit additional data as requested.

The specific *kan*^{r} gene used by Calgene was cloned from Tn5 and encodes an enzyme that confers resistance to the antibiotics neomycin, kanamycin and geneticin (G418). The complete nucleotide sequence for the *kan*^{r} gene has been published (Beck et al. 1982). The specific form of neomycin phosphotransferase encoded by the *kan*^{r} gene is aminoglycoside 3'-phosphotransferase II (APH(3')II) (Beck et al. 1982). This enzyme catalyzes an ATP-dependent phosphorylation of the 3'-hydroxyl group on the

aminohexose moiety of several aminoglycoside antibiotics. Phosphorylation of the antibiotics interferes with their uptake and ability to bind to cellular ribosomes, thereby rendering the cells resistant to the antibiotics. APH(3')II, also known as neomycin phosphotransferase II (NPTII), is used by Calgene in the development of the following genetically engineered plant varieties and plant products:

- Tomatoes with improved agronomic and quality traits (such as the FLAVR SAVR tomato);
- Cotton with the BXN™ (bromoxynil resistant) gene conferring tolerance of the crop to the herbicide bromoxynil for improved weed control;
- Cotton resistant to specific insect pests such as the cotton bollworm and tobacco budworm; and
- Rapeseed varieties that will produce specialty edible and industrial oils. Edible rapeseed oil is produced from canola varieties in the U.S., Canada and Europe.

Table 1 provides a complete list of submissions on the *kan*^{r} selectable marker gene to the FDA.

Calgene's approach to demonstrate the safety of the APH(3')II selectable marker is based on the scientific principles on which the FDA policy is based (FDA Statement of Policy: Foods Derived from New Plant Varieties, Fed. Reg. 57:22984, May 29, 1992) and the recommendations of the International Food Biotechnology Council (IFBC 1990) and the Joint FAO/WHO Consultation (1991). The data demonstrate that APH(3')II is a safe marker protein for selection of transgenic tomato, cotton, and rapeseed.

The FDA's conclusion that FLAVR SAVR tomatoes are as safe as tomatoes bred by conventional means and the FDA's final rule allowing the use of APH(3')II as processing aid in the development of new varieties of tomato, oilseed rape and cotton represent a major advance in development of new

TABLE 1. Review of Submissions to FDA

Date of submission	Subject/Results	In response to
11/26/90	Safety of *kan*^{r} gene and APH(3')II demonstrated	Consultations with agency
5/1/91-7/30/91	Only 43 public comments received on *kan*^{r} document	FDA requests public comments
5/31/92	*kan*^{r} gene used in human therapy, APH(3')II is not toxic or allergenic and is degraded in the gut	Consultations with agency
10/30/92	APH(3')II will not compromise antibiotic therapy	Consultations with agency
1/4/93	*kan*^{r} document converted to Food Additive Petition for APH(3')II	Consultations with agency
6/3/93	Revised environmental assessment (EA) for APH(3')II	Consultations with agency
7/16/93-8/16/93	No public comments submitted on APH(3')II EA	FDA requests public comments
8/19/93	Neomycin in animal feed not inactivated	Consultations with agency

foods using genetic engineering techniques. This is the first time a whole food, developed using genetic engineering, has been approved for commercialization.

SAFETY ISSUES

Based on the FDA Statement of Policy and from consultations with the FDA, a number of safety questions arose which are summarized below.

Calgene has provided responses to each question to demonstrate the safety of the APH(3')II marker gene.

- Is APH(3')II a toxin or allergen?
- Could the *kan*^{r} gene move from the plant genome via horizontal gene transfer?
- If the *kan*^{r} gene could move via horizontal transfer, what would be the impact?
- Could APH(3')II compromise an oral dose of kanamycin or neomycin in human antibiotic therapy?
- Could APH(3')II inactivate antibiotics in animal feed?

SUMMARY OF RECENT LITERATURE

Reviews on Selectable Marker Genes

In their review of selectable marker genes, Flavell et al. (1992) concluded the following:

- The NPTII (*kan*^{r}) gene product (APH(3')II) is not toxic to humans or animals.
- Eating APH(3')II does not compromise oral kanamycin and neomycin therapy in humans.
- Transfer of the gene from plants to pathogenic bacteria to humans or other species is highly unlikely and would not compromise antibiotic therapy in humans or animals if it did happen.
- Potential gene movement from genetically modified plants will not cause environmental damage.
- "Overall, the ubiquity of the gene in nature and its benign properties make it ideal as a selectable marker in plant transformation."

In their review on the biosafety of kanamycin-resistant transgenic plants, Nap et al. (1992) concluded:

- "The [*kan*^{r}] gene used for resistance is an excellent choice because of the high substrate specificity of the enzyme encoded."
- "Human or veterinary antibiotic therapies will not be compromised. ... At no point will there be any interference with modern antibiotic therapies. Even the probability that the *eu-apha 2* [*kan*^{r}] gene would interfere with the efficacy of the veterinary applications of kanamycin and neomycin is negligible."
- "The physico-chemical characteristics of the antibiotic exclude the existence of selective conditions in the environment. Therefore, a transgenic plant or any other organism that might have acquired the gene will not get any selective advantage because of this gene."
- "There is no toxicity or predictable harm of both gene or gene product for human or animal consumption."
- "Full legislative clearance of this transgenic trait is therefore acceptable."
- "Kanamycin-resistant transgenic plants can generally be recognized as safe."

In his review on the ecology of transgenic plants, Crawley (1992) concluded:

- "There is a view that the ecology of genetically engineered organisms is somehow different from the ecology of conventional organisms, and that the intentional release of genetically engineered organisms poses a greater threat to the Balance of Nature than do other kinds of organisms bred by man. This view is mistaken. ... The ecological rules are the same for transgenics as for non-transgenics."
- "Release of genetically engineered crop plants does not pose substantial new threats to the environment."

These recent reviews provide additional support for Calgene's conclusion that the *kan*^{r} selectable marker gene can be safely used in food crops.

Human Gene Therapy

The *kan*^{r} gene (from Tn5) has been recently used in two clinical studies involving human gene therapy (Kasid et al. 1990, Blaese et al. 1990). These clinical studies represent a human *in vivo* safety evaluation of the *kan*^{r} selectable marker gene. No adverse effects of the *kan*^{r} selectable marker gene or gene product have been observed.

The first study involves insertion of the *kan*^{r} selectable marker gene into TIL (tumor-infiltrating lymphocyte) cells infused into cancer patients. In rodent experiments, TIL cells (incubated with interleukin 2) were observed to be 50-100 times more potent in mediating tumor rejection than lymphokine-activated killer (LAK) cells. Early human experiments showed that TIL cells recognize the patient's own cancer cells and are not effective in another patient. Of the first 25 patients treated with TIL therapy, each of whom had advanced melanoma or kidney cancer that did not respond to other treatments, half showed tumor regression of 50% or more (Potera 1989). In the clinical studies, the *kan*^{r} selectable marker was incorporated into the TIL cells to serve as a "tag" allowing the distribution of these cells to be monitored.

The second use of the *kan*^{r} gene in humans involves severe combined immune deficiency (SCID), which is a lethal genetic disease characterized by the virtual absence of all T and B cell immune function (Kantoff et al. 1988). Most affected infants die of opportunistic infections before the age of two. In one-fourth of SCID cases, a deficiency of the purine catabolic enzyme adenosine deaminase (ADA) has been identified. ADA catalyzes the conversion of adenosine (Ado) and deoxyadenosine (dAdo) to inosine and

deoxyinosine. In the absence of this enzyme, dAdo accumulates intracellularly and is phosphorylated to dATP (which is normally present only at low levels in mammalian cells). The high levels of dATP are thought to result in cellular toxicity. T cells contain the highest levels in the body of the enzymes that phosphorylate dAdo to dATP, which likely accounts for the observed selective toxicity toward the immune system. Human gene therapy for this disease involves removal of some T lymphocytes from the patient, insertion of the missing ADA gene using a vector containing the *kan*^{r} selectable marker gene, selecting for those T cells that have acquired the ADA gene, growing the cells to large numbers in culture and reintroducing them into the patient. The clinical protocol was approved by the NIH Institutional Biosafety Committee.

Anderson et al. (1987) in a preclinical data document submitted to the NIH Recombinant DNA Advisory Committee commented that "since eukaryotic cells are already resistant to the aminoglycosides used clinically, there appears to be no risk from expression of the *neo*^{r} [*kan*^{r}] gene" in the transformed bone marrow cells. No clinically observable adverse side-effects resulting from the presence of the *kan*^{r} selectable marker gene and gene product have been noted in the studies performed to date. Blaese and Anderson (1990) reported that they "have observed no untoward effects of expression of the *neo*^{r} gene in T cells either *in vitro* or in mice, monkeys, or humans who have received T cells expressing this enzyme." In comments presented to the NIH Institutional Biosafety Committee (March 7, 1990), Dr. W. French Anderson stated that no antibodies to the *kan*^{r} gene product had been detected in patients receiving the TIL therapy. He did indicate that sensitive assays for the *kan*^{r} gene product, not currently available, are needed. In comments before the same committee, related to both the TIL and ADA(-) SCID studies, Dr. Michael Blaese reported that "no evidence of gene product toxicity has been observed with the genes inserted to date."

Occurrence of *kan*^{r} Genes in the Environment

Kanamycin-resistant genes occur naturally in the environment (Henschke and Schmidt 1989). Van Elsas and Pereira (1986) found background levels of kanamycin-resistant *Bacillus* ranging from 3300 to 30,000 CFUs per gram of soil in several different Brazilian soils. In this same study, kanamycin-resistant *Bacillus* populations were observed to comprise from 1 to 3 percent of the total *Bacillus* population within the different soil types examined. Therefore, although kanamycin-resistant bacteria represent a small percentage of the total soil microflora, they are present at detectable background levels.

Relative to the environmental impact of released genetically engineered microorganisms, Stotzky (1989) states, "Preliminary studies on the effects of adding to soil high concentrations (e.g., 10^8 CFUs per gram of soil) of various strains of *E. coli*, *E. cloacae* and *P. aeruginosa*, with and without plasmids carrying antibiotic-resistance genes, have not shown any consistent and lasting effects on the gross metabolic activity (as measured by CO_2 evolution), transformation of fixed nitrogen, activity of soil enzymes (phophatases, arylsulfatases, dehydrogenases) and species diversity of the soil microbiota." This statement lends support to the conclusion that the introduction of crop plants containing an antibiotic-resistant selectable marker is not likely to result in adverse environmental impacts.

Horizontal Gene Flow

The probability of horizontal gene flow is vanishingly small for the following reasons (IFBC 1990):

- The introduced genes are permanently incorporated into the plant chromosome.
- Transgenic plants do not contain genes required for transport of DNA into bacteria.

- If bacteria acquired the DNA through passive uptake from decomposed plant tissue, the bacterial cell could not "read" the genes since they do not have bacterial promoters.

No mechanism for transfer of genes from plants to microorganisms is known and no cases of such transfer have been adequately documented. Carlson and Chelm (1986) argued for an eukaryotic (plant) origin of glutamine synthetase II in bacteria, albeit over an evolutionary time period. They suggested that this was evidence that horizontal gene flow from plants to microorganisms had occurred at one point in evolution. However, their paper was directly refuted by Shatters and Kahn (1989) who concluded that "the GS [glutamine synthase] proteins are highly conserved and the divergence of these proteins is proportional to the phylogenetic divergence of the organisms from which the sequences were determined. No transfer of genes across large taxonomic gaps is needed to explain the presence of GSII in these bacteria." Other so-called "evidence" that horizontal gene flow occurs from plants to microorganisms involves transient changes (non-heritable) such as transencapsidation of chloroplast DNA (Rochon and Siegel 1984) or possibly endocytosis (Bryngelsson et al. 1988), neither of which have been shown to result in actual transfer of genes from plants to microorganisms. No mechanism by which plant DNA could be incorporated from plants into the genomes of the microorganisms has been proposed. For *Agrobacterium*-mediated transformation, Zambryski et al. (1982) provided evidence that once inserted DNA is integrated into the plant host genome, it cannot be remobilized even if acted on again by *vir* genes.

Recently, Smith et al. (1992) presented arguments for the occurrence of horizontal gene transfer. Two cases of transfer from eukaryotes to prokaryotes, and two cases of transfer from prokaryotes to eukaryotes were considered by the authors as likely to have occurred over an evolutionary

time period. If their assertions are true (and these authors admit that "horizontal gene transfers are difficult to prove" and that additional data "may also lead us to reconsider some of the cases that at present appear to be sound"), these occurrences can still be considered to be extremely rare.

RESPONSES TO FDA CONSULTATIONS

November 26, 1990 Submission

kan^r Gene: Safety and Use in the Production of Genetically Engineered Plants (Request for Advisory Opinion, U.S. Food and Drug Administration, Docket #90A-0416, November 26, 1990). In this document, detailed calculations were made using worst-case scenarios to determine whether there could be any adverse effect on human or animal health and on the environment from the use of APH(3')II in tomato, cotton and rapeseed. This included 1) the impact on the environment resulting from the commercial production of tomatoes, cotton and rapeseed plants containing the *kan*^{r} gene; 2) any potential increase in human exposure to kanamycin and neomycin resistant bacteria from the direct consumption of food containing the *kan*^{r} gene or indirectly from an increase in kanamycin and neomycin resistant soil bacteria that could result in the compromised efficacy of kanamycin or neomycin; 3) the potential for compromised efficacy from exposure to APH(3')II in humans taking oral therapeutic doses of kanamycin and neomycin; and 4) the potential toxicity of APH(3')II when ingested by humans.

Under worse case and extremely rare conditions, the potential increase in the number of human gut bacteria that could become resistant to kanamycin is 0.00000000000026%. This means that for every 380 humans that consume the mean level of genetically engineered tomatoes, one gut bacterium

susceptible to kanamycin may become resistant to the antibiotic (see also Redenbaugh et al. 1993).

The document provided the following conclusions on the *kan*^{r} selectable marker gene and APH(3')II:

- There would be no significant increase above background in human exposure to kanamycin- or neomycin-resistant bacteria from the consumption of food containing the *kan*^{r} gene.
- There would be no significant increase above background in kanamycin- or neomycin-resistant soil bacteria resulting from the commercial growing of tomatoes containing the *kan*^{r} gene.
- There would be no risk to the environment from the APH(3')II contained in tomatoes, cotton or rapeseed plants grown commercially.
- There would be no risk of compromising efficacy of oral therapeutic use of kanamycin and neomycin due to APH(3')II present in food.
- APH(3')II is degraded and inactivated in the human gastrointestinal tract to a level that does not represent a risk of toxicity.

May 1-July 30, 1991 First Public Comment Period

The FDA provided a 90-day public comment period on the use of the *kan*^{r} selectable marker gene in tomato, cotton, and rapeseed (Federal Register 56:20004-20005, May 1, 1991). There were 43 comments from various individuals, organizations, and institutions. Of these, only five expressed general concern about the use of genetically engineered food crops and four raised specific issues, principally on potential toxicity of APH(3')II and potential for compromising the efficacy of oral use of kanamycin. Of secondary concern were the potential for horizontal gene transfer, potential for immunological effects of APH(3')II, and whether quality control could be done to determine the number of *kan*^{r} genes and the level of APH(3')II.

The issues raised had all been addressed in the original submission to the FDA. Although the FDA did not request a response, Calgene provided additional details on the specific issues.

Specifically, horizontal gene transfer is not a risk for two reasons. First, no mechanism for transfer of genes from plants to microorganisms is known and no cases of such transfer have ever been reported. Second, using worst case probability estimates for hypothetical gene transfer, the additive effect of a *kan*^{r} gene entering the microbial flora from genetically engineered plants is insignificant when compared to the population of kanamycin resistant microorganisms naturally present.

There is no evidence that the APH(3')II protein is toxic to humans. The protein occurs naturally, being produced by bacteria in the human gut.

Experiments examining survival of active APH(3')II have shown that the enzyme is inactivated by pepsin in simulated gastric fluids and by simulated intestinal fluids, as is the case for any other typical protein. Even if not degraded, APH(3')II would be inactive in the absence of the energy producing cofactor ATP and under the low pH conditions of the gut. Glycosylation and subsequent increase in the antigenic capacity of APH(3')II would not occur because APH(3')II does not contain the necessary sequence information for transport to the subcellular locations at which glycosylation reactions take place.

Finally, quantitation of the number of *kan*^{r} genes and the amount of APH(3')II protein is precise, as demonstrated in the FLAVR SAVR tomato document.

<u>May 31, 1992 Submission</u>

Calgene provided additional data in response to consultations with the FDA on APH(3')II. This included further reports on the use of the *kan*^{r} gene

in human gene therapy, which showed that "the *neo*^{r} gene could be integrated with stability into the TIL genome, that high levels of neomycin phosphotransferase were produced, that transduced cells could be selected in G418, and that the growth patterns, cell-surface phenotype, and cytolytic functions of the TIL were not consistently altered by gene transduction [and that] such cells represented a minimal risk to the patient and posed no risk to health care personnel or the public" (Rosenberg et al. 1990). The conclusions were that "no safety problems of any kind" have been revealed (Rosenberg 1992) and "that retroviral mediated gene transduction [is] a feasible and safe means for introducing genes into humans," including the use of the *kan*^{r} gene (Rosenberg 1991).

In addition, data were submitted to the FDA which showed:

- APH(3')II is degraded under simulated human gastric and intestinal conditions.
- APH(3')II does not compromise efficacy of kanamycin.
- APH(3')II was shown to not have significant homology with known toxins.
- APH(3')II was shown to not have significant homology with known allergens.
- The *kan*^{r} gene has been used safely in human gene therapy.

October 30, 1992 Submission

Calgene provided additional data in response to consultations with the FDA concerning the effect of ATP on APH(3')II activity. A document, "The *kan*^{r} Selectable Marker: Evaluation of the Potential for Compromised Efficacy of Antimicrobial Therapy," was prepared which demonstrated that compromising the efficacy of oral neomycin therapy due to ingestion of

transgenic foods containing APH(3')II simultaneously with fruits and vegetables rich in ATP would be extremely unlikely. The results of this evaluation demonstrated that only a small fraction of neomycin could be inactivated under a worst case scenario, which is based on the assumptions below, several of which are extremely unlikely to occur.

Assumptions

1) 95th percentile consumption, at a single sitting, of specific fruits or vegetables high in ATP content.
2) Calculations based on Pao et al. (1982) survey of a three-day consumption period.
3) Stoichiometric reaction of 100% of the ATP in ingested food with orally administered neomycin (this is highly unlikely).
4) Administration of neomycin simultaneously with consumption of a transgenic food containing APH(3')II and with other fruits or vegetables rich in ATP.
5) Presence of intact, functional APH(3')II enzyme, which requires a buffered stomach environment (pH 7).
6) Stability of ATP in the stomach environment.

Oral doses of kanamycin are administered only for two medical problems: portal systemic encephalopathy and bowel surgery. During preoperative preparation for bowel surgery, it is highly unlikely that patients would be consuming any solid foods.

Calculations of potential inactivation were made for patients with portal systemic encephalopathy who consume fresh fruits and vegetables. Under these rare conditions where the maximum amount of neomycin could be inactivated, the loss of antibiotic efficacy would be an inactivation of 1.5% of a 1 g dose of neomycin for tomatoes.

January 4, 1993 Submission

Calgene requested the FDA Commissioner to issue a food additive regulation for aminoglycoside 3'-phosphotransferase II (APH(3')II) encoded by the *kan*^{r} gene, which is a selectable marker, used as a processing aid in the production of transgenic tomatoes, cotton and *Brassica napus* rapeseed. This request was based on the previous data submitted to the FDA. The FAP was assigned the number 3A4364.

The reasons for the FAP request were not related to any safety issues since the FDA was already conducting a thorough review, but rather related to a number of public relations and political issues:

- FAP requires a "formal" review process, which is more easily understood by the public.
- FAP reduces risk of lawsuits, since it is legally defensible.
- FAP eliminates risk of a new U.S. Administration overturning the advisory opinion.
- FAP sends a clear message to the consumer that FDA conducted a thorough safety review.
- Such approval does not preclude self-GRASing (Generally Recognized as Safe) for APH(3')II for these or other products at some future date.
- FAP is understood by the U.S. Congress.
- This FAP does not trigger a food product label or labeling, since APH(3')II is a processing aid that becomes a component of food.

Although the conversion to an FAP would normally start a new review period, this was not the case since APH(3')II had already been under extensive review since November 26, 1990. The FDA did request that Calgene revise and update the Environmental Assessment (EA) to meet the requirements of a food additive petition.

June 3, 1993 Submission

Calgene revised and submitted to the FDA an Environmental Assessment on APH(3')II. The concern with the fate of antibiotic resistance gene products is that as a consequence of the increase in antibiotic resistance genes, gene product (i.e., APH(3')II) could reach concentrations in the environment that may be toxic to plants, soil microflora, or wildlife. However, this is not likely due to the lack of persistence and low toxicity of most proteins (i.e., gene products) in the environment and due to the narrow substrate specificity of most enzymes, including APH(3')II (Nap et al. 1992). In general, natural organic materials are rapidly degraded in the soil, usually within three weeks (Tisdale and Nelson 1975). Furthermore, APH(3')II is not and has never been intended to be used as a toxic agent in the same manner that pesticides or herbicides are used.

APH(3')II levels were determined using Western analysis and were significantly lower than 0.1% of the total protein in all cases. As an example, the APH(3')II signal from 600 µg of BXN™ cottonseed protein had a weaker Western analysis signal than 16 ng pure APH(3')II. Therefore, the concentration of APH(3')II was less than 0.003% of the total seed protein. Assuming seed protein is approximately 22% of seed weight (Ensminger et al. 1990), the maximum amount of APH(3')II in 1 gram of unprocessed cottonseed is

= (0.22 g protein/g seed)(0.003% APH(3')II)

= 6.6 µg APH(3')II/g cottonseed

Cottonseed meal was also subjected to Western blot analysis. The APH(3')II signal from 600 µg of meal protein was less than the 8 ng purified APH(3')II signal in the western blots. Therefore, the concentration of APH(3')II was less than 0.0014% of the total meal protein. Assuming meal is approximately 41% protein (Ensminger et al. 1990), the maximum amount of

APH(3')II in 1 gram of processed cottonseed meal is

= (0.41 g protein/g meal)(0.0014% APH(3')II)

= 5.8 µg APH(3')II/g cottonseed meal

Based on these measurements, the levels of APH(3')II was less than 0.003% of the protein in unprocessed cottonseed and less that 0.0014% of the protein in processed cotton meal.

Similarly, levels of APH(3')II were measured in ripe FLAVR SAVR fruit (Redenbaugh et al. 1992) and in seed and processed meal of High Stearate rapeseed. The APH(3')II signals from 1200 µg of rapeseed protein and rapeseed leaves were less than the 10 ng signal of the purified APH(3')II in the western blots and the signal for 1200 µg of processed meal protein could not be detected (level of detection was 1 ng). Therefore, the levels of APH(3')II in High Stearate rapeseed whole seed and leaves are less than 0.0008% of the protein and less than 0.00008% of protein in the processed meal.

Calculations were made for potential release of the *kan*^{r} gene and gene product, APH(3')II, in the environment which show that even under the worst case, uptake, incorporation and expression of the gene would not be significant, since the numbers of naturally occurring kanamycin resistant microbes in the soil environment would continue to exceed any newly resistant forms by 7 orders of magnitude.

At worst, kanamycin-resistant transformants resulting from plant DNA left in the fields would constitute not more than 1/10,000,000 of the existing kanamycin-resistant soil population. The most realistic estimate is that kanamycin-resistant bacteria resulting from transformation by plant DNA would represent an addition of about one organism to the total number of kanamycin-resistant soil microorganisms present in one hectare.

There are several reasons why the levels of APH(3')II estimated to be released into the environment are of little concern. First, the estimated

releases of APH(3')II calculated in this section make use of several "worst-case" assumptions and, in addition, the estimates presented are not estimates of exposure, but estimates of soil "loading" at the field site. APH(3')II will undergo significant degradation in the soil similar to other "free" proteins released into the environment (Stotzky 1989). Also, released APH(3')II may become adsorbed (or sequestered) onto soil particles making it unavailable. Thus, degradation and sequestering would most probably reduce these release estimates, resulting in much lower actual exposure levels.

Another reason for little concern from release of APH(3')II involves a structure-activity argument. APH(3')II is an enzyme which catalyzes a specific reaction, an ATP-dependent phosphorylation of the 3'-hydroxyl group on the aminohexose moiety of a narrow spectrum of aminoglycoside antibiotics. Phosphorylation of the antibiotics interferes with the uptake and binding of these antibiotics to cellular ribosomes, thereby rendering the cells resistant to the antibiotics. This specifically defined activity functions only on antibiotic substrates and thus, would not be expected to produce any toxic effects in plants or animals. In addition, APH(3')II needs specific substrates in order to perform its catalytic activity. These substrates, ATP and aminoglycoside antibiotics, are not prevalent in the environment.

July 16-August 16, 1993 Second Public Comment Period

Because the Environmental Assessment had been revised and updated, the FDA provided a 30-day public comment period on the EA portion of the APH(3')II Food Additive Petition (Federal Register 58:38429-38430, July 16, 1993). Interestingly, no comments were received by the agency. This is a positive indication that all issues were previously resolved and that the conversion to an FAP was beneficial to public understanding and acceptance.

August 19, 1993 Submission

After consultation with FDA's Center for Veterinary Medicine, Calgene submitted a data package concerning the potential inactivation of neomycin in animal feed during storage due to activity of APH(3')II ("Stability of Antimicrobial Activity Attributable to Neomycin Sulfate in Cottonseed Meal Prepared from BXN Cotton and in Rapeseed Meal Prepared from Transgenic High Stearate and High Laurate Lines of *Brassica napus*").

The *kan*^{r} gene product has the potential, if not degraded, if active, if in the presence of sufficient ATP and if under suitable environmental conditions, to inactivate neomycin sulfate added to animal feed. An experiment was conducted to determine the stability of antimicrobial activity of neomycin sulfate, as determined by bioassay, in cottonseed meal and rapeseed meal prepared from transgenic lines containing the *kan*^{r} gene.

Key aspects of the experimental design were:

- Meal of the transgenic lines was prepared from freshly harvested field grown seed to assure the highest possible initial levels of the APH(3')II enzyme.
- Meal was prepared using pilot scale conditions that simulate, as near as possible, commercial-scale crush and extraction conditions.
- Mixing of the neomycin with meal was carried out using equipment/facilities typically used by veterinarians to prepare animal diets at the University of California, Davis, Department of Nutrition.
- The study protocol was developed in consultation with Dr. Tom Graham, DVM, of the University of California, Davis, School of Veterinary Medicine, Department of Nutrition.
- Samples were stored under controlled environmental conditions within the temperature optimum for activity of the APH(3')II enzyme.

- Neomycin bioassays were performed on coded samples at Barrow Agee Laboratories (Memphis, TN).

Using sensitive immunological methods for detection and measurement of the APH(3')II protein, Calgene determined that transgenic lines of cotton and rapeseed containing the *kan*^{r} gene produced detectable levels of the APH(3')II protein in seed. After 8 weeks of storage at 37°C, meal prepared from transgenic lines of cotton and rapeseed showed no significant decline in neomycin levels that could be attributed to the presence of the APH(3')II enzyme. This strongly indicates that commercial processing of rapeseed and cottonseed effectively denatures and degrades the APH(3')II protein, rendering it nonfunctional in the meal. Thus, there is no discernible risk that use of meal from transgenic cottonseed or rapeseed containing the *kan*^{r} gene would compromise the efficacy of neomycin in animal feed.

To further validate this study, meal samples were assayed for the presence of active APH(3')II enzyme, using cotton meal as representative of the transgenic cotton and rapeseed meals following processing. No activity was observed. These data support the position that there is no discernible risk that use of meal from transgenic cottonseed or rapeseed containing the *kan*^{r} gene will compromise the efficacy of neomycin in animal feed.

Conclusions of FDA's Safety Assessment of APH(3')II and the FLAVR SAVR Tomato

At the FDA food advisory committee meeting on April 6-8, 1994, scientists at CFSAN presented their safety assessment of the FLAVR SAVR tomato and the use of the *kan*^{r} selectable marker gene and gene product APH(3')II (Piercey 1994). CFSAN concluded that the FLAVR SAVR tomato was as safe and nutritious as other commonly consumed tomatoes. The food advisory committee made the following conclusions (Federal Register 59:26700):

- "The committee members generally expressed the view that the approach used by FDA to evaluate the safety of the tomato, including the safety of the *kan*^{r} gene, was appropriate and that all relevant scientific questions had been adequately addressed."
- "Transfer of the *kan*^{r} gene consumed as a component of tomatoes to microorganisms in the GI tract was highly unlikely."
- "The potential for transfer of the *kan*^{r} gene from plants to microorganisms in the environment is highly unlikely."
- If transfer could take place, "it would not cause a significant environmental impact."

On May 18, 1994, the FDA concluded that "the proposed use of APH(3')II as a processing aid in the development of new varieties of tomato, oilseed rape, and cotton is safe" (Federal Register 59:26700) and that the "FLAVR SAVR™ tomatoes have not been significantly altered when compared to varieties of tomatoes with a history of safe use" (Federal Register 59:26646). The following conclusions were provided by the FDA (Federal Register 59:26700):

- "FDA has determined that there is no need to set a tolerance for the amount of APH(3')II that will be consumed because the agency knows of no reason why this protein would have any properties that would distinguish it toxicologically from any other phosphorylating enzymes in the food supply."

Direct Effects of Ingestion of APH(3')II

- "FDA agrees with Calgene that the characteristics of APH(3')II do not raise a safety concern."
- "APH(3')II is not known to be toxic."
- "APH(3')II is a phosphorylating enzyme" and "does not possess any properties that would distinguish it toxicologically from other phosphorylating enzymes."

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- "APH(3')II has been shown to be rapidly degraded under simulated gastric conditions."
- "The estimated dietary exposure to APH(3')II is very low (480 µg APH(3')II per person per day, or 0.16 parts per million."
- "Probability of transfer of the *kan*^{r} gene to gut microflora is remote."
- "The *kan*^{r} gene will not compromise the efficacy of antibiotic treatment."
- "There is no evidence that free DNA containing the *kan*^{r} gene, even if present, can transform cells lining the GI tract."

Biological Activity of APH(3')II

- "Processed products that contain tomatoes with the *kan*^{r} gene are unlikely to contain any enzymatically active APH(3')II."
- "The biological activity of APH(3')II is destroyed during gastric and intestinal phases of digestion."
- "Any active APH(3')II that might remain would not significantly inactivate kanamycin or neomycin in the gut."
- "FDA concludes that the presence of APH(3')II in food will not compromise the therapeutic use of orally administered kanamycin or neomycin."
- "Oils derived from transgenic cottonseed and rapeseed modified using the *kan*^{r} gene would not be expected to contain active or inactive APH(3')II."
- "There was no significant difference with respect to neomycin stability between medicated cottonseed and rapeseed meals prepared from transgenic cottonseed and rapeseed containing APH(3')II."
- "Transgenic strains of cottonseed and rapeseed containing APH(3')II have no apparent untoward effect regarding the stability of neomycin and that the therapeutic efficacy of neomycin in animal feed will not be affected."

Environmental Assessment of APH(3')II

- "No increase in kanamycin-resistant soil microorganisms."

- "Even if such transfer could occur, the rate at which it could occur is such that it would not result in a detectable increase over the existing background population of kanamycin-resistant bacteria."
- "The use of the *kan*^{r} gene does not pose safety concerns in terms of increase in the population of antibiotic-resistant pathogens due to the potential for horizontal transfer of the gene."
- "Introduction of the *kan*^{r} gene will not confer a competitive advantage upon a plant receiving it."
- "Transfer of the *kan*^{r} gene to other crops or related weeds will have no significant adverse environmental effects."

This completes the safety assessment by the FDA on the FLAVR SAVR tomato and use of APH(3')II in tomato, cotton and oilseed rape. The FDA's review was specific for tomato, cotton and oilseed rape and they have stated that "approval of Calgene's petition would not mean that developers could use the *kan*^{r} gene in crops other than those identified in the petition" (Federal Register 59:26700. The FDA will continue to evaluate the use of the *kan*^{r} gene in other crops, as well as the use of other antibiotic resistance marker genes, on a case-by-case basis.

CONCLUSION

The U.S. Food and Drug Administration has concluded that "the characteristics of APH(3')II do not raise a safety concern." All evidence supports the conclusion that APH(3')II is not a toxin or allergen. Calculations were made to show that there is no risk of compromising efficacy of oral therapy of antibiotics for human or animal use. Enzymes, which include APH(3')II, generally do not raise safety concerns since very few toxic agents have enzymatic properties (Pariza and Foster 1983).

Emission of APH(3')II into the environment was shown to be of no concern. It is very unlikely that the *kan*^{r} gene which encodes APH(3')II could move from the plant genome via horizontal gene transfer; certainly, no mechanism of such transfer has been demonstrated. Even if such transfer could occur, there would still be no concern of emission of APH(3')II into the environment, since calculations using the most favorable assumptions and probabilities demonstrate lack of significant changes in the potential numbers of microorganisms containing the *kan*^{r} gene or potential impact on the level of APH(3')II in the environment.

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U.S. Food and Drug Administration

Food

Use of Antibiotic Resistance Marker Genes in Transgenic Plants

September 4, 1998

Guidance for Industry
Use of Antibiotic Resistance Marker Genes in Transgenic Plants
Draft Guidance

This guidance document is being distributed for comment purposes only.

Draft released for comment on: September 4, 1998.

Comments and suggestions regarding this draft document should be submitted by December 7, 1998, to Dockets Management Branch (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville MD 20852. For questions regarding this draft document, contact Carrie Hendrickson at (301) 436-1202 (regarding human food issues) or William Price at (301) 827-6652 (for animal feed issues).

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Guidance For Industry
Use of Antibiotic Resistance Marker Genes in Transgenic Plants

Antibiotic resistance genes are present in transgenic plants as a result of use as marker genes to select transformed plant cells. These genes are under the control of eukaryotic promoters and are expressed in the transgenic plant. Transgenic plants may also contain antibiotic resistance genes that are under the control of prokaryotic promoters, and therefore, not expressed. The latter are incorporated into plant genomes because they are present on constructs used to transform plant cells, having been used for selection in bacteria in earlier steps. In either case, crop developers should evaluate the safety of use of antibiotic resistance marker genes present in transgenic crops.

An evaluation of the safety of use of an antibiotic resistance marker, if it is expressed, should include an assessment of the safety of the protein or enzyme encoded by the gene, if present in food. Regardless of whether it is expressed, crop developers should evaluate the potential for therapy with antibiotics to be compromised through transfer of the gene from plants to microorganisms in the gut of man or animal, or in the environment.

Safety evaluation of a protein encoded by an antibiotic resistance marker gene should include 1) an assessment of potential toxicity of the protein, 2) an assessment of whether the protein has the potential to elicit allergenic reactions, and 3) an assessment of whether the presence in food of the enzyme or protein encoded by the antibiotic resistance marker gene would compromise the therapeutic efficiency of orally administered antibiotic.

FDA acknowledges that the likelihood of transfer of an antibiotic resistance marker from plants to microorganisms in the gut or in the environment is remote and that, such transfer, if any, would likely be insignificant when compared to transfer between microorganisms, and in most cases, would not add to existing levels of resistance in bacterial populations in any meaningful way. Nonetheless, FDA believes that developers should evaluate the use of antibiotic resistance marker genes in crops on a case-by-case basis taking into account information on 1) whether the antibiotic is an important medication, 2) whether it is frequently used, 3) whether it is orally administered, 4) whether it is unique, 5) whether there would be selective pressure for transformation to take place, and 6) the level of resistance to the antibiotic present in bacterial populations. If a careful evaluation of the data and information suggests that the presence of the marker gene or gene product in food or feed could compromise the use of the relevant antibiotic(s), the marke gene or gene product should not be present in the finished food or feed. FDA notes that certain antibiotics are the only drug available to treat certain clinical conditions (e.g., vancomycin for use in treating certain

staphylococcal infections). Marker genes that encode resistance to such antibiotics should not be used in transgenic plants.

The guidance represents the agency's current thinking on the use of antibiotic resistance marker genes in transgenic plants. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. An alternative approach may be used if such an approach satisfies the requirements of the applicable statute, regulations, or both.

Report on Consultations Regarding Use of Antibiotic Resistance Marker Genes in Transgenic Plants

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Consultations Regarding the Use of Antibiotic Resistance Marker Genes in Transgenic Plants

I. Need for Consultations

In 1992, the Food and Drug Administration (FDA) issued a policy statement regarding foods derived from new plant varieties including those derived using genetic engineering techniques (U.S. FDA, 1992). In this policy statement, FDA specifically discussed antibiotic resistance selectable marker genes(1) and noted that both the antibiotic resistance gene and gene product, unless removed, are expected to be present in foods(2) derived from plants developed using the markers. The agency acknowledged that selectable marker gene-encoded enzymes that inactivate certain clinically useful antibiotics, when present in food, theoretically might reduce th therapeutic efficacy of antibiotics administered orally. Thus it is important to evaluate such concerns with respect to commercial use of antibiotic resistance marker genes in food, especially those that will be widely used. In addition, the agency believes it is important to consider the possibility that resistance to antibiotics in microorganisms might spread through potential horizontal transfer of antibiotic resistance marker genes from plants to microorganisms in the gastrointestinal tract or in the environment.

Since FDA's decision regarding the use of the kanamycin resistance (*kan*^{r}) gene product, aminoglycoside 3'-phosphotransferase II (APH(3')II, also known as neomycin phosphotransferase II or nptII) in the developmen of transgenic tomato, cotton, and oilseed rape (U.S. FDA, 1994, see also Appendix 1), the agency has continued to receive inquiries regarding the safety and regulatory status of antibiotic resistance marker genes Therefore, FDA sought to develop sound scientific principles regarding the safety of the use of antibiotic resistance marker genes in the development of transgenic plants for food use and to provide sound scientific guidance to crop developers regarding the safe use of antibiotic resistance marker genes. Towards this end, FDA undertook several consultations with outside experts between November, 1996 and February, 1997. The purpose of the consultations was to determine whether circumstances exist under which FDA should recommend that a given antibiotic resistance gene not be used in crops intended for food use, and if so, to delineate the nature of those circumstances.

In order to facilitate the consultations, the agency developed several questions to form the basis of the discussions with the outside experts. These included: What are the therapeutic uses of the antibiotics that th marker gene products inactivate and how widely are they used? How prevalent is resistance to these antibiotics among bacteria naturally found in the gut or in the environment? For each antibiotic resistance marker gene that is expressed in a transgenic plant, what is the likelihood that clinical therapy could be compromised due to inactivation of the oral dose of the antibiotic from consumption of processed or unprocessed food derived from the transgenic plant? What is the likelihood that events such as rearrangement, recombination, and translocation, would result in changing the expression of the antibiotic resistance marker gene? What is the likelihood that the therapeutic use of an antibiotic could be compromised from transfer of the antibiotic resistance gene from food to gut epithelium with subsequent expression? What is the likelihood that antibiotic resistance marker genes could be transferred from transgenic plants to soil microorganisms with subsequent expression of the gene? How meaningful is the potential rate of transfer, if any, of an antibiotic resistance gene to pathogenic microorganisms thereby rendering them refractory to the antibiotic? Are there any other issues or other information regarding antibiotic resistance marker genes that the agency should take into account in developing guidance to industry on the selection and use of these genes?

II. Format of the Consultations and List of Experts

The experts came to Washington, DC, to engage in separate discussions with a team of scientists from FDA's Center for Food Safety and Applied Nutrition (CFSAN), Center for Veterinary Medicine (CVM), and Center for Drug Evaluation and Research (CDER). Prior to their arrival, the experts were provided with background material including 1) FDA's policy regarding foods derived from new plant varieties, which was published in the May 29, 1992 issue of the *Federal Register,* 2) the final rule authorizing the use of the *kan*^{r} gene product, APH(3')II, in the development of transgenic tomato, cotton and oilseed rape, which was published in the May 23, 1994 issue of the *Federal Register,* 3) the proceedings of a World Health Organization workshop on antibiotic resistance marker genes that took place on September 21-24, 1993, in Copenhagen, Denmark, 4) a document by the UK Advisory Committee on Novel Foods and Processes on the use of antibiotic resistance markers in transgenic plants, 5) a Nordic Council of Ministers document entitled "Health Effects of Marker Gene in Genetically Engineered Food Plants," 6) proceedings of a Joint FAO/WHO Expert Consultation on Biotechnology and Food Safety that was held from September 30 to October 4, 1996, 7) an Environmental Protection Agency final rule that established an exemption from the requirement of a tolerance for residues of

APH(3')II in transgenic plants when used as a plant pesticide inert ingredient, 8) the consultation procedures FDA has been following for foods and feeds derived from transgenic plants, and 9) a list of products on which such consultations have been completed, together with a recent example of a memorandum to the file, and a letter that is issued to the crop developer to signify official closure of the consultation.

Each consultation began with an overview of FDA's food biotechnology policy, the marker genes used in transgenic plants and those that the agency has seen in its consultations to date, and a review of FDA's evaluation of the food safety and environmental safety of *kan*^{r} gene and its gene product, APH(3')II, for use in developing transgenic tomato, cotton, and oilseed rape.

The following experts participated.

- Mitchell L. Cohen, M.D., Director, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA.
- Michael Gilmore, Ph.D., Professor, Department of Medicine, University of Oklahoma, Oklahoma City, OK.
- Donald Lein, D.V.M., Head, Diagnostics Laboratory, Cornell University College of Veterinary Medicine, Ithaca, NY.
- Abigail Salyers, Ph.D., Professor, Dept of Microbiology, University of Illinois, Urbana, IL.
- Kenneth Wilson, M.D., Professor of Medicine, Duke University, Infectious Diseases Section, VA Medical Center, Durham, NC.

III. Summary of the Consultations

A. Direct Effects of Ingestion of Enzymes Encoded by Antibiotic Resistance Marker Genes

Throughout the consultations, the potential effects due to ingestion of enzymes encoded by antibiotic resistance genes as components of food raised little concern in comparison to potential health effects from transfer of antibiotic resistance genes to microorganisms. The potential safety issues, whether toxicity or allergenicity of the gene product, or potential inactivation of an orally administered antibiotic by the gene product, could be addressed by considerations similar to those the agency undertook in its evaluation of the safety of APH(3')II (U.S. FDA, 1994).

For example, digestibility of the protein product by digestive enzymes, functional similarity to other proteins in the diet, lack of sequence homology to known toxins in the sequence databases, and lack of reported toxicity in the literature could be used as indications that the protein does not have toxic properties. In addition, since most of these proteins are likely to be from sources that are not known to be allergenic, *in vitro* or *in vivo* tests for allergenicity would not be useful. However, it was agreed that a determination that the protein does not have any of the properties that are common to allergenic proteins, such as resistance to digestion, heat or acidic pH, coupled with lack of homology to known allergenic proteins in the protein and nucleic acid sequence databases, can give some assurance that a protein encoded by an antibiotic resistance marker gene will not elicit allergenic reactions when consumed as a component of food.

With respect to whether an antibiotic resistance gene product found in food could compromise therapy with a clinically useful antibiotic by inactivating it *in situ*, some assurance against this possibility could be obtained by showing that the gene product is readily digestible or is inactivated by processing methods used by food producers. In addition, if the gene product requires a cofactor in order to inactivate the antibiotic, one could examine whether the concentration of cofactor would be limiting through theoretical calculations. Cofactor requirements could also be addressed through direct experimentation. Finally, in the event of some potential inactivation of antibiotic, advice could be provided that the antibiotic likely to be affected should not be taken together with food. In cases where this is unavoidable and some inactivation is anticipated, increased doses could be given to make up for the fraction of antibiotic that would be inactivated provided that amount can be determined.

In the case of animal feed, it is likely that production methods will denature the enzyme thereby rendering it inactive against the antibiotic. Further, it was noted that most antibiotics are delivered through drinking water rather than feed due to cost considerations. Where antibiotic is added to animal feed, the potential for antibiotic inactivation could be determined by preparing medicated feed from the transgenic plant and analyzing for any loss of potency of antibiotic. This procedure was used by Calgene Inc., for feed containing neomycin prepared from transgenic oilseed rape and cotton containing APH(3')II (U.S. FDA, 1994).

B. Potential Transfer of Antibiotic Resistance Marker Genes to Gut Epithelial Cells

Several issues were considered by FDA in its approval of Calgene's food additive petition for the use of APH(3')II with respect to why potential transfer and expression of the *kan*^{r} gene in gut epithelial cells do not raise a safety concern. Transfer is considered to be unlikely because the DNA is degraded by nucleases and even if some were to survive digestion and were transferred, integrated and expressed, epithelial cells are short lived and would slough off to be replaced by untransformed cells. The above considerations would also be applicable to evaluation of other antibiotic resistance marker genes. Although a large amount of food-derived DNA regularly passes through the gastrointestinal tract, there are no published reports on the

transfer, integration or expression of genes in cells lining the gut. Some experts cautioned that one should assume that DNA can get into the cells lining the gut[3]; however, the critical factor is the lack of selective pressure. Without selective pressure, it is highly unlikely that genes taken by these cells would be expressed even if integrated into the genome. In addition, these cells are sloughed regularly and replaced by new cells. Finally, crypt cells are not sloughed off; however, even if DNA containing an antibiotic resistance gene could ge into these cells and integrate into their genomes, lack of selective pressure makes it unlikely that the gene would be expressed.

C. Potential Transfer of Antibiotic Resistance Marker Genes to Gut Microorganisms

It is highly unlikely that antibiotic resistance genes could be transferred from plant genomes to gut microorganisms. First, there are no known mechanisms for the direct transfer of plant genomic DNA to microorganisms. Second, there are several barriers to potential transfer. These include degradation by acid and nucleases in the stomach and intestines, the bacterial restriction and modification systems that destroy foreign DNA that enters the cell, the absence of homologous ends for efficient integration into the bacterial genome, and lack of selective pressure.

In addition, when any DNA (including antibiotic resistance genes) is integrated into plant genomes, the codon usage may have been altered for more efficient expression in the plant and the gene may have picked up methylation patterns of the plant. If this DNA is now taken up by a bacterium, it would be recognized as foreign and degraded by the microorganism's restriction endonucleases, thus making integration into the genome and subsequent expression even more unlikely. Moreover, transfer between bacteria, even among broadly different phylogenetic lines, is far more likely than transfer from plants to bacteria. Finally, since uptak is usually not sequence-specific, the antibiotic resistance gene would be competing for transfer into a bacterium with the rest of the DNA in the plant genome and DNA from other sources in the diet[4].

Nonetheless, the possibility was raised that unlikely events could take place given sufficient selective pressure and that, because of the short generation times of bacteria, clonal expansion of the transformed bacteria coul take place. For these reasons, and because some antibiotics are so important clinically, it is prudent for developers to ensure that marker genes that encode resistance to clinically important antibiotics are not present in food or feed derived from new plant varieties. For example, vancomycin was cited as a drug of last resort for some staphylococcal and enterococcal infections. Additional critical antibiotics mentioned by some of the experts were other glycopeptides, fluoroquinolones, tetracycline, gentamicin and the later derivatives of *beta*-lactam antibiotics[5].

Overall, the arguments made concerning the improbability of the transfer of the *kan*^{r} gene to gut microorganisms in the case of the Flavr Savr^{TM} tomatoes (U.S. FDA, 1994) could also be applied to other antibiotic resistance marker genes. However, while the possibility of transfer from plants to microorganisms is remote for all marker genes, crop developers should consider the following factors in evaluating whether an antibiotic resistant gene is suitable for use as a selectable marker: 1) whether the antibiotic that may be affected is clinically important, 2) whether it is frequently used, 3) whether it is administered orally, 4) whether it is unique or acceptable alternative antibiotics exist, 5) whether there would be selective pressure for transformants to be selected, and 6) whether there already is resistance to the antibiotic in the environment.

FDA's analysis (U.S. FDA, 1994) showed that the *kan*^{r} gene that was used in the case of the Flavr Savr^{TM} tomato passes the paradigm outlined above. Neomycin and kanamycin are infrequently used antibiotics, neither is unique for any use, and rarely are administered orally. Thus, selective pressure would be minimal fo development of resistant bacteria because the drugs are not used in humans or in animals to any great extent. Similarly, these antibiotics are not used in agriculture or aquaculture to any great extent. Therefore, they would not provide the selective pressure required to select for the *kan*^{r} determinant if transfer from plant to soil microorganisms were to take place. In addition, existing resistance levels far exceed any transfer that may take place from transgenic plants to microorganisms.

However, different circumstances may apply to other antibiotics. For example, with regard to the presence or absence of selective pressure, streptomycin and oxytetracycline may provide selective pressure in the environment because of their use as pesticides in agriculture. On the other hand, ampicillin may provide selective pressure in the human gut when used in the clinical setting but not in the environment.

The notion that it may be possible to construct a list of antibiotic resistance marker genes that are acceptable for use in the development of transgenic crops was discussed. The *kan*^{r} gene can be placed on such a list. Some experts suggested that the hygromycin resistance gene may be included on such a list because of its limited use in humans. It was noted, however, that it may have important veterinary uses. Other experts would include the *beta*-lactamase gene of pUC18 (that confers resistance to a narrow spectrum of *beta*-lactam antibiotics), and the tetracycline resistance gene on such a list. There is so much resistance to these antibiotic already in the environment that any potential transfer from transgenic plants to microorganisms, especially when compared to transfer among bacteria, is unlikely to add to the existing levels of resistance in any

meaningful way.

The idea was put forth that antibiotic resistance genes could be ranked on a continuum with the *kan*^{r} gene on one end as the most acceptable, and the vancomycin resistance gene on the other. It was further suggested that use of marker genes beyond the *kan*^{r} and hygromycin resistance genes might be acceptable on the basis of studies to address potential transfer and a commitment to conduct post-market surveillance for transfer of the gene in question (see below).

D. Potential Transfer of Antibiotic Resistance Marker Genes to Microorganisms in the Environment

Some experts noted that some soil microbes may be naturally transformable and that they may take up and incorporate DNA causing genomic rearrangements that might help them occupy particular ecological niches. Some experts felt that it does not make sense to expand the availability of the resistance gene in the environment because abundance of marker genes may compress the typical 4- 5-year time lag between first use of new antibiotic and the emergence of resistance in hospitals. Others felt that the risk of transfer from plant genome to soil microorganisms is not a significant one. This latter group felt that DNA from plant debris would be unavailable for transfer because it would be degraded by nucleases when the plant cell lysed; in addition, there would be no selective pressure in most cases although there are exceptions such as when streptomycin and tetracycline are used as pesticides to prevent fire blight in fruit trees, or when manure is used as fertilizer following use of antibiotics as growth promoters in animals. It was noted that transfer from bacteria to bacteria accounts for the wide dissemination of certain antibiotic resistance markers in soil bacteria

E. Approaches to Assessing Potential for Transfer of Antibiotic Resistance Marker Genes and Conducting Surveillance for Resistance

Participants in the consultations discussed a study conducted by a crop developer wherein plant DNA containing the *beta*-lactamase gene from an insect-resistant transgenic corn line (intact or nuclease degraded) was incubated with competent *E. coli*. The study was intended to determine if any bacteria were transformed and acquired ampicillin resistance; the experiment showed that transformation did not occur above a frequency of 1 in 6.8 x 10^{19}. Some experts said that if transformation were to take place, it would be more likely to do so in experiments using competent bacteria in the laboratory than in nature because competent bacteria have the highest transformation frequency. They added that if transformation was not observed in the laboratory, especially if the experiment was carried out in gram negative and gram positive bacteria (an *Enterococcus* strain and an *E. coli*), the results would suggest that such transfers may not take place in the natural setting to the extent that they would raise health or safety concerns.

Other experts stated that an *in vitro* experiment does not give them much confidence because it does not reflect the complex ecological system that exists in nature. In addition, a monoculture of *E. coli*, is an artificial system that would not be a strong basis on which to assess risk.

The types of experiments that are conducted should be commensurate with the importance of the antibiotic that may be compromised. While an *in vitro* model would suffice for an antibiotic that is relatively unimportant clinically, studies in animals may be warranted for important antibiotics. Some experts suggested that the conclusion could be made that transfer does not take place if a large number of animals were fed bioengineered plants containing an antibiotic resistance gene under intense selective pressure, and new resistant microorganisms with this genotype were not observed.

Some experts also suggested that FDA might consider requiring crop developers to monitor for the spread of resistance due to use of an antibiotic resistance marker in a transgenic plant, especially if the gene confers resistance to a clinically important antibiotic. However, it would be a difficult task to document transfer due to the high levels of resistance that already exist. Monitoring, if conducted, should be at the genetic level rather than at the phenotypic level and, given that all resistance genes originate from microorganisms, it should distinguish a gene that has been transferred from a plant (for example, by looking for unique sequences that may be embedded in the gene in question). It was noted that examining areas with a high concentration of transgenic biomass would increase the chances of finding the rare transfer event. Alternatively, monitoring antibiotic resistance markers where the antibiotic is used in feed as a growth promoter would increase the chance of finding a microbial transformant because there would be selective pressure.

Because the argument is often made that the rate of transfer of an antibiotic resistance marker gene to microorganisms in the gut would be so low as to be meaningless when compared to existing levels of antibiotic resistance, some experts suggested that a survey should be undertaken of present levels of antibiotic resistance among several microbial populations. While such data exist for some resistance genes and for some microorganisms, it was noted that there are gaps in the database. Such a survey would involve isolating DNA from about 100 representatives from each major genus (*Bacteroides, Eubacterium, Bifidobacterium, Peptostreptococcus*, *etc.*). Many of these organisms can be obtained from various laboratories and institutes, and probed for antibiotic resistance marker genes. To look for those genes that occur less commonly, PCR analysis of rectal swabs from humans and animals could be done. Data obtained from these studies would provide evidence concerning the presence of a specific antibiotic resistance gene, and if present, how prevalent it is in the general population of naturally occurring intestinal bacteria.

IV. Conclusions

- The approach taken by FDA in its evaluation of the safety of the use of the *kan*^{r} gene and its product, APH(3')II, in the development of transgenic tomato, cotton, and oilseed rape, is scientifically sound and included all relevant parameters. These included: 1) evaluation of the safety of the protein with respect to toxicity and allergenicity, 2) an assessment of whether presence in food of APH(3')II would compromise the therapeutic efficiency of orally administered neomycin and kanamycin, and 3) an assessment of whether therapy with antibiotics might be compromised through transfer of the *kan*^{r} gene from plants to microorganisms in the gut or in the environment or to the cells lining the gastrointestinal tract.
- The presence in food of proteins encoded by antibiotic resistance genes is not of great concern. They can be evaluated with respect to toxicity and allergenicity and with respect to potential to compromise therapy with antibiotics (in similar fashion to the approach taken by FDA for APH(3')II).
- Similarly, the potential transfer of antibiotic resistance genes from foods derived from transgenic plants to cells lining the gastrointestinal tract does not raise a safety concern. Most DNA is degraded in the gut and thus, would be unavailable for transfer, and even if some DNA survived and was available for transfer into these cells, it would not be integrated and expressed due to lack of selective pressure. Additionally, because these cells are continuously sloughed off and replaced by new cells, a cell that incorporated an antibiotic resistance gene would not be long-lived and present a safety hazard with respect to compromising therapy with antibiotics.
- The likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of man or animal, and in the environment is remote. Several barriers operate against such transfer. In addition, the rate of such transfer, if any, would be insignificant when compared to transfer between microorganisms, and would not add to existing levels of resistance in bacterial populations in any meaningful way. Nonetheless, caution should be the rule for antibiotic resistance markers that inactivate clinically important antibiotics.
- The *kan*^{r} gene is safe to use as a selectable marker in the development of transgenic crops. Some experts also felt that there would be little concern with use of the hygromycin resistance gene as a selectable marker. However, hygromycin may have important veterinary uses and, therefore, its use should be carefully evaluated in those crops that have animal feed applications.
- There are varying levels of concern with use of other antibiotic resistance genes as selectable markers in transgenic plants, with the highest level of concern for those genes that confer resistance to antibiotics such as vancomycin, an antibiotic viewed as a drug of last resort for some infections. Overall, use of these antibiotic resistance marker genes should be evaluated on a case-by-case basis with the evaluation taking into account information on 1) whether the antibiotic is an important medication, 2) whether it is frequently used, 3) whether it is orally administered, 4) whether it is unique, 5) whether there would be selective pressure for transformation to take place, and 6) whether there already is resistance to the antibiotic in bacterial populations.
- Some experts suggested that surveying the current levels of resistance to various antibiotics would be important in order to gauge the impact of any potential transfer of antibiotic resistance genes from transgenic plants to microorganisms. In addition, some experts recommended that developers of transgenic crops might be encouraged to conduct surveillance to ascertain that transfer of antibiotic resistance genes from transgenic plants to microorganisms is not taking place. Such surveillance would safeguard against the occurrence of drug resistance from use of marker genes in transgenic plants and provide the public health community and the regulatory agencies an opportunity for early intervention to prevent adverse impact on public health.

V. References

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VI. Notes

(1) Transformation of plant cells by introducing exogenous genes is an inefficient process and only a small proportion of cells successfully take up, integrate, and express the new genetic material. Because the new cells

that do so are not readily distinguishable from the vast majority that do not, developers frequently link an antibiotic resistance marker gene to the gene(s) of interest to allow them to distinguish between transformed and nontransformed cells. Plant cells that are not transformed die when grown in medium containing the antibiotic while those that are transformed live because incorporation and expression of the antibiotic resistance marker gene enables them to counter the action of the antibiotic. Those cells, which contain the marker gene and other gene(s) of interest are subsequently regenerated into transgenic plants.

(2) In this report, the term "food" encompasses both human food and animal feed.

(3) A recent paper (Schubbert, et al., 1997) demonstrated, using phage DNA, that DNA ingested in food is not completely degraded in the gastrointestinal tract of mice, can reach other organs via the intestinal wall mucosa, and can be covalently linked to mouse DNA in these organs.

(4) The fact that an antibiotic resistance gene is under a eukaryotic promoter in the plant is frequently cited as a barrier because, should transfer take place, the eukaryotic promoter would not be able to direct its expression in the microorganism. However, the experts noted that rearrangements, especially under selective pressure, can easily bring a prokaryotic promoter in front of the gene leading to expression.

(5) The pUC19 *beta*-lactamase gene typically seen in recombinant plant genomes under the control of a bacterial promoter would not pose a health hazard should it be transferred and expressed. Unlike the *beta*-lactamase genes that confer resistance not only to a wide variety of *beta*-lactam antibiotics but also to *beta*-lactamase inhibitors that have been used to "recycle" antibiotics (*e.g.* ampicillin) and are currently causing problems in hospitals, this *beta*-lactamase poses no clinical problems because there are many antibiotic formulations that easily control bacterial strains producing it (Salyers, 1996).

Appendix 1. Evaluation of the Safety of the Kanamycin Resistance Gene as a Selectable Marker

I. Background

The kanamycin resistance (*kan*^{r}) marker gene is one of the most widely used selectable marker genes. The *kan*^{r} gene, which was originally isolated as a component of transposon Tn5 from the bacterium *Escherichia coli* (Beck et al., 1982) encodes aminoglycoside 3'-phosphotransferase II (APH(3')II)(6). APH(3')II is an enzyme with an apparent molecular weight of 25,000 that catalyzes the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of aminoglycoside antibiotics including neomycin, kanamycin, paromomycin, ribostamycin, gentamicins A and B, as well as butirosins, thereby inactivating the antibiotics (Davies et al., 1978; Goldman et al., 1976). Of the antibiotics that are inactivated by APH(3')II, only neomycin and kanamycin are currently in therapeutic use for humans and animals in the United States (U.S. Pharmacopeia, 1990; Prescott et al., 1988)(7).

On November 26, 1990, Calgene, Inc., of Davis, California, submitted to FDA, a request for advisory opinion regarding whether the *kan*^{r} gene may be used as a selectable marker in the production of genetically engineered tomato, cotton, and oilseed rape plants intended for human food and animal feed uses (*kan*^{r} Gene Safety and use in the production of genetically engineered plants, Docket Number 90A-0416). In the May 1, 1991 issue of the *Federal Register*, FDA announced that the request had been received and solicited comments from interested persons (U.S. FDA, 1991). The data submitted to the agency with the request for advisory opinion and the comments received were made available to the public at the Dockets Management Branch.

Subsequently, in January 1993, Calgene requested that FDA convert its request for advisory opinion to a food additive petition under section 409 of the Act. The FDA then announced in the *Federal Register* of July 16, 1993 that a food additive petition had been filed by Calgene, proposing that the food additive regulations be amended to provide for the safe use of APH(3')II as a processing aid in the development of new varieties of tomato, oilseed rape, and cotton (U.S. FDA, 1993).

Calgene had also sought an advisory opinion from the agency on its Flavr Savr^{TM} tomato in a letter dated August 12, 1991. After it completed its evaluation of the safety of the Flavr Savr^{TM} tomato and the use of APH(3')II in the development of tomato, oilseed rape and cotton, FDA, in May, 1994, convened its Food Advisory Committee to deliberate on its evaluation of Calgene's Flavr Savr^{TM}. Because the *kan*^{r} gene is the selectable marker used in the Flavr Savr^{TM}, the Food Advisory Committee also deliberated on FDA's evaluation of the safety of the use of the selectable marker in the tomato. Following these deliberations FDA amended the food additive regulations to permit the use of APH(3')II in the development of genetically modified tomatoes, oilseed rape, and cotton intended for food use (U.S. FDA, 1994).

Only the product of the *kan*^{r} gene, APH(3')II, and not the gene itself, was regulated as a food additive. The DNA that makes up the *kan*^{r} gene does not differ from any other DNA and does not itself pose a safety concern as a component of food. As the 1992 policy statement made clear, because DNA is present in the cells of all living organisms, including every plant and animal used for food by humans or animals, and is efficiently digested, FDA does not anticipate that DNA would itself be regulated as a food additive. However, as

discussed below, because of the property of the *kan*^{r} gene to confer antibiotic resistance, the agency considered the possibility that the gene might be transferred to other organisms.

II. Issues Considered by The FDA

Safety issues associated with use of APH(3')II in the development of transgenic tomato, cotton and oilseed rape that the agency considered can be divided into two areas: (1) Those associated with the direct effects of ingestion of the protein, including the possibility of allergenicity; and (2) those associated with the biological activity of APH(3')II (*i.e.*, the effect of the enzyme on the therapeutic efficacy of orally administered antibiotics) The agency also evaluated whether there are any safety concerns from potential horizontal transfer of the *kan*^{r} gene in tomato, oilseed rape, and cotton.

A. Direct Effects of Ingestion

The FDA evaluated data that showed that, unlike most allergenic proteins, APH(3')II is rapidly inactivated by stomach acid, is degraded by digestive enzymes (Fuchs et al., 1993), and is not glycosylated when produced i the transgenic tomato, oilseed rape, and cotton. In addition, enzymes such as APH(3')II are heat labile. Thus, APH(3')II does not possess any of the characteristics associated with allergenic proteins such as proteolytic stability, glycosylation, or heat stability (Taylor et al., 1987). In addition, protein and DNA sequence comparisons using sequences in four separate databases (GenBank, EMBL, PIR 29, and Swiss-Prot) showed that APH(3')II does not have significant homology to any proteins listed as food allergens or toxins in these databases.

The agency also noted that it was not aware of any reports that indicate that APH(3')II might be toxic and further noted that all plants and animals that are part of the food supply contain phosphorylating enzymes such as APH(3')II that have been consumed without adverse consequences. Further, acute mouse feeding studies showed that feeding extremely high doses of purified APH(3')II caused no deleterious effects (Fuchs e al., 1993). Finally, FDA calculated that the estimated dietary exposure (EDI) to APH(3')II is very low (480 µg APH(3')II per person per day[(8)], or 0.16 part per million in the diet, based on a 100-percent market share for tomatoes containing APH(3')II). For these reasons, the agency concluded that there are no allergenicity or toxicity concerns from ingestion of the enzyme.

B. Effects on the Therapeutic Efficacy of Orally Administered ntibiotics

1. APH(3')II in human foods

In assessing whether there could be effects on the therapeutic efficacy of orally administered antibiotics, FDA considered only APH(3')II from fresh tomatoes to be relevant because it is the only form that is enzymatically active. Processed tomato products are subjected to high temperatures that would be expected to inactivate the APH(3')II enzyme. For edible oils extracted from cottonseed and rape seed, high temperature treatment, solvent extraction, and subsequent purification steps generally included in the processing of such oils would also be expected to inactivate APH(3')II.

The FDA evaluated several studies intended to address whether APH(3')II consumed as a component of fresh tomatoes could render orally-administered kanamycin ineffective. These experiments were performed under simulated gastric and intestinal conditions (*i.e.*, appropriate pH, reagent concentrations, temperature, and reaction times) chosen to reflect conditions expected *in vivo*. In some studies both tomato extract and nonfat milk were added to determine whether the presence of additional food-source proteins in the simulated gastri and intestinal fluids might slow the proteolytic degradation of APH(3')II by competition. After evaluating data on the loss of immunologically detectable APH(3')II, FDA concluded that, under normal gastric and intestinal conditions, APH(3')II would be effectively degraded before the enzyme could inactivate kanamycin or neomycir and therefore, APH(3')II would not interfere with orally administered kanamycin or neomycin therapy.

In addition, FDA evaluated the results of *in vitro* degradation studies performed under simulated abnormal gastric conditions, such as may exist in patients treated with drugs that reduce stomach acidity. These studies showed that APH(3')II is not degraded in neutralized (pH 7.0) simulated gastric fluid and thus, APH(3')II may remain active in such abnormal gastric conditions. However, FDA concluded that, even under those conditions, APH(3')II would not be expected to inactivate significant amounts of orally administered kanamycin or neomycin because the concentration of ATP, which the enzyme requires to inactivate kanamycin and neomycin would be limiting. The FDA evaluated data from the published literature on ATP levels in fresh fruits and vegetables, estimated ATP intake, and calculated the fraction of neomycin that would be phosphorylated even making the conservative assumption that all of the ATP will survive the intestinal phosphatases and be available to react with the antibiotic. Even under the worst-case situation (high intake of ATP-containing food, low dose of antibiotic) FDA determined that only a small fraction (no more than 1.5 percent) of the antibiotic would be inactivated. This conclusion was supported by data from an *in vitro* study that showed that no significant inactivation of kanamycin was observed when tomato extract containing APH(3')II and kanamycin was incubated over a 4-hour period.

In addition, the agency also considered the patient population likely to be exposed to aminoglycoside antibiotics. Oral aminoglycosides are most commonly administered to either pre-operative patients (prior to bowel surgery) or patients with hepatic encephalopathy. Neither patient population would be expected to be ingesting tomatoes or any other fresh fruits and vegetables; therefore there is little or no risk of inactivating the oral antibiotic in these patients. For these reasons, FDA concludes that the presence of APH(3')II in food will not compromise the therapeutic use of orally administered kanamycin or neomycin.

2. APH(3')II in animal feed

The FDA also considered the potential inactivation of neomycin that is used in animal feeds manufactured usin cottonseed meal and rape seed meal obtained from transgenic plants. The transgenic tomato was not considered because very little tomato and tomato byproducts are used in the animal feed industry. Further, neomycin is primarily used to treat calves and swine whereas tomato byproducts, to the extent that they are used in animal feed, are primarily used as ingredients in cattle diets.

The agency reviewed data on neomycin levels both in nontransgenic medicated cottonseed and rape seed meals and in transgenic medicated cottonseed and rape seed meals over a storage period of 56 days (considered a worst-case situation) and concluded that there was no significant inactivation of neomycin and thus, the therapeutic efficacy of neomycin in animal feed will not be affected. The agency also considers this conclusion applicable to other aminoglycoside antibiotics inactivated by APH(3')II, when orally administered.

C. Potential Transfer of the Kanamycin Resistance Gene

The agency also evaluated issues relevant specifically to the safety of the use of the *kan*^{r} gene in tomato, oilseed rape, and cotton. In particular, FDA evaluated the potential for horizontal transfer of the gene and subsequent expansion of the population of antibiotic-resistant pathogens. The agency evaluated whether efficacy of oral antibiotic treatment of humans or animals could be compromised by consumption of food containing the *kan*^{r} gene either because of transfer of the gene from food to resistant intestinal microflora or to cells lining the intestinal lumen. In addition, the agency considered the possible transfer of the *kan*^{r} gene from transgenic plants to soil microorganisms and expansion of the antibiotic-resistant bacterial population.

1. Potential transfer of the *kan*^{r} gene to intestinal microorganisms and cells lining the intestinal lumen.

The agency evaluated theoretical and experimental evidence that demonstrate that the potential for compromise of antibiotic therapy by horizontal transfer of the *kan*^{r} gene to gut microorganisms or intestinal epithelial cells is not of significant concern. The agency considered potential transfer of the *kan*^{r} gene only fron fresh tomatoes because processing is expected to inactivate the *kan*^{r} gene in processed tomato products and in food and feed products derived from cotton and oilseed rape.

The agency also evaluated *in vitro* data that showed that only 0.1 percent of DNA could be detected as fragments of 1,000 base pairs or longer after exposure to simulated stomach fluids for 10 minutes and to simulated intestinal fluids for another 10 minutes. Thus most of the DNA remaining after digestion would be smaller than the *kan*^{r} gene which is about 1,000 base pairs long and would be unavailable for potential transformation of gut microorganisms. In addition, in animals, even if DNA was not completely degraded by processing during feed production, any remaining DNA would be degraded by the digestive processes. For example, studies have shown that nucleic acids introduced into the rumens of calves, or incubated with calf, sheep, or cow rumen contents *in vitro*, were rapidly and completely degraded to nucleotides and nucleosides (McAllan et al., 1973). Similar results were obtained when DNA was infused into the duodenum of steers (McAllan, 1980). Moreover, many rumen bacterial strains have nuclease activity, which degrades DNA and provides yet another barrier to transformation (Flint and Thompson, 1990).

Finally, Calgene calculated and FDA agreed that, even using worst-case assumptions such as all microorganisms in the intestine being transformation competent, in a person consuming fresh tomatoes at the 90th percentile level, the transformation frequency of intestinal microorganisms with the kan^r gene will be approximately $3x10^{-15}$ transformants per day. This transformation frequency is more than 5 orders of magnitude less than the frequency of mutation to kanamycin resistance per bacterial replication, *i.e.*, 10^{-9} (Davies, 1986). Thus, for every 300,000 bacteria that mutate to kanamycin resistance per replication (generall a matter of hours), there would be, at most, under worst-case conditions, one kanamycin-resistant bacterium per day added to that number due to transformation.

The potential for food-producing animals to experience decreased efficacy of antibiotic therapy as a result of pathogenic intestinal microflora incorporating and expressing the *kan*^{r} gene would be similar to that described for humans, *i.e.*, equally improbable because the worst-case transformation scenario described above for human gut microorganisms also applies to microorganisms found in the gut of food-producing animals.

With respect to epithelial cells lining the intestinal lumen, no transformation of human epithelial cells has been demonstrated *in vivo* (Hoskins, 1978). In addition, even if transformed, intestinal epithelial cells are terminally differentiated (*i.e.*, do not divide) and have a relatively short life span, and thus would continually be shed and

replaced by nontransformed cells.

2. Potential transfer of the kanamycin resistance gene to soil microorganisms

The FDA also considered the possibility that the *kan*^{r} gene might be transferred to soil microorganisms, thereb increasing the level of antibiotic-resistant organisms in the environment. A major barrier to transformation is expected to be the rapid degradation of plant DNA by plant nucleases that takes place when the cell breaks up. Similar calculations to the above showed that, under worst-case assumptions, kanamycin-resistant transformants resulting from plant DNA left in the fields would represent not more than one in 10 million of the existing kanamycin-resistant soil population. Biosafety of use of the *kan*^{r} marker gene in transgenic plants has also been discussed elsewhere (Nap et al., 1992, Redenbaugh et al., 1994).

D. Food Advisory Committee Discussions Regarding Potential Horizontal Transfer of the *kan*^{r} Gene

The FDA's Food Advisory Committee has discussed the possibility that the *kan*^{r} gene might be transferred to microorganisms in the GI tract and in the environment. The committee members concluded that transfer of the *kan*^{r} gene consumed as a component of tomatoes to microorganisms in the GI tract was highly unlikely based on published data in the scientific literature. Similarly, the committee members judged that the potential for transfer of the *kan*^{r} gene from plants to microorganisms in the environment is highly unlikely based on current knowledge of mechanisms of gene transfer. In addition, members of the committee pointed out that the rate at which such transfer could take place, if at all, was of so small a magnitude that, coupled with the high prevalence of kanamycin resistant organisms already present in the environment, it would not cause a significant environmental impact.

Some members of the committee, while convinced by the information presented at the meeting that the transfer of the *kan*^{r} gene from tomato plants to microorganisms in the soil was improbable, expressed concern regarding the use of the *kan*^{r} gene in other crops that may be grown on a wide scale. In addition, some committee members were concerned that a determination of safety with regard to the use of *kan*^{r} gene in Calgene's tomato might signal to producers that it is now permissible to use the *kan*^{r} gene in other crops. In light of such concerns, these committee members advised that use of the *kan*^{r} gene in other crops should be evaluated on a case-by-case basis.

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IV. Notes

(6) Other names for this enzyme include neomycin phosphotransferase II (NPT II), neomycin phosphotransferase and kanamycin phosphotransferase II.

(7) Gentamicin, which is used for human therapeutic use, is composed of a complex mixture of the antibiotic substances produced by *Micromonospora purpurea* and contains primarily gentamicin C_1 (25-50%), gentamicin C_{1a} (10-35%), and gentamicins C_{2a} and C_2 (25-55%) (9). Gentamicins A and B are at most minor components of the commercial drug. Thus, APH(3')II does not confer resistance to gentamicin that is used therapeutically (Davies, 1986).

(8) Because oils produced from transgenic cottonseed and rape seed would not contribute APH(3')II to the human diet, the exposure estimate was derived exclusively for tomatoes. The agency made several conservative assumptions in arriving at its EDI for APH(3')II of 480 µg/person/day. For example, FDA assumed that all tomatoes contain APH(3')II at a level of 0.1 percent of total protein although, of the two lines intended for commercialization by Calgene, one contains less than 0.01 percent and the other less than 0.002 percent of APH(3')II (as a percentage of total protein). Second, FDA included APH(3')II in processed products in its estimate although high temperature treatment used in the production of processed tomato products would be expected to result in loss of enzymatic activity of APH(3')II.

Appendix 2. Review of FDA/Industry Consultations Regarding New Plant Varieties and Selectable Markers Used to Develop Them

I. Purpose of the Consultations

Following evaluation and approval of the Flavr Savr™ tomato in 1994, FDA has not found it necessary to conduct comprehensive scientific reviews of foods derived from bioengineered plants based on the attributes of these products, but consistent with its 1992 policy, FDA expects developers to consult with the agency on safety and regulatory questions. Such consultations will facilitate resolution of safety and regulatory issues. Many firms have initiated consultations with the agency early in the research and development phase of their product.

At some stage in the process of research and development, a firm will have accumulated the information that i believes is adequate to ensure that the product is safe and complies with the relevant provisions of the Act. The firm will then be in a position to conclude any ongoing consultation with FDA. To inform FDA about bioengineered foods that are intended for commercial distribution, the agency recommends that the crop developer submit to FDA, a summary of the safety and nutritional assessment that has been conducted, and if necessary, meet with agency scientists to discuss the scientific data and information that support the summar of the safety and nutritional assessment.

The safety and nutritional assessment summary typically contains sufficient information for agency scientists to understand the approach the firm has followed in identifying and addressing relevant issues and includes,

1. The name of the bioengineered food and the crop from which it is derived.
2. A description of the various applications or uses of the bioengineered food, including animal feed uses.
3. Information concerning the sources, identities, and functions of introduced genetic material.
4. Information on the purpose or intended technical effect of the modification, and its expected effect on th composition or characteristic properties of the food or feed.
5. Information concerning the identity and function of expression products encoded by the introduced genetic material, including an estimate of the concentration of any expression product in the bioengineered crop or food derived thereof .
6. Information regarding any known or suspected allergenicity and toxicity of expression products and the basis for concluding that foods containing the expression products can be safely consumed.
7. Information comparing the composition or characteristics of the bioengineered food to that of food derived from the parental variety or other commonly consumed varieties with special emphasis on important nutrients, and toxicants that occur naturally in the food.

8. A discussion of the available information that addresses whether the potential for the bioengineered food to induce an allergic response has been altered by the genetic modification.
9. Any other information relevant to the safety and nutritional assessment of the bioengineered food.

The goal of FDA's evaluation of information on new plant varieties provided by developers during the consultation process is to ensure that human food and animal feed safety issues or other regulatory issues (*e.g.* labeling) are resolved prior to commercial distribution. During the consultation process, FDA does not conduct a comprehensive scientific review of data generated by the developer. Instead, FDA considers, based on agency scientists' evaluation of the available information, whether any unresolved issues exist regarding the food derived from the new plant variety that would necessitate legal action by the agency if the product were introduced into commerce. Examples of unresolved issues may include, but are not limited to, significantl increased levels of plant toxicants or anti-nutrients, reduction of important nutrients, new allergens, or the presence in the food of an unapproved food additive. The FDA considers a consultation to be completed when all safety and regulatory issues are resolved.

In 1994, FDA discussed this consultation process during a public joint meeting of the agency's Food and Veterinary Medicine Advisory Committees, which consist of food and feed safety experts from outside the agency. At this meeting, FDA summarized safety and nutritional information provided by developers on seven genetically engineered foods: three improved softening or ripening tomatoes, a virus-resistant squash, an insect-resistant potato, and herbicide-tolerant cotton and soybean. The committee members agreed with FDA that, based on the types of bioengineered foods and feeds under development, the consultation procedures provide an appropriate level of government oversight.

II. Bioengineered Foods on Which Consultations Have Been Completed

In addition to the Flavr Savr™ tomato, the agency has completed thirty consultations; seven each in 1994 an 1995, ten in 1996, and six in 1997. These consultations are listed below and are categorized by crop. The trai of the new variety, the gene responsible for the trait, and the source organism of the gene are also given. In addition, the firms that undertook the consultations with FDA and the year in which the consultations were completed are given in parenthesis. A list of consultations that are completed as well as the consultation procedures can be accessed at FDA's home page on the World Wide Web at HTTP://WWW.FDA.GOV under Center for Food Safety and Applied Nutrition. Note that the listed products may have pending regulatory issue with EPA or USDA/APHIS.

A. New Corn Varieties

1. Glufosinate Tolerant
   a. Phosphinothricin acetyltransferase gene from *Streptomyces viridochromogenes* (AgrEvo, 1995)
   b. Phosphinothricin acetyl transferase gene from *Streptomyces hygroscopicus* (Dekalb Genetics, 1996)
2. Male Sterile
   a. Barnase gene from *Bacillus amyloliquefaciens* (Plant Genetics System, 1996)
3. Insect-Protected
   a. Cry1A(b) from *Bacillus thuringiensis* subsp. *kurstaki* (two consultations by Monsanto, 1996; Northrup King, 1996; Ciba-Geigy, 1995)
   b. CryIA(c) gene from *Bacillus thuringiensis* (Dekalb Genetics, 1997)
4. Insect-Protected and/or Glyphosate-Tolerant
   a. CryIA(b) from *Bacillus thuringiensis* subsp. *kurstaki* and/or enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (Monsanto, 1996

B. New Tomato Varieties

1. Modified Fruit Ripening
   a. S-adenosylmethionine hydrolase from *E. coli* bacteriophage T3 (Agritope, 1996)
   b. Fragment of aminocyclopropane carboxylic acid synthase gene from tomato (DNA Plant Technology, 1994)
   c. Aminocyclopropane carboxylic acid deaminase gene from *Pseudomonas chloraphis* strain 6G5 (Monsanto, 1994)
2. Delayed Softening
   a. Fragment of polygalacturonase gene from tomato (Zeneca Plant Sciences, 1994

C. New Oilseed Rape Varieties

1. Male Sterile/Fertility Restorer
    a. Barnase (male sterile) and barstar (fertility restorer) from *Bacillus amyloliquefaciens* (Plant Genetics System, 1996)
2. Glufosinate-Tolerant (two lines)
    a. Phosphinothricin acetyltransferase from *Streptomyces viridochromogenes* (two consultations by AgrEvo, 1995 and 1997)
3. Laurate Canola
    a. 12:0 acyl carrier protein thioesterase from *Umbellularia californica* (Calgene, 1995)
4. Glyphosate-Tolerant
    a. Enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (Monsanto, 1995)

D. New Cotton Varieties

1. Sulfonyl Urea-Tolerant
    a. Acetolactate synthase from *Nicotiana tabacum* (Du Pont, 1996)
2. Glyphosate-Tolerant
    a. Enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (Monsanto, 1995)
3. Insect-Protected
    a. CryIA(c) from *Bacillus thuringiensis* subsp. *kurstaki* (Monsanto, 1995)
4. Bromoxynil-Tolerant
    a. Nitrilase gene from *Klebsiella ozaenae* (Calgene, 1994)

E. New Potato Varieties

1. Insect-Protected
    a. CryIIIA from *Bacillus thuringiensis* (two consultations by Monsanto, 1994 and 1996)

F. New Soybean Varieties

1. High-Oleic Acid
    a. Sense suppression of endogenous Delta-12 desaturase (GmFad2-1) gene (Du Pont, 1997)
2. Glyphosate-tolerant
    a. Enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (Monsanto, 1994)

G. New Squash Varieties

1. Virus-Resistant
    a. Coat protein genes of cucumber mosaic virus, zucchini yellow mosaic virus, and watermelon mosaic virus 2 (Seminis Vegetable Seeds, 1997)
    b. Coat protein genes from watermelon mosaic virus 2 and zucchini yellow mosaic virus (Asgrow Seed, 1994)

H. New Papaya Variety

1. Virus-Resistant
    a. Coat protein gene from the ringspot virus (University of Hawaii and Cornell University, 1997)

I. New Radicchio Variety

1. Male Sterile
    a. Barnase from *Bacillus amyloliquefaciens* (Bejo Zaden, 1997)

III. Antibiotic Resistance Markers Seen in Consultations to Date

The FDA understands that crop developers are seeking alternative markers for selection of transformants that have the desired trait. Methods are also being developed to excise out antibiotic marker genes after they have been used in the selection process (Dale and Ow, 1991; Ebinuma, 1997). However, development of many of the transgenic plants that are on the market and those that are approaching commercialization was initiated

several years ago at which time antibiotic resistance selection methods were widely used. Thus, at the preser time, and for the foreseeable future, there will be a need to address the safety of the use of antibiotic resistance marker genes in transgenic crops used for food or animal feed.

Although many types of selectable markers, including antibiotic resistance genes, herbicide tolerance genes, metal tolerance genes, genes involved in amino acid metabolism, genes influencing phytohormone production, and screenable or reporter genes, are used in the development of transgenic crops (WHO, 1993; Karenlampi, 1996), by far the most prevalent are those conferring resistance to antibiotics. Antibiotic resistance was used as a selectable marker in 31 out of 52 consultations the agency has had regarding transgenic crops to date. Neomycin phosphotransferase II (nptII, APH(3')-II) accounted for 27 of these cases, and hygromycin phosphotransferase (aphIV, hpt) accounted for the rest.

In addition to markers that are used for selection, transgenic crops frequently have antibiotic resistance markers under bacterial promoters incorporated into their genome. These markers are necessary for passage of the constructs in bacterial systems, and most often end up in the genomes of transgenic plants because biolistics or gene gun methods using the entire construct are used to transform the plant cells. However, because the genes are under the control of bacterial promoters, they are functional only in the prokaryotes that were used in developing the gene constructs; they are not expressed in the plant even though the entire gene or multiple copies of the gene may be present.

In the 52 consultations the agency has had to date regarding plants developed using rDNA technology, there have been 19 consultations where the transgenic crop has one or more antibiotic resistance marker genes under a bacterial promoter incorporated into the plant genome (*beta*-lactamase (bla) in 12, aminoglycoside adenyltransferase (aad) in 4, nptII in 3, neomycin phosphotransferase III (nptIII) in 1, chloramphenicol acetyltransferase gene (cat) in 1, and a tetracycline resistance gene (tet^R) in 1). In some cases, only partial fragments of the genes were incorporated.

By the end of 1997, 30 out of 52 consultations that were initiated by developers have been completed. Table lists the antibiotic resistance markers found in the transgenic plants (both expressed and those under bacterial promoters). The consultations are arranged by crop.

Antibiotic Resistance Markers in Completed Consultations through 1997

Crop	No. of Consultations	Antibiotic Resistance Markers Used for Selection	Antibiotic Resistance Genes under Bacterial Promoters
Corn	9	---	3 *nptII* 5 *bla*(9) 1 *cat*(10)
Tomato	4	4 *nptII*	---
Oilseed Rape	5	3 *nptII*	---
Cotton	4	3 *nptII*	2 *aad*
Potato	2	2 *nptII*	1 *aad*
Soybean	2	1 *nptII*(11)	1 *bla*
Squash	2	2 *nptII*(12)	---
Papaya	1	1 *nptII*	*tetR*(13)
Radicchio	1	1 *nptII*	---

I V. References

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4. World Health Organization, "Health aspects of marker genes in genetically modified plants: report of a WHO workshop," Geneva, Switzerland, 1993.

V. Notes

(9) The -lactamase gene in one of the lines is truncated.

(10) The chloramphenicol acetyl transferase gene is truncated and rearranged.

(11) Although nptII was used for selection, the gene segregated out in subsequent generations and the

modified soybean variety on which consultations were concluded did not contain nptII.

(12) In one of the two transgenic squash lines, nptII was used for selection but gene subsequently segregated out. Thus the new squash variety that was the subject of a consultation did not contain nptII.

(13) Only a partial fragment of the tetracycline resistance gene is present.

Appendix 3. Review of Positions by Other Government Agencies and International Bodies on Antibiotic Resistance Marker Use in Transgenic Plants

I. The U.S. Environmental Protection Agency

Pesticides are regulated by the Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide, and Rodenticide Act and under section 408 of the Federal Food, Drug, and Cosmetic Act. Thus, in the case of a new variety that has been modified to have a pesticidal trait, the safety of the pesticide, as well as the genetic material needed to express the pesticide, and marker genes used to confirm the presence of the pesticidal substance are evaluated by the EPA. EPA sets tolerances for pesticides and pesticide inert ingredients or exempts them from tolerance requirements. In the *Federal Register* of September 28, 1994, EPA established an exemption from the requirement of a tolerance for residues of neomycin phophotransferase II and the genetic material necessary to produce it when produced in plants as a plant pesticide inert ingredient (U.S. EPA, 1994).

II. The U.K. Advisory Committee on Novel Foods and Processes

The Advisory Committee on Novel Foods and Processes (ACNFP) of the United Kingdom's Ministry of Agriculture Fisheries, and Foods recently published a document entitled "The Use of Antibiotic Resistance Markers in Genetically Modified Plants for Human Food: Clarification of Principles for Decision-Making" (ACFNP, 1996). In that document, the ACNFP stated that the evaluation of food/feed from genetically modified plants containing antibiotic resistance marker genes will be conducted on a case-by-case basis and will be determined to a large extent by considerations of the possibility of the transfer, maintenance and expression of the marker gene in gut or rumen microorganisms, the clinical use and importance of all the antibiotics for which resistance is encoded and the potential toxicity or allergenicity of the gene product. Since the ACNFP considers the safety issues associated with the antibiotic resistance markers on a case-by-case basis, it does not publish prescriptive lists of "acceptable" and "unacceptable" antibiotic resistance marker genes.

The ACNFP considers that there is a finite, albeit a very low probability of transfer, maintenance and expression of the gene in rumen or gut microorganisms. ACNFP considers this of little concern for antibiotic resistance markers with plant regulatory sequences but not those with bacterial regulatory sequences. It states that where transfer is considered to be possible and subsequent expression likely and the antibiotic which would be affected is of major clinical importance, *e.g.*, ampicillin, use of the marker would be unlikely to be approved. With respect to toxicity and/or allergenicity of the gene product, the ACNFP believes that thorough characterization of the antibiotic resistance marker gene allows the comparison of sequence data with genes known to code for toxic or allergenic gene products. In addition, *in vitro* degradation studies in gastric fluid can be used to indicate the likelihood for persistence of the gene product in the digestive tract and provide further reassurance about the safety of the antibiotic resistance marker gene.

Recently, the ACNFP recommended that approval be denied for use of unprocessed insect-resistant corn developed by Ciba-Geigy for animal feeds (Letter from the Ministry of Agriculture, Fisheries and Foods, to Ciba-Geigy Limited, April 29,1996). This transgenic corn contains an intact *beta*-lactamase gene with a promoter and an origin of replication (*ori*) derived from the pUC18 vector. Unlike the *ColE1 ori* vector found in nature which generates 4 to 18 copies per cell, the pUC *ori* generates over 600 copies per cell. The ACNFP believed that there is a finite risk of transfer of the *beta*-lactamase gene from the transgenic corn to bacteria in the rumen or gut of livestock and that as a consequence of the presence of a promoter and the pUC ori on the *beta*-lactamase gene, such transfer would have extremely grave consequences for therapy with *beta*-lactam antibiotics(14).

III. The European Commission Scientific Committees for Food, and Animal Nutrition

In December, 1996, the European Union's Scientific Committee for Food (EC/SCF) issued an opinion regarding the potential for adverse health effects from the consumption of genetically modified maize (modified for insect resistance through insertion of the Bt-endotoxin gene, and for increased tolerance to the herbicide glufosinate ammonium) including any potential adverse effects from the non-expressed *beta*-lactamase gene (EC/SCF, 1996). The committee concluded that the possibility that the product would add significantly to the already widespread occurrence of ampicillin resistant bacteria in animals and man is remote. This conclusion was based on evidence derived from theoretical considerations, laboratory studies, a step-wise assessment regarding the gene construct itself, its distribution and persistence in maize and products derived from this crop, the possibility of transfer of the gene from maize to gram negative bacteria, and the possibility that it would function in such bacteria. All of this evidence led to the conclusion that the risk of bacterial transformation is extremely low. Further, the committee concluded that even if transfer were to take place, it would have no

detectable additional effect as the *beta*-lactamase gene is already widespread in nature including human and animal gastrointestinal tracts. The committee stated that it proposes to scrutinize the future needs and applications of antibiotic resistance marker genes.

Similarly, with respect to the presence of the *beta*-lactamase gene, the European Commission's Scientific Committee for Animal Nutrition (EC/SCAN) concluded that the probability of the transfer of a functional gene construct from the genetically modified maize into bacteria is virtually zero, and that if the virtually impossible event occurred, it would have no clinical significance. There is no evidence of a risk of causing *beta*-lactam antibiotic resistance in the animal gut bacteria from the use of the genetically modified maize (EC/SCAN, 1996)

IV. The Nordic Working Group on Food Toxicology and Risk Assessment

The Nordic Working Group on Food Toxicology and Risk Assessment under the auspices of the Nordic Council o Ministers issued a document entitled " Health Effects of Marker Genes in Genetically Engineered Food Plants" i 1996 (Karenlampi, 1996). The report deals with the food safety aspect of genes conferring resistance to the antibiotics kanamycin, hygromycin and streptomycin, and to the herbicides glufosinate, glyphosate and chlorsulfuron as well as the *gus* reporter gene. Based on the detailed safety analysis that has been conducted to date, it suggests a positive list of marker genes acceptable in the genetic engineering of food plants, namely the *kan*^{r} gene and the glyphosate tolerance marker gene encoding 5-enolpyruvylshikimate-3-phosphate synthase. It also suggests that the glufosinate tolerance gene encoding phosphinothricin acetyltransferase might be the next marker gene to be added to such a list.

V. World Health Organization/Food and Agriculture Organization

The World Health Organization (WHO) has conducted a workshop on health aspects of marker genes in genetically modified plants (WHO, 1993). The workshop recognized the need for marker genes and that it was impractical at present to remove marker genes from modified plants once they have fulfilled their function. The WHO workshop concluded that many of the general safety issues raised about other genes apply to marker genes as well. These include potential toxicity and allergenicity of the gene product and possible secondary and pleiotropic effects of the insertion. The workshop concluded that in assessing the safety of the proteins expressed by marker genes, the focus of the assessment should be on the function of the protein rather than its structure. It also concluded that there is no reason to suppose that marker gene proteins pose a particular allergenic concern but that, if the gene is obtained from a source known to cause allergy, the allergenicity of the gene product should be investigated. The workshop concluded that in addition to safety issues common to all introduced proteins, there are safety issues specific to antibiotic resistance markers. These include potential inactivation of an oral dose of antibiotic due to the presence of a marker gene product in food, and horizontal transfer of the gene to gut microorganisms.

The workshop concluded that there is no recorded evidence for transfer of genes from plants to microorganisms in the gut and that if transfer did occur, any health concern would depend on many factors, including the ability of the transformed microorganisms to replicate in the gut and to express the gene product

This conclusion was reaffirmed in the 1996 joint Food and Agriculture Organization (FAO)/WHO Expert Consultation on Biotechnology and Food Safety (WHO, 1996), which stated that the most relevant food safety issue concerning gene transfer is the potential consequence of the transfer of an introduced gene from material derived from a genetically modified organism to microorganisms in the gastrointestinal tract, in such a way that the gene can be successfully incorporated and expressed, and impact human or animal safety. The consultation added that there is no recorded evidence for the transfer of genes from plants to microorganisms in the gut and that there are no authenticated reports of such bacterial transformation in the environment of the human gastrointestinal tract.

The consultation cited the events that would need to occur for gene transfer - survival of the DNA in the hostile environment of the GI tract, need for recipient organisms to be transformation-competent, need for the DNA to bind to the recipient organism and translocate across the cell membrane, survival of the DNA from degradation by the restriction/modification system of the microorganism DNA, and need for the DNA to integrate into the host genome or plasmid which requires sequences homologous to the host DNA at both ends of the foreign DNA- and concluded that the possibility of gene transfer is vanishingly small. The consultation further concluded that data on such transfer will only be needed when the nature of the marker gene is such that, if transfer were to occur, it would give rise to a health concern. In assessing any potential health concerns, the human or animal use of the antibiotic and the presence and prevalence of resistance to the same antibiotic in gastrointestinal microflora should be considered. As an example, the consultation noted that the antibiotic vancomycin is critical in the treatment of certain bacterial diseases where multiple antibiotic resistance is prevalent, and there is lack of alternatives.

VI. References

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7. U.S. Environmental Protection Agency, "Neomycin Phosphotransferase II; Tolerance Exemption," *Federal Register*, 56, 49351, 1994.
8. World Health Organization, "Health aspects of marker genes in genetically modified plants: report of a WHO workshop," Geneva, Switzerland, 1993.
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VII. Note

(14) On the other hand, in a conference sponsored by Tufts University and the Foundation for Nutritional Advancement (1996), a group of scientists discussed Ciba-Geigy's transgenic corn containing the *beta*-lactamase gene and concluded that use of this particular type of *beta*-lactamase gene constitutes an insignificant to near zero risk of causing ampicillin resistance in either animals or humans because 1) the probability of DNA survival in segments large enough to be taken up by bacteria is very low, 2) the probability of bacteria taking up or incorporating DNA into the bacterial genome is virtually zero, and 3) even if the DNA from the marker gene was to be incorporated in the bacterial genome, there is a low probability that it would be expressed. In addition, they concluded that the clinical significance is virtually zero because it is ubiquitous, already occurs in nature, and can be overcome by antibiotics other than ampicillin.

Links on this page:

CHEMICAL CONSTITUENTS OF TOBACCO LEAF AND DIFFERENCES AMONG TOBACCO TYPES

John C. Leffingwell, Ph.D.

In 1960, a little over 200 chemical constituents had been identified in tobacco leaf of all types and less than 450 had been reported in smoke. Today, approximately 3000 have been identified and characterized in tobacco leaf and some 4000 in smoke. Estimates are that the total number of chemical constituents in leaf exceeds 4000 and there are over 6000 in tobacco smoke. It is not the purpose of this section to comprehensively review all of the known constituents, but rather to provide an insight into the known composition and chemistry of tobacco types that impact tobacco quality and differentiate tobacco types. Emphasis will be placed on the major tobaccos utilized commercially: Virginia (flue-cured), Air-cured (burley and cigar), and Oriental.

The physical and chemical properties of leaf tobacco are influenced by genetics, agricultural practices, soil type and nutrients, weather conditions, plant disease, stalk position, harvesting and curing procedures. A change in any of these factors can markedly alter the chemical composition of leaf and thus affect smoking quality (50).

It is now generally accepted that the metabolic carbon-nitrogen balance in living plants is due to continuing transformations based on the Krebs tricarboxylic acid cycle . In the Krebs cycle, carbon dioxide from air is assimilated through photosynthesis in the tobacco leaf while inorganic nitrogen (nitrate and/or ammonia) is assimilated through the roots from the soil. Soil nitrate is converted to ammonia which is utilized in the Krebs cycle to form amino acids which serve as a nitrogen pool for the formation and transformation of a multitude of nitrogenous chemicals important in the development of aroma and flavor quality. Dawson (13) has suggested a concept based on the Krebs cycle to account for inherited and culturally induced variations in gross tobacco composition. Using this concept, he rationally suggested that for tobaccos where the nitrogen supply is abundant, such as in cigar and burley tobacco

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TABLE 1

Composition of Cigarette Tobaccos (26)

Representative Analyses of Cigarette Tobaccos

(Leaf Web after Aging, Moisture free Basis)

Component, %[a]	Flue-cured, type 13	Burley, type 31	Maryland, type 32	Oriental[b]
Total volatile bases as ammonia	0.282	0.621	0.366	0.289
Nicotine	1.93	2.91	1.27	1.05
Ammonia	0.019	0.159	0.130	0.105
Glutamine as ammonia	0.033	0.035	0.041	0.020
Asparagine as ammonia	0.025	0.111	0.016	0.058
α-Amino nitrogen as ammonia	0.065	0.203	0.075	0.118
Protein nitrogen as ammonia	0.91	1.77	1.61	1.19
Nitrate nitrogen as NO_3	trace	1.70	0.087	trace
Total nitrogen as ammonia	1.97	3.96	2.80	2.65
pH	5.45	5.80	6.60	4.90
Total volatile acids as acetic acid	0.153	0.103	0.090	0.194
Formic acid	0.059	0.027	0.022	0.079
Malic acid	2.83	6.75	2.43	3.87
Citric acid	0.78	8.22	2.98	1.03
Oxalic acid	0.81	3.04	2.79	3.16
Volatile oils	0.148	0.141	0.140	0.248
Alcohol-soluble resins	9.08	9.27	8.94	11.28
Reducing sugars as dextrose	22.09	0.21	0.21	12.39
Pectin as calcium pectate	6.91	9.91	12.41	6.77
Crude fiber	7.88	9.29	21.79	6.63
Ash	10.81	24.53	21.98	14.78
Calcium as CaO	2.22	8.01	4.79	4.22
Potassium as K_2O	2.47	5.22	4.40	2.33
Magnesium as MgO	0.36	1.29	1.03	0.69
Chlorine as Cl	0.84	0.71	0.26	0.69
Phosphorus as P_2O_5	0.51	0.57	0.53	0.47
Sulfur as SO_4	1.23	1.98	3.34	1.40
Alkalinity of water-soluble ash[c]	15.9	36.2	36.9	22.5

a In % except for pH and alkalinity. (Harlan and Mosley, !955)

b Blend of Macedonia, Smyrna, and Samsun types.

c Milliliters of lN acid per 100 g tobacco.

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production, there should be an abundant formation of protein, amino acids and nicotine. For Oriental tobacco, where growth is maintained with limited supplies of nitrogen nutrients and water, there is an accumulation of acetate in the Krebs cycle resulting in the biosynthesis of terpenoids via mevalonic acid as well as a higher production of carbohydrates, "aromatic" acids, and resins at the expense of nitrogen constituents. Flue-cured tobacco is intermediate in that the phytochemistry during the plants life cycle is balanced by a moderate supply of nitrogen which is depleted as the plant reaches maturity.

Examination of representative analyses of the major cigarette tobacco types as presented by Harlan and Moseley (26) (Table 1) provides an overview of the major differences in aged flue-cured, burley, Maryland and Oriental tobaccos.

Although average reducing sugar content in flue-cured and Oriental cigarette tobaccos today rarely runs as high as reported in Table 1, the basic analytical trends still remain valid. Thus, in air-cured burley, Maryland and cigar tobaccos the carbohydrates have been virtually depleted via metabolism of the living cells, whereas the protein and (alpha-amino nitrogen are obviously higher than in flue-cured or Oriental tobaccos. Conversely, the flue-cured and Oriental tobaccos possess significant amounts of reducing sugars (which are virtually absent in the air-cured tobaccos) and lesser amounts of protein and alpha-amino nitrogen. Substantial changes in the chemical composition of tobacco leaf occur following harvest and during subsequent processes.

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TABLE 2
Changes in Composition of Virginia Tobacco
During the Flue-Curing Process (4)
(Percent of Dry Weight)

Constituents	Green	Yellowed	Cured
Starch	29.30	12.40	5.52
Free Reducing Sugars	6.68	15.92	16.47
Levulose	2.87	7.06	7.06
Sucrose	1.73	5.22	7.30
Crude Fiber	7.28	7.16	7.24
Total Nitrogen	1.08	1.04	1.05
Protein Nitrogen	0.65	0.56	0.51
Nicotine	1.10	1.02	0.97
Ash	9.23	9.24	9.25
Calcium	1.37	1.37	1.37
Oxalic Acid	0.96	0.92	0.85
Citric Acid	0.40	0.37	0.38
Malic Acid	8.62	9.85	8.73
Resins	7.05	6.53	6.61
Pectinic Acid	10.99	10.22	8.48
pH of Tobacco	5.55	5.64	5.55

(Bacon, et. al., 1952)

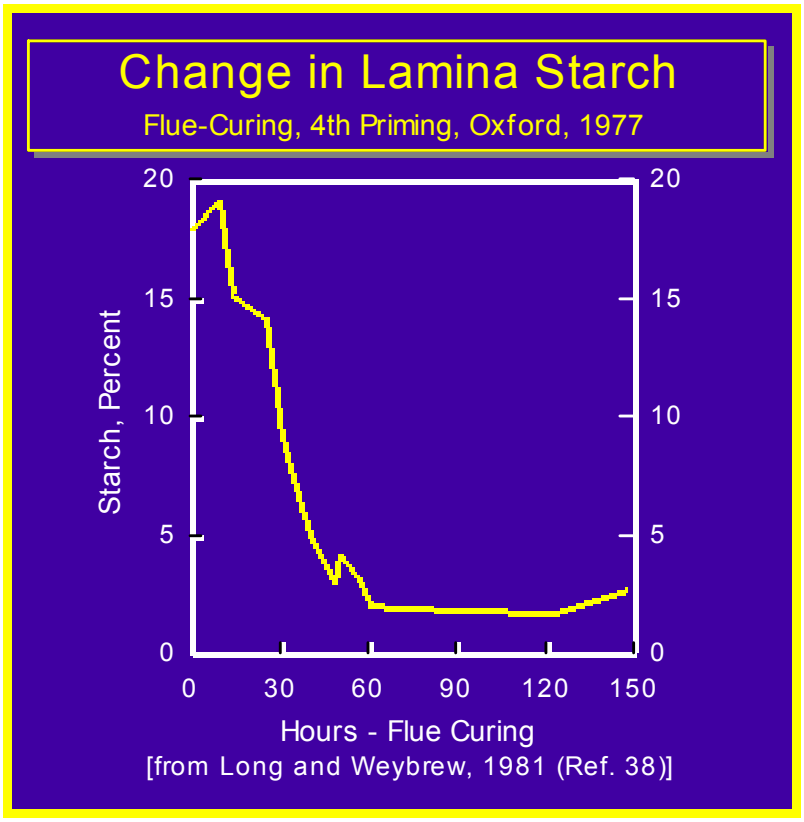
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Carbohydrates: Starch, Sugars, Sugar Esters, Cellulose, and Pectin

Starch & Sugars:

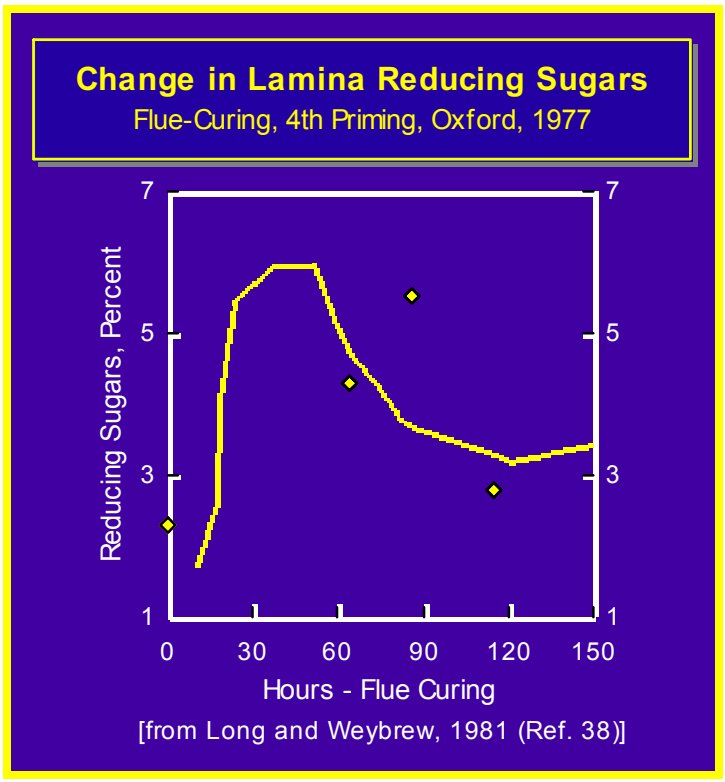
In flue-cured tobacco, respiration in the primed leaf is arrested by the controlled dessicative dehydration during flue-curing which causes enzyme inactivation. Nevertheless, considerable change has occurred (Table 2) in the leaf (during the period after priming and early stages of curing) as starch loss occurs via enzymatic hydrolysis with a concomitant increase in reducing sugar (4). This is illustrated for the starch depletion during cure of Virginia tobacco (Figure 1) and the concurrent generation of reducing sugars (38) (Figure 2).

FIGURE 1



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FIGURE 2



In burley tobacco the starch accumulation during growth is only about 25% the amount in Virginia tobacco (53) and this is depleted nearly completely during the catabolic respiration of the plant during air-curing leaving negligible sucrose, and reducing sugars in the cured leaf.

Starches are generally polymers of two polysaccharides: amylose and amylopectin. Corn starch has approximately 27% amylose and 73% amylopectin whereas tobacco has been found to have approximately 23% amylose and 77% amylopectin (31). The amylose portion is estimated to have a chain length of forty to forty-seven anhydroglucose units while the amylopectin has about twenty-six glucose units.

Sugar Esters:

The first report of sugar esters in Oriental tobacco came in 1970 with the isolation, structure elucidation and synthesis of 6-0-acetyl-2,3,4-tri-0-[(+)-3-methylvaleryl]-beta-D-

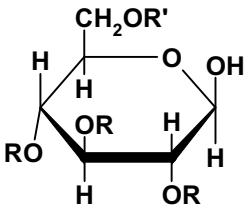
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glucopyranose (a glucose tetraester) by Schumacher (40). This along with the more predominant sucrose tetraesters of lower carboxylic acids (Figure 3) are now considered to be some of the most important aroma precursors responsible for Oriental flavor (35).

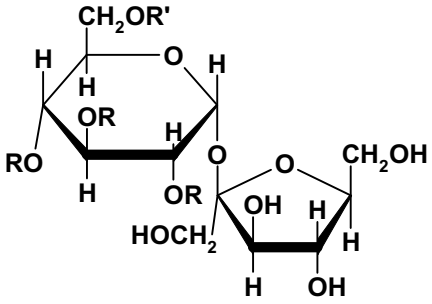
FIGURE 3

SUGAR ESTERS PRESENT IN ORIENTAL TOBACCO

(Carboxylic Acid Smoke Aroma Precursors)



GLUCOSE TETRAESTERS



SUCROSE TETRAESTERS

R = C_3 -C_8 Carboxylate

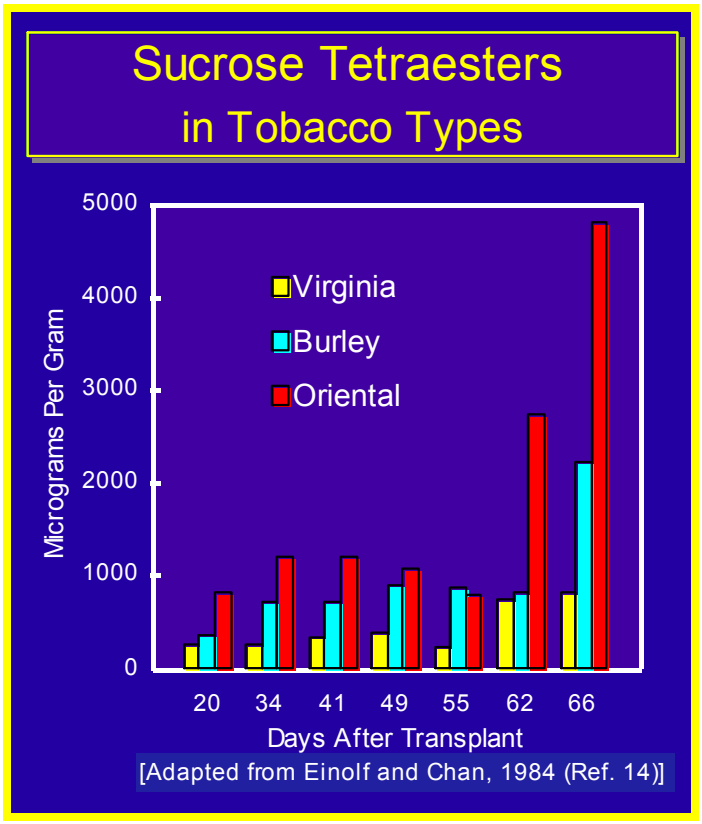
R' = Acetate

In 1981, Severson et. al., found that the cuticular waxes of a tobacco budworm resistant tobacco contained a series of sucrose tetraesters (STE) which are the probable precursors of 6-0-acetyltriacylglucopyranosides [Glucose Tetraesters (GTE)] isolated from Oriental tobacco (42-44).

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Einolf and Chan have quantified the accumulation of STE for Coker 319 (Virginia), Kentucky 14 (burley) and Smyrna (Oriental) tobaccos utilizing flue-curing, air-curing and sun-curing respectively to mimic the field curing practices for each tobacco type (14). The graphic representation (35) (Figure 4) shows that accumulation of STE for Oriental and burley is greater than for Virginia tobacco, with the Oriental and burley maximums occurring at full maturity.

FIGURE 4



It has also been demonstrated that the tetraesters of Oriental STE tended to have higher amounts of the more aromatic C_5 and C_6 carboxylic acids (44).

From a smoke aroma and flavor standpoint, it is interesting that GTE and STE readily release free carboxylic acids on thermolysis while totally esterified sucrose and glucose esters

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(such as glucose pentaisovalerate and the sucrose octaesters) do not (easily) release their acid moieties on thermolysis.

Glucosides:

The suggestion that non-volatile aroma precursors of tobacco existed in the form of glucosides was made by the observation that materials such as 3-hydroxydamascone and 3-keto-ionol were released from tobacco by hydrolysis (22). Very little information on the hydrolytic release of aroma compounds from fruits and other plant materials by either the acid or enzymatic hydrolysis of glycosidically bound terpenoid and norterpenoid materials was known prior to the mid-1970's. Today, it is clear that many plants, including tobacco, which have sugars (mono- and disaccharide's) generate nonvolatile materials with an inter-sugar link to aroma materials arising from the oxidative and metabolic transformation of carotenoid pigments (55). Many tobacco identical aroma compounds have now been found in fruits, wines and other products, primarily bound as glucosides. Heckman, et. al., demonstrated that a series of tobacco aglucone isolates are significantly increased by enzymatic hydrolysis of glucosides a from Virginia tobacco (27) (Table 3).

The commercial enzymatic hydrolysis of tobacco scrap and dust has been used to generate tobacco aroma materials from the glucosidically bound precursors in tobacco by one of the major flavor companies. The use of glucosides for flavoring tobacco has been patented (2) and commercial use of the glucoside of ethyl vanillin (30) (an artificial flavor about 3X more powerful than vanillin) for improving sidestream aroma was employed at one time in the U.S. in cigarettes.

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TABLE 3
Aglucone Increase from Enzymatic Hydrolysis
of Glucosides in Virginia Tobacco (27)
(micrograms/100 grams)

Aglucone	Virginia Control	Enzyme-Treated	Percent Increase
3-Mehylbutanol	0	104	N/A
Benzyl alcohol	1815	3167	74
2-Phenylethanol	887	1744	97
2-Methoxy-4-vinylphenol	498	792	59
4-Vinylphenol	1023	1255	23
3-Hydroxydamascone	2469	2697	9
4-Vinylsyringol	1615	2850	77
3-Keto-α-ionol	7257	10993	52
4-(4-Hydroxy-2,6,6-trimethyl-1-cyclohexen-1-yl)-3-butyn-2-ol	1783	1805	1
3-Hydroxy-5,6-epoxy-β-ionol	248	698	181
4-(3-Hydroxybutenylidene)-3,5,5-trimethylcyclohex-2-en-1-one	426	1146	169
3-(2-Hydroxyethyl)-phenol	155	1207	679
6-Hydroxy-3-keto-α-ionone	67	109	63
Vomifoliol	416	701	69

(Heckman, et. al., 1981)

Cellulose:

It has been estimated (23) that cigarette blends contain approximately 10% of cellulose and hemicellulose which corresponds closely to the values obtained for a series of flue-cured varieties (10). However, it must be noted that the tobacco stalk, stem and midrib portion of leaf are much higher in cellulose than the lamina portion of the leaf.

Cellulose is a polysaccharide of the general formula $(C_6H_{10}O_5)_n$ usually consisting mainly of glucose units. Acid hydrolysis of cellulose fractions from Japanese Bright

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(Virginia) tobacco leaf gave only glucose from one fraction and another leaf fraction cellulose gave mainly galactose, while the stem cellulose gave mainly arabinose and glucose (31).

Normally, high cellulose content in a tobacco blend is a negative to smoking quality in that it tends to impart a sharp stinging harshness and a "burnt paper" odor to the smoke. The cellulose content of stems plays an important part in the manufacture of reconstituted tobacco in that the fiber provides structural strength. The degree of polymerization (DP) of cellulose from leaf lamina varies considerably from that of mid-rib and stems (DP of 1100-1650 for lamina versus 1600-1800 for leaf mid-ribs). These values are considerably lower than that found for wood fiber (DP of about 3000). In addition, the DP for Virginia tobacco tends to be lower than for air-cured tobaccos (48). Pyrolysis studies on the generation of formic acid from tobaccos also suggests that Virginia cellulose may have a shorter mean chain length than burley (19).

As tobacco stems play an important role in the modern economics of tobacco manufacture, being employed as both cut rolled expanded stems and as a cellulose source in reconstituted tobacco, we must note that Virginia or flue cured stems are preferred to air-cured stems for their smoking quality. This may be due to the difference in cellulose polymerization, as well as other materials present (such as higher nitrate [and combustibility]) in air-cured stems.

Hemicellulose:

Hemicelluloses are a group of polysaccharides found in the cell walls of all plants in association with lignan. Whether lignan and hemicellulose are bonded or the hemicellulose is mechanically entrapped by lignan is unclear (3). The hemicellulose of Japanese Bright (Virginia) tobacco leaf was separated into two fractions which on hydrolysis gave mainly arabinose, galactose and glucose, along with lesser amounts of xylose (31). Hemicellulose is subject to extreme variations as its composition can change with growth and maturation and is influenced by cultural and environmental factors.

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Pectin:

The tobacco pectins are the "glue" that holds tobacco leaf together. Pectin comprises 6-12% of tobacco weight and as such contributes importantly both to the structural stability of leaf and to pyrolysis products that contribute to the smoke chemistry.

The pectin molecule is basically a polymeric carbohydrate comprised of 1,4-linked α-D-galactouronic acids with various degrees of methylation of the carboxylic acid groups. Dispersed within the polygalacturonic acid backbone are neutral sugar moieties such as galactose, rhamnose and arabinose.

Bokelman and coworkers (49) found that the main pectic polysaccharide fraction from tobacco has a backbone of 1,4-linked α-D-galactopranosyluronic acid interspersed with 2-linked L-rhamnopyranosyl residues. Approximately 22% of the galacturonic acid was methylated. The main pectic chain was determined to be branched at carbon-4 of rhamnose with the neutral side chains containing terminal and 4-linked β-D-galactopyranosyl and α-L-arabinofuranosyl residues.

Lauterbach and Moldoveanu (34) determined the degree of both methylation and amidation by pyrolysis GC-MS for the soluble pectic fractions of commercial grades of cured Virginia, burley and Oriental tobacco with the following results:

	% Methoxylation	% Amidation
Virginia Tobacco	20%	24%
Burley Tobacco	4%	34%
Oriental Tobacco	12%	16%

It has long been known that the pectin fractions of tobacco are modified chemically by curing procedures (especially air-curing and cigar fermentation where ammonia can interact with the pectic substances present).

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From a commercial aspect, pectins play an important role in the manufacture of reconstituted tobacco. One of the two types of major commercial reconstituted tobacco processes involves the treatment of a tobacco slurry with diammonium phosphate to solubilize the tobacco pectin (57). When the tobacco slurry is then cast on to a hot metal belt, a portion of the ammonia is volatilized "setting" the tobacco pectin to "glue" the reconstituted sheet together. Because ammonia generated plays an important role in natural tobacco flavor formation, this reconstituted process not only provides a means of extending tobacco utilization, it also provides a superior reconstituted tobacco from a smoking quality standpoint by increasing the flavorful pyrazines. The role of ammonia in tobacco will be discussed below.

Carbohydrates and Smoke Chemistry

Carbohydrates in the forms previously described can account for 40-50% of tobacco weight and contribute significantly to smoking quality. As mentioned the sugar esters are a major contributor to the Oriental tobacco flavor in that they thermally release the potent lower fatty acids characteristic of Turkish smoke. In addition, it has been noted that of the 147 pyrolysis products reported from cellulose, starch, mono & di-saccharides and pectic substances that 121 have been found in tobacco smoke (27).

Sensory Role of Carbohydrates, Ammonia and Nicotine - pH of Tobacco Smoke:

Pyrolysis of carbohydrates in tobacco smoke has long been known empirically to change the sensory impression of certain types of tobaccos. Even before cigarettes became popular, sugars were added to air-cured tobaccos used for pipe smoking to mitigate the harshness of nicotine in the smoke. And, today, as 50 years ago, tobaccos are purchased and blended based on certain chemical criteria (sugar & nicotine content) as well as the more subjective criteria of appearance and smoke flavor. In addition, it is a common practice to add sugars (usually invert sugar [Glucose + fructose] or partially inverted sugar which still contains some sucrose) in American blend cigarettes. Sugars are known to "balance" the smoke flavor, primarily by modifying the sensory impact of nicotine and other tobacco alkaloids (37).

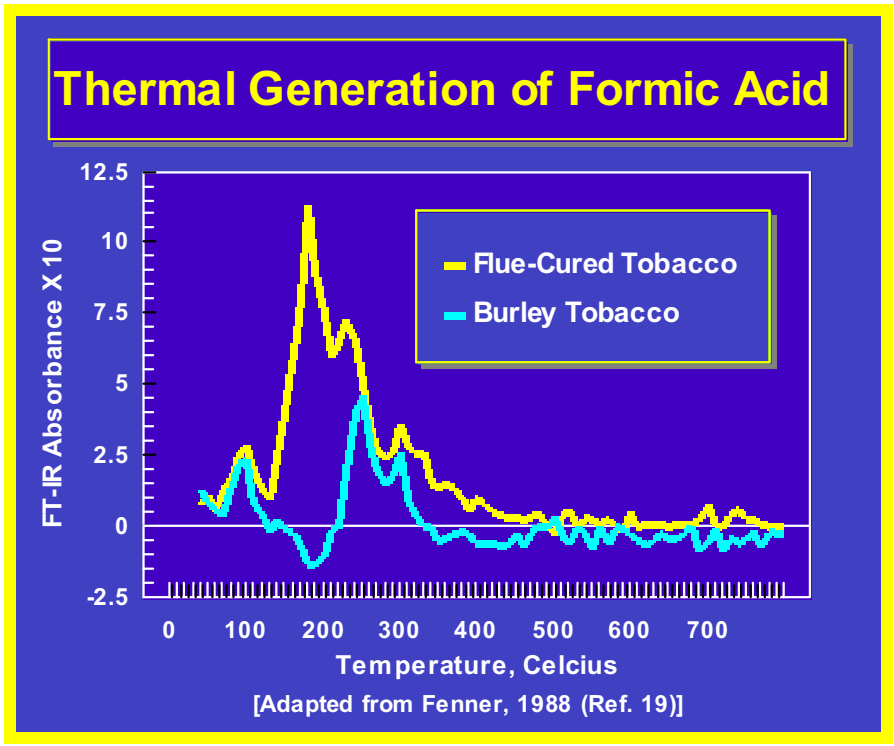
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In the section on nitrogenous constituents of tobacco, we will discuss the interaction of amino acids and ammonia in the development of important compounds in both Virginia and air-cured tobaccos (including amino-sugar compounds).

However, at this point a brief discussion on the effect of the major acid forming constituents (carbohydrates) of tobacco smoke and the interaction with the major basic substances of tobacco and smoke (nicotine & ammonia) is in order.

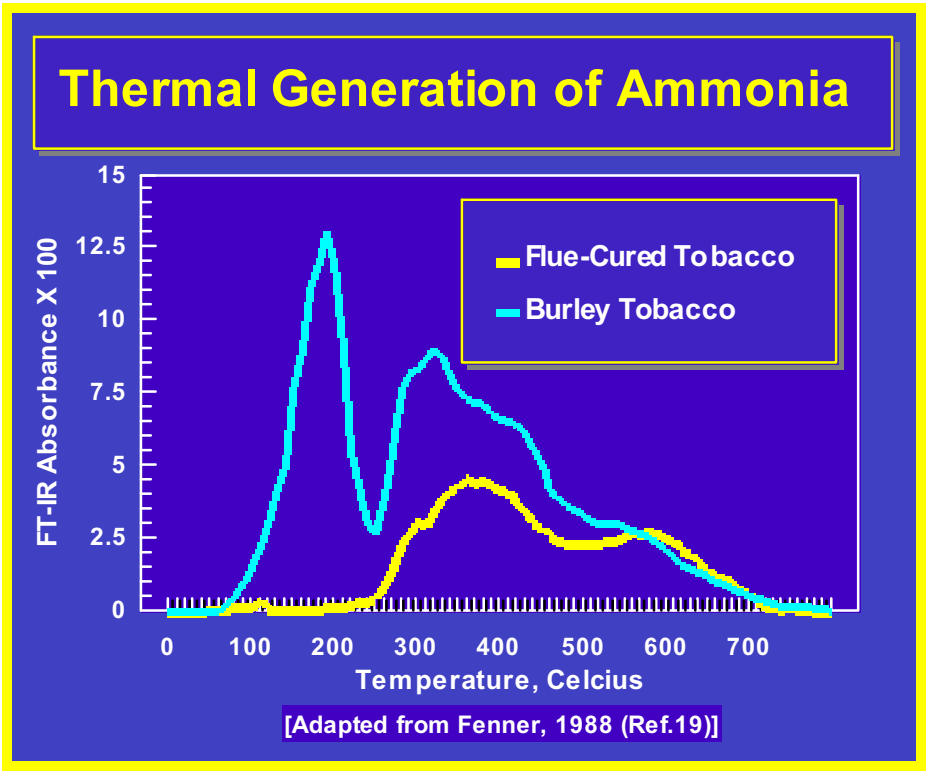
In 1988, Fenner (19) showed the striking differences in the generation of both formic acid (Figure 5) and ammonia (Figure 6) from Virginia and burley tobaccos on pyrolysis over the temperature range encountered in a cigarette.

FIGURE 5



In Figure 5, we see the generation of formic acid in Virginia tobacco to be far greater than for burley, with the large transition at 190°C. being due to simple sugars while the transition at 250°C. is due to pectin & hemicellulose and the transition at 310°C. is due to cellulose.

FIGURE 6



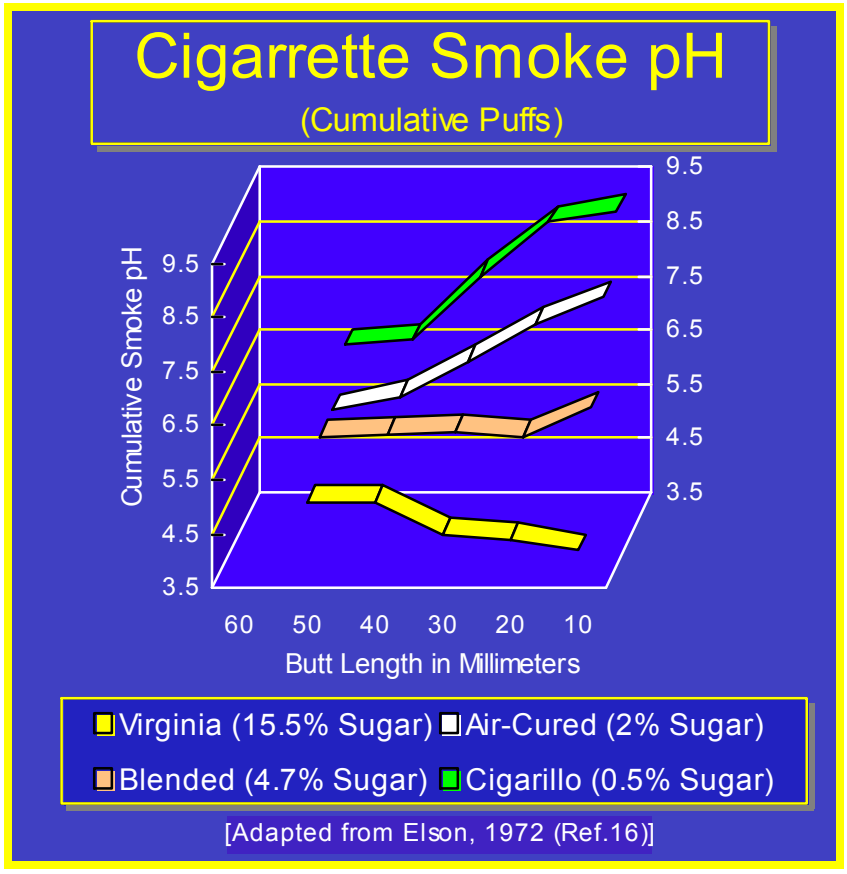
Conversely, Figure 6 demonstrates that burley tobacco generates significantly more ammonia than Virginia. The ammonia transition at 190°C. may simplistically be considered as a labile or exchangeable ammonia source (e.g., ammonium salts), while the 350°C. transition is amine derived and the 550°C. transition is amide-derived ammonia.

The role of acid forming constituents (carbohydrates) and basic constituents (ammonia) can have a significant effect on smoke pH. Figure 7 shows the cumulative effect of successive puffs on the pH of water through which smoke has been passed. In a study of over 150 brands of cigarettes, Elson, et. al. (16), concluded that brands with low sugar content

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generated more alkaline smoke than those with high sugar content (which become progressively more acidic).

FIGURE 7



As nicotine is a major volatile base present in cigarette smoke, the pH of smoke plays an exceedingly important role on the sensory impression imparted.

The higher ammonia levels of air-cured tobacco containing low or negligible sugar content would presumably have two major effects. First. it has an effect on the ratio of nicotine/nicotine salts delivered on smoking relative to high sugar and/or more acidic tobacco types wherein the nicotine and other alkaloids are complexed (more) as salts in the smoke.

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Acids----->
NICOTINE ALKALOID **<=======>** **NICOTINE ALKALOID SALT**
<---Ammonia
(Free Base) (Salt Complex)

Since pH of smoke in air-cured tobacco is considerably more alkaline than flue-cured or Oriental, the ratio of nicotine base to nicotine salts increases. This causes the sensory and physiological perception of increased nicotine strength (and harshness). It should also be noted that the pH of air-cured tobacco smoke increases with succeeding puffs from such cigarettes. Accordingly, the increased alkalinity of straight air-cured cigarettes renders them virtually unacceptable to nearly all smokers as the high smoke pH imparts an alkaloid harshness with a flavor distortion which can be unpleasant. Conversely, many American blend cigarette smokers find the “acidic” smoke of straight Virginia cigarettes to be unbalanced. "...Nicotine alone does not determine smoking flavor..." (37).

Nitrogenous Constituents

The data in Table 4 for air-cured cigar tobacco shows the gross chemical changes in nitrogen compounds during air-curing (20).

In general, there occurs some loss in total nitrogen, a reduction in protein via hydrolysis to amino acids, and formation of amino acid amides (as reflected by an increase in soluble nitrogen). Changes occurring in burley tobacco curing (24,25) are similar to those in stalk-cured cigar tobacco.

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TABLE 4
Changes of Nitrogenous Compounds in Air-Cured Cigar Tobacco (20)
(Percent of Harvested Dry Weight)

	Primed Leaf		Stalk Cured	
Type of Nitrogen	**Before Curing**	**After Curing**	**Before Curing**	**After Curing**
Total	5.61	5.34	4.70	3.80
Protein (Insoluble)	3.69	1.65	3.80	1.85
Soluble	1.92	3.69	0.90	1.95
Amino	0.23	0.80	0.15	0.15
Ammonia plus Amide	0.15	1.07	0.05	0.80
Alkaloid	0.35	0.32	0.40	0.40
Nitrate	0.63	0.77	0.20	0.25
Remainder	0.56	0.73	0.10	0.35

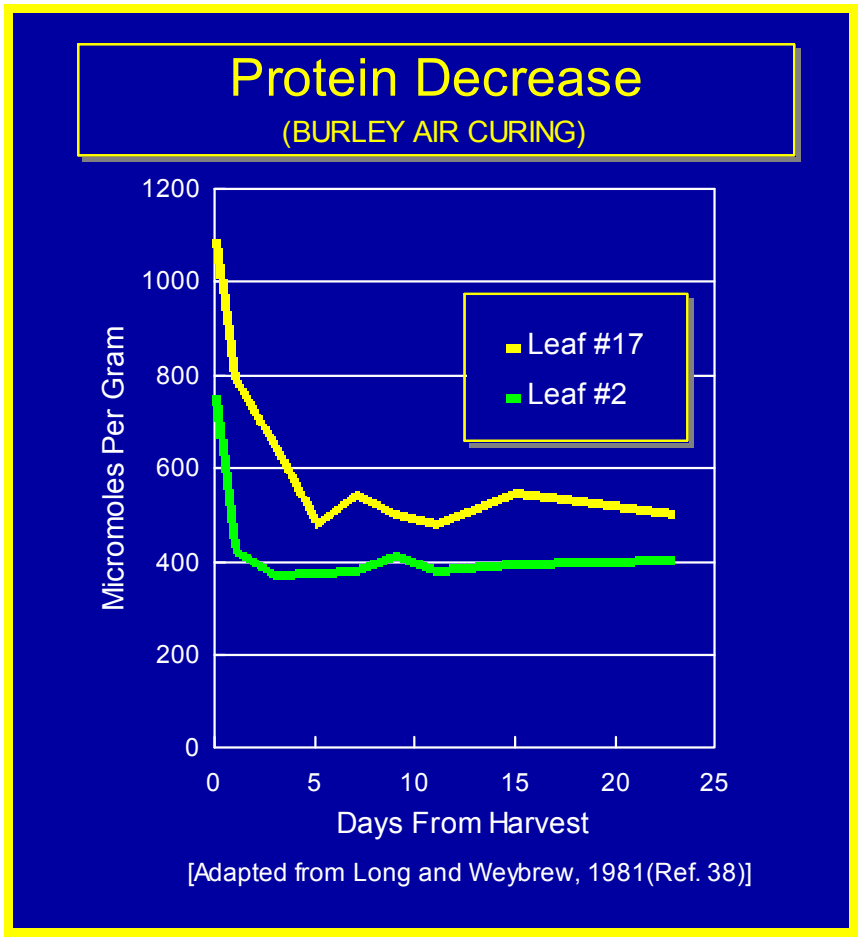
(Frankenburg, 1946)

Flavor quality for both flue-cured and burley tobaccos is dependent on a variety of complex interacting factors related to genetics, agricultural practices, soil types, nutrients, weather conditions, plant disease, stalk position as well as harvesting and curing practices. In general, elevated levels of proteins and amino acids appear to contribute negatively to smoke flavor. This is particularly true of burley tobaccos grown in cool moist climates with insufficient sunshine, which are over-fertilized, harvested and air-cured prematurely. In such cases the normal catabolic changes during senescence and curing which result in the enzymatic hydrolysis of Fraction 1 protein to amino acids (which then can undergo further catabolic changes) are at least partially arrested. Such immature tobaccos possess a proteinaceous aroma on smoking which is unpleasant. Even secondary fermentation of such poor tobaccos often cannot provide sufficient improvement to make them useable. By comparison to normal burley, in which approximately 50% of total protein undergoes hydrolysis to amino acids, such poor tobaccos may exhibit less than 15% protein hydrolysis during air-curing (29).

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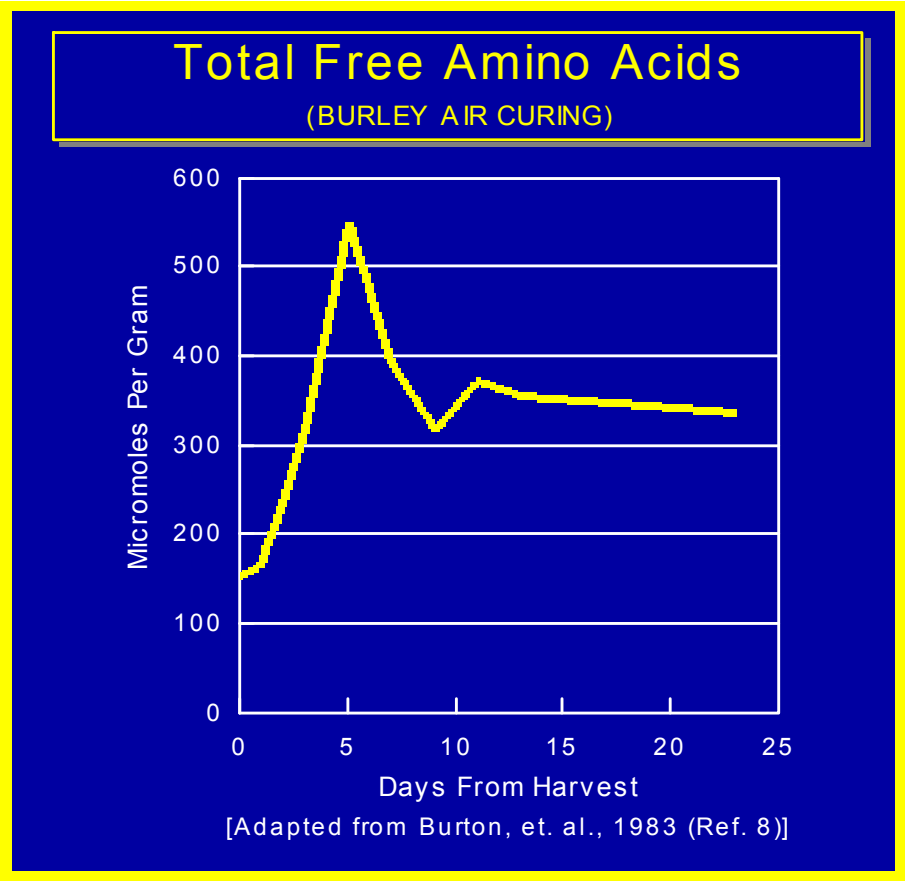
Normal air-curing of fully mature burley shows a rapid drop in protein (Figure 8)(24,38) with a concomitant initial increase in free amino acids for the first few days in cure (Figure 9) (8).

FIGURE 8



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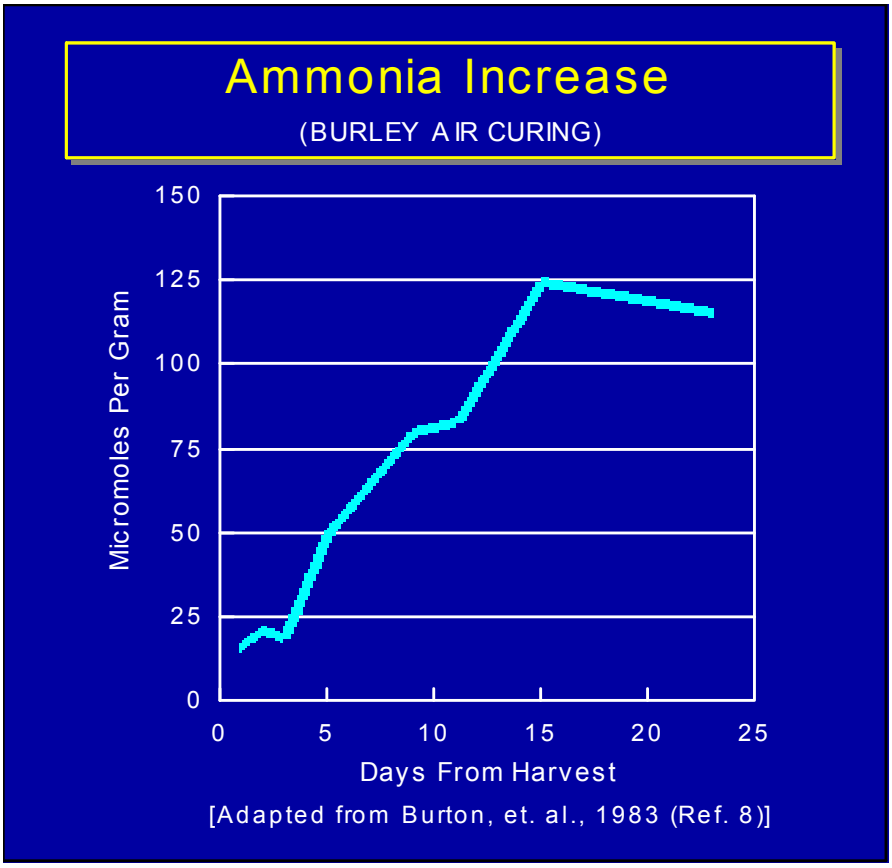
FIGURE 9



Thereafter, there is a decrease and finally a leveling off in total free amino acid concentration. The decrease in free amino acid content after the 5th day of air-curing is due to both metabolic deamination and decarboxylation as well as nitrogen translocation. Even though a slight decrease from a maximum in total free amino acids occurs during the cure, some individual amino acids show a net decrease while others have a net increase (8). It should be noted that the ammonia level of air-cured tobacco rapidly increases during this period (Figure 10) (8).

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FIGURE 10



The role of ammonia on smoke pH and nicotine perception has been discussed above, but ammonia also plays an important role in the formation of aroma and taste compounds.

It is important to remember that, before curing, air-cured burley or cigar tobaccos may contain 3-4% protein nitrogen, while flue-cured tobacco contains only 15-20% of that amount. In addition, about 50% of the protein in burley may undergo hydrolysis during air-curing while only about 20% of the protein is hydrolyzed in curing flue-cured tobaccos. Thus, burley tobacco possesses a relative abundance of free amino acids compared to flue-cured tobacco. Representative free amino acid analysis (Table 5) of high smoking quality blends of flue-cured and burley tobaccos with similar nicotine content illustrate this dramatically (37). In burley, we find the main free amino acids present to be aspartic acid, asparagine and glutamic acid, while for flue-cured, proline, asparagine and glutamine are dominant. In total, 43 amino acids have been found in tobacco leaf.

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TABLE 5
Free Amino Acids (37)
High Quality Smoking Grade Blends
(milligrams per gram)

Amino Acid	Flue-Cured	Burley
Aspartic Acid	0.13	7.84
Threonine	0.04	0.43
Serine	0.06	0.17
Asparagine	1.12	10.30
Glutamic Acid	0.10	1.78
Glutamine	0.82	0.38
Proline	4.11	0.45
Glycine	0.02	0.14
Alanine	0.32	0.35
Valine	0.06	Trace
Isoleucine	(-)	0.06
Leucine	Trace	0.10
Tyrosine	0.68	0.84
Phenylalanine	0.24	0.50
Lysine	0.03	0.33
Histidine	0.11	0.45
Arginine	(-)	0.26
Tryptophan	(-)	0.50

(Leffingwell, 1976)

Cigarette tobaccos are almost never utilized in consumer products unless they have undergone a period of aging ranging from 12-60 months. Of course, inventory control is an important factor requiring maintenance of adequate stocks to allow periodic blend changes to occur; *but, freshly cured and redried leaf is not used primarily because the smoke changes dramatically from a raw, somewhat irritating and disagreeable taste to a smoother, more rounded flavor during the aging process*. Aging is ordinarily carried out at ambient temperatures and about 12% moisture. In areas with relatively long-term high ambient

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temperatures the desired aging effects are produced in shorter periods than in colder climates (5), and it is common for companies located in cold climate regions to store and age their tobaccos in southern locales. The role that nitrogen compounds play in the improvement of tobacco flavor quality with aging is important and the reasons for this will become apparent.

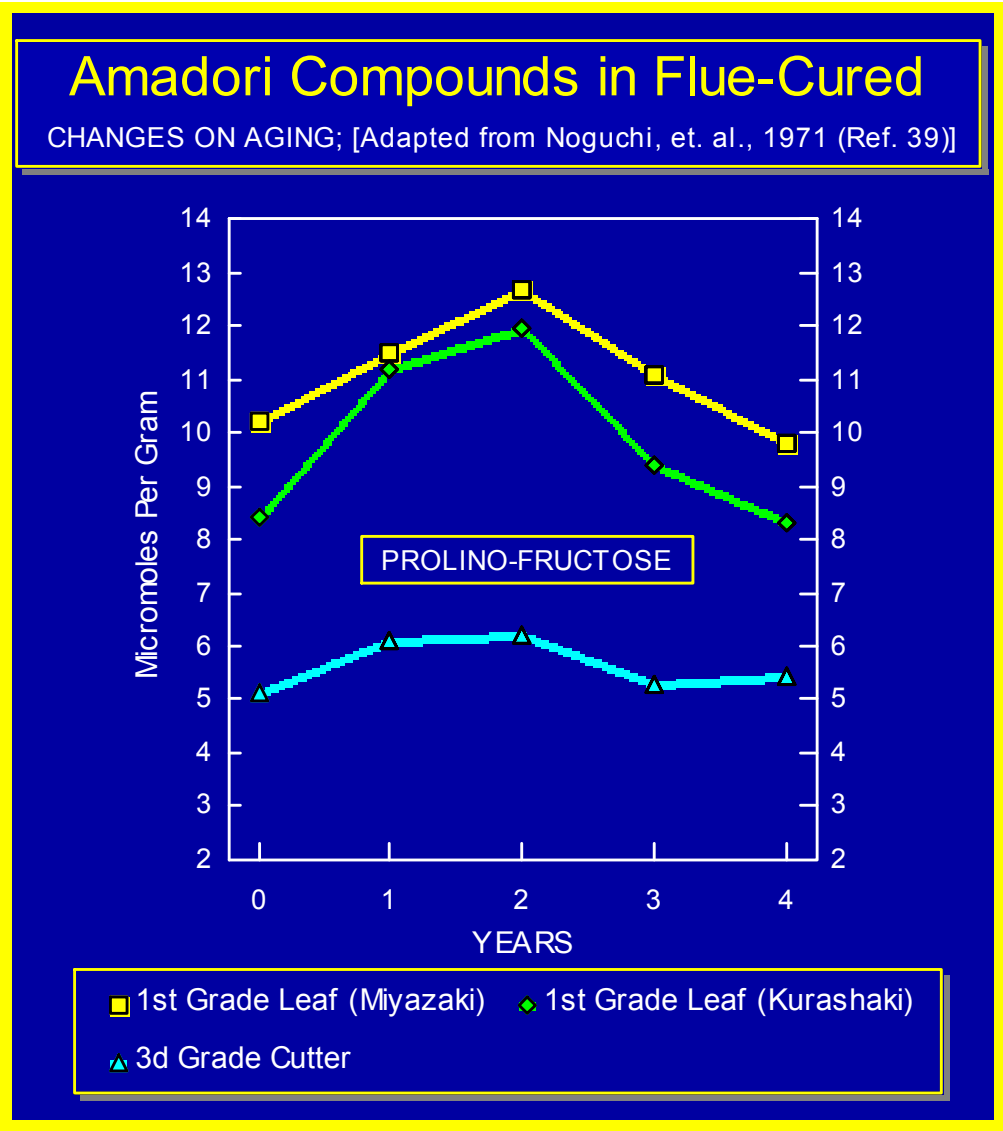
Let us now discuss briefly the role of certain amino acids in flue-cured tobacco. Protein hydrolysis with formation of free amino acids occurs also at the yellowing and early stages of drying during flue-curing**,** although flue-cured tobacco contains only about 20-25% of the total amount of protein/amino acids as burley tobacco. The major amino acid in flue-cured tobacco, proline, seems to be an anomaly in that as much as a 25 fold increase has been observed during the curing schedule for both flue-cured and air-cured (Maryland) tobaccos (54). This appears to be greater than can be accounted for by proteolysis of fraction 1 protein - a hypothesis (24) being that proline is partially formed by a metabolic conversion of the pyrrolic portion of chlorophyll (which is rapidly decreasing during this stage).

In flue-cured tobaccos, proline reacts with the reducing sugar, glucose, to form l-deoxy-l-(L-prolino)-D-fructose (39), an Amadori compound, which along with other amino-sugars comprises as much as 1.5-2.0% of the dry aged tobacco weight. This compound is significant in that it has been shown to undergo low temperature degradation primarily to the probable precursor (2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one [DDMP]) of the important volatile flavorant, maltol, found in Virginia tobacco (35). It has also been shown that the smoking properties of DDMP are identical to that of maltol (37).

Figures 11 and 12 show the change in two of the major Amadori compounds in Virginia tobacco grown in Japan over a four year aging period (39).

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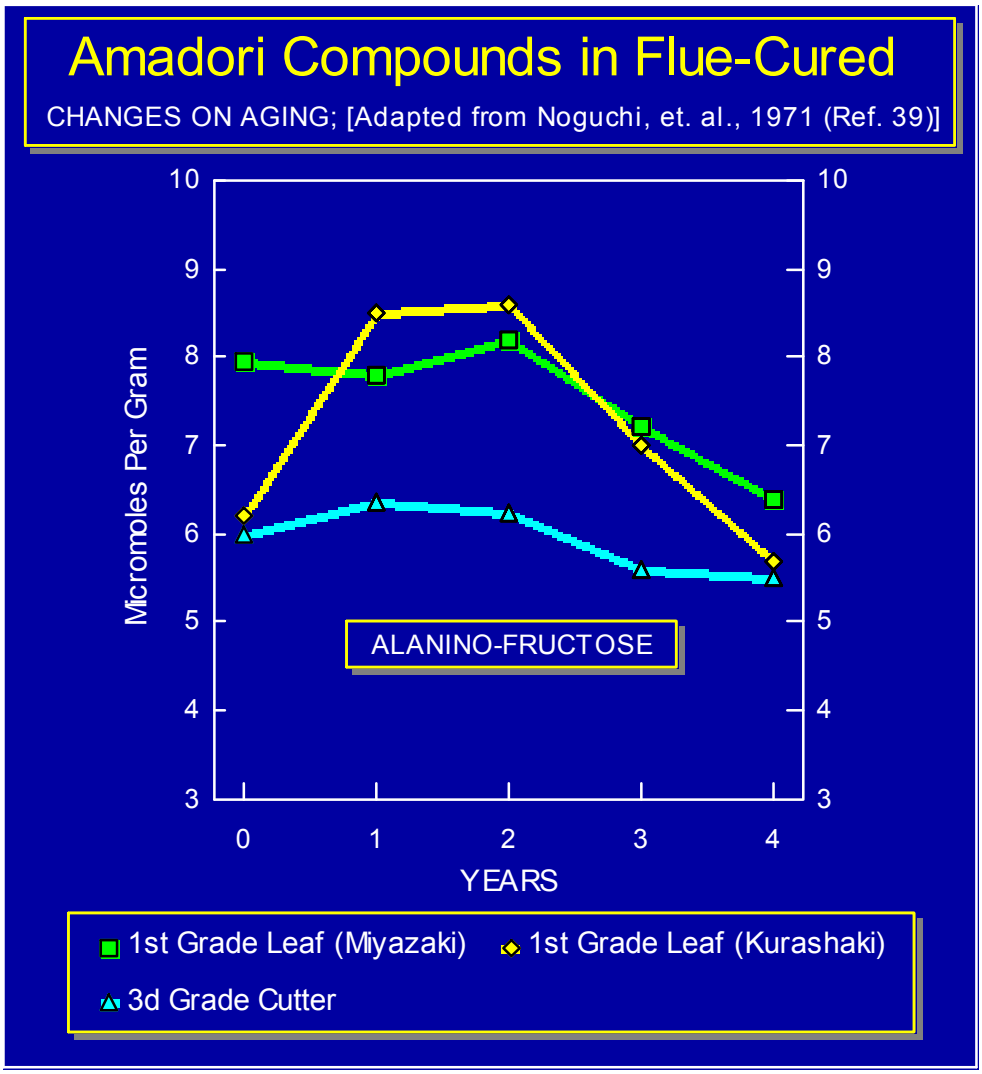
FIGURE 11



The major Amadori compounds present tend to show a gradual increase peaking at about two years, and then a decrease. During the same period, the amino acid content of the tobaccos show decreasing values each year. Of course, it is also known that the flavor of Virginia tobacco improves with aging, it rarely being used for cigarettes prior to 12-14 months aging, and preferably 18-24 months, which interestingly corresponds quite well to these graphs. Amadori compounds are also known to generate a number of flavorful pyrazines and pyrroles on pyrolysis.

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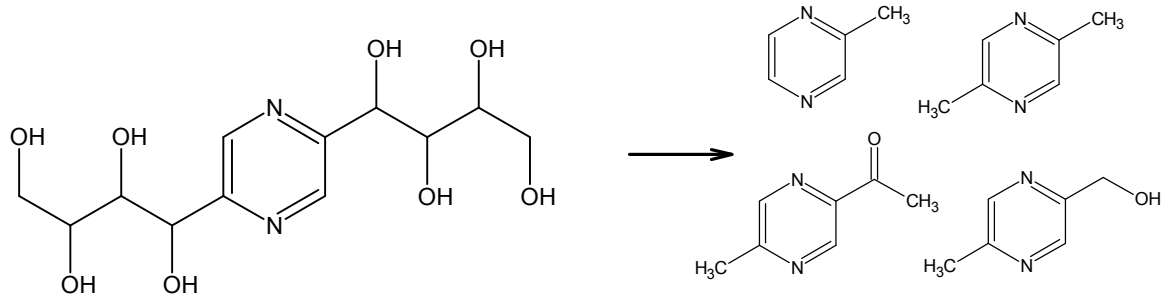
FIGURE 12



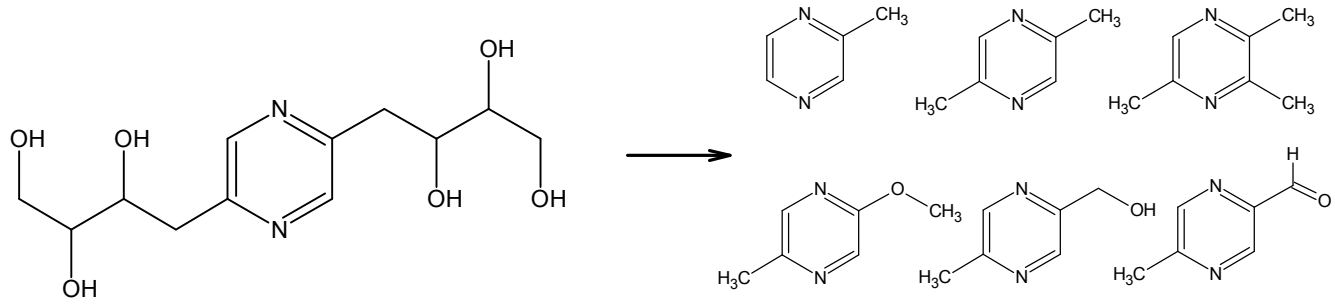
Amino acids as well as ammonia interactions with sugars or carbonyl compounds can form a variety of chemical entities. For example, a number of groups have reported the isolation of fructosazines and deoxyfructosazines from Virginia tobacco (27,46) and these are known to be formed by the interaction of ammonia with glucose and fructose under weakly acidic conditions.

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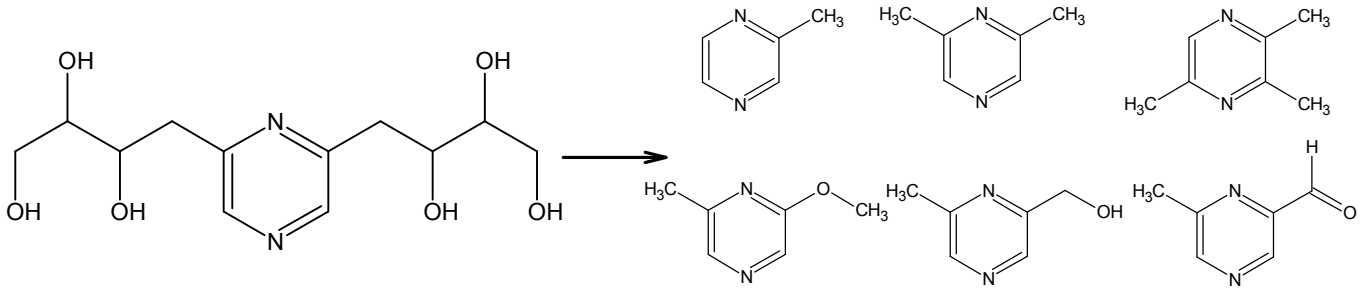
MAJOR PYRAZINES FROM PYROLYSIS OF FRUCTOSAZINE & DEOXYFRUCTOSAZINES (27, 59)



FRUCTOSAZINE



2,5-DEOXYFRUCTOSAZINE



2,6-DEOXYFRUCTOSAZINE

On pyrolysis, these polyhydroxypyrazines (including glucosamine) generate products such as water, acetic acid, acetol, furans, pyrroles and a number of simple pyrazines (including some of the major tobacco leaf pyrazines, 2,5- & 2,6-dimethylpyrazine, trimethylpyrazine and tetramethylpyrazine) (27). Although pyrazines as a class represent a very small percentage of tobacco weight, they are known to be very important to the flavor and aroma of roasted foodstuffs and tobacco. It is noteworthy that reconstituted tobacco prepared by the Philip Morris process (57) is characterized by the presence of 2-hydroxymethyl-5-methyl pyrazine

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(along with lesser amounts of 2-hydroxymethyl-6-methyl pyrazine) derived from these polyhydroxypyrazines (58). Moldoveanu and Alford have shown that the deoxyfructosazine major pyrolysates are the corresponding methylpyrazine carboxaldehydes and of hydroxymethylmethyl pyrazines (59).

Bright and co-workers demonstrated that the weight percentages of five amino acids were significantly reduced by heat treatment, while the roasting of tobacco causes dramatic increases in pyrazine levels (especially those high in ammonia or ammonia precursors) under conditions where percent of nicotine remained constant (Tables 6 & 7) (6).

TABLE 6
Change in Amino Acid Levels
of Tobacco After Roasting (6)
(Complete Cigarette Blend)

Amino Acid	Before(%)	After(%)	Δ%
Aspartic Acid	1.39	0.82	- 41
Proline	0.65	0.31	- 52
Lysine	0.32	0.10	- 69
Histidine	0.24	0.16	- 33
Arginine	0.16	0.06	- 62

(Bright, et. al., 1975)

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TABLE 7

Effects of Roasting on Dimethylpyrazine Concentration (6)

	Conc. Prior To Roasting (ppb)	Conc. After Roasting (ppb)
Virginia	0.1	0.3
Turkish	0.1	0.8
Burley	0.1	460
Reconstituted Leaf	0.1	200

(Bright, et. al., 1975)

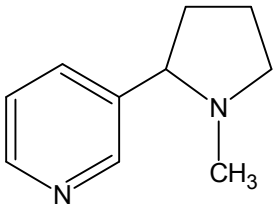
Tobacco Samples Were Shredded, Without Additives, Roasted 4 Hours At 120° $\pm$ 3° C.

Wahlberg, et. al., have examined the chemical changes occurring on the flue-curing and aging of Virginia tobacco (52). One of the observations was that a number of pyrazines, pyrroles and pyridines increased during the flue-curing and aging process (18). As pyrroles, pyridines and pyrazines can arise in tobaccos by a number of chemical pathways ranging from the heating or aging of proteins and amino acids to the interaction of amino acids and / or ammonia with sugars or carbonyl constituents, suffice it to say that no single pathway is involved and that this complex transformation is dependent on a variety of factors and chemical mechanisms (35). The generation of certain pyridines (e.g., methyl nicotinate, 3-cyanopyridine and 3-acetylnicotine) can also be derived from degradation of nicotine.

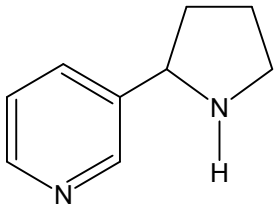
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Tobacco Alkaloids

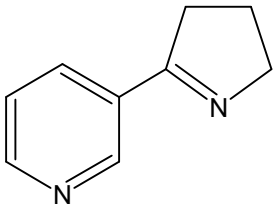
The major tobacco alkaloids are nicotine, cotinine, nornicotine, myosmine, nicotyrine, anabasine and anatabine.



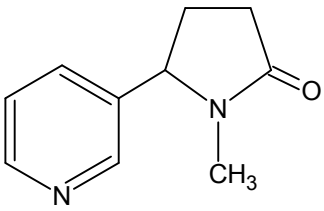
NICOTINE



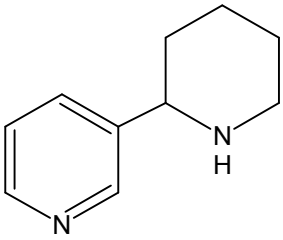
NORNICOTINE



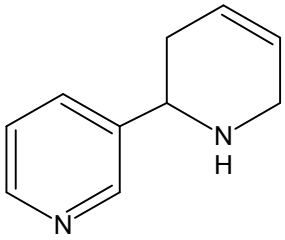
MYOSMINE



COTININE



ANABASINE



ANATABINE

This subject is covered in greater detail reference 18 and will not be discussed here except to note that nicotine can range in concentration from 0.5 to 8% in the major cultivated tobacco species, N. tabacum and N. rustica. The interaction of smoke pH and the sensory perception of nicotine is discussed above.

Plastid Pigments

The major pigments of tobacco are the chlorophylls and carotenoid pigments, plastid pigments found in a wide variety of plants. The chlorophyll and carotenoid contents and changes during the latter stages of growing (and the curing processes) have been studied in good detail for both burley and Virginia tobaccos.

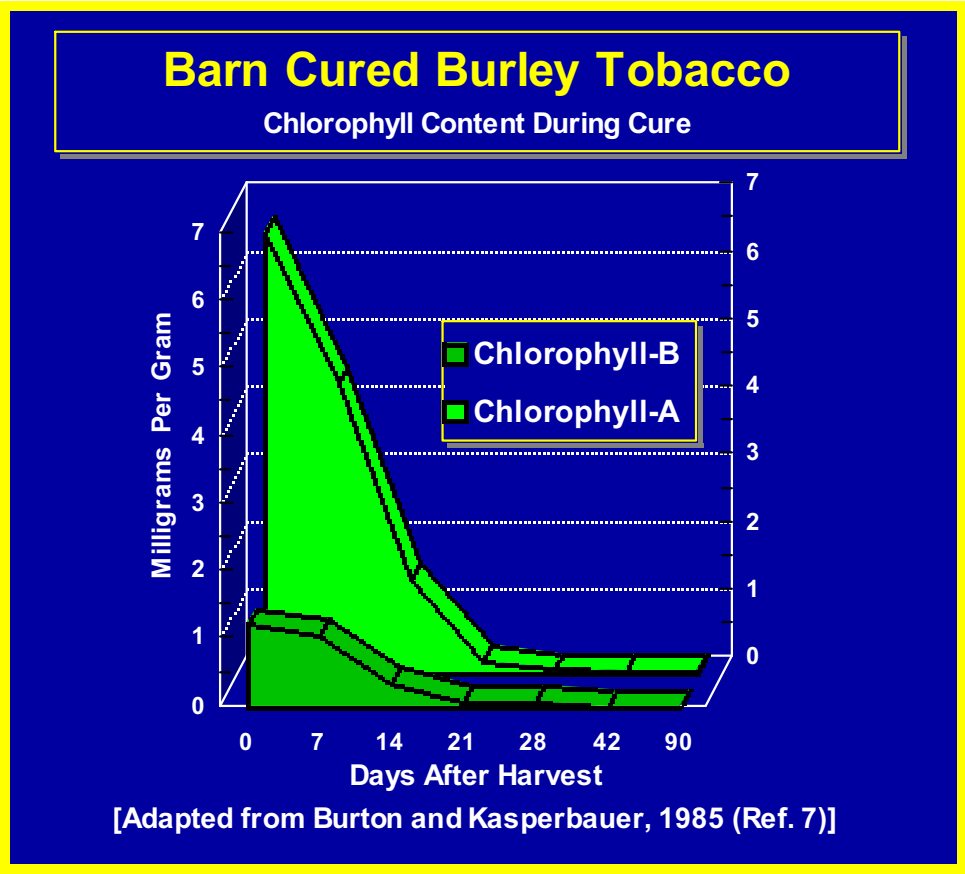
Chlorophylls:

As shown in Figure 13 for burley tobacco, the major green pigments are chlorophyll-A and chlorophyll-B, both of which decrease as the tobacco reaches maturity, proceeds into

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senescence, and continues to decrease during barn curing (7). Studies on Virginia tobacco show similar trends as the tobacco reaches maturity (11).

FIGURE 13



Leaf color in the field as well as for cured leaf are considered to be important in judging the quality of leaf and this has been extensively studied (50).

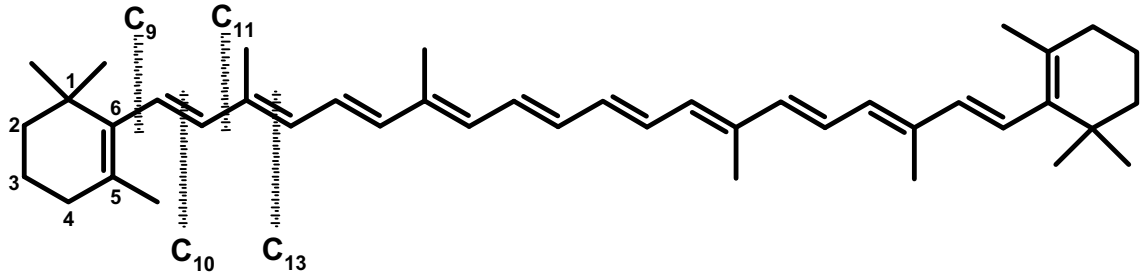
Carotenoid Pigments:

The major carotenoid pigments in tobacco are lutein, β-carotene, neoxanthin and violaxanthin. In addition to being major color pigments (red-orange to yellow), the carotenoids are the precursors to many of the volatile aroma components of tobacco.

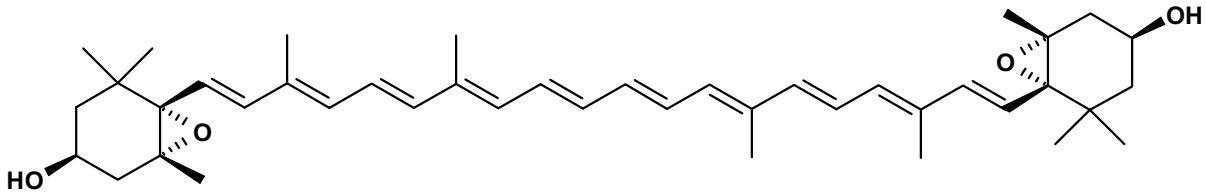
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FIGURE 14

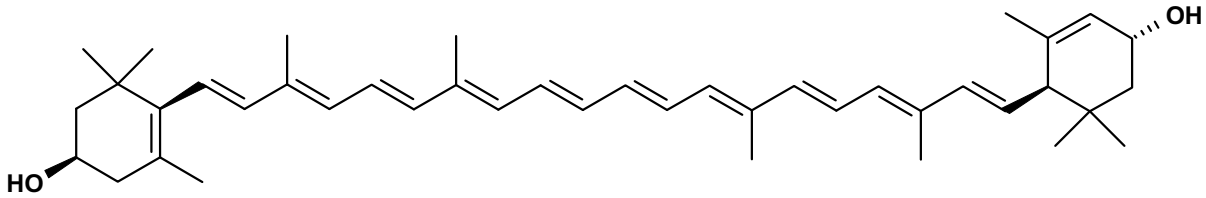
MAJOR CAROTENOID PIGMENTS IN TOBACCO



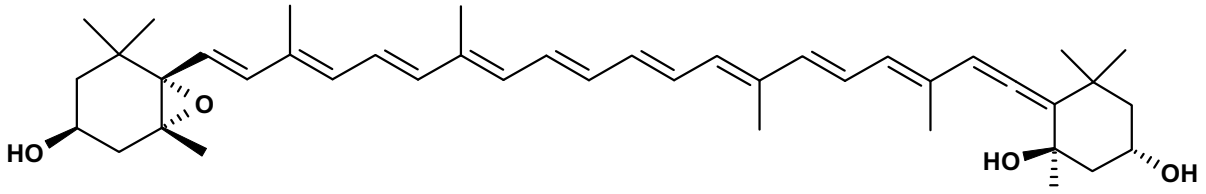
β-CAROTENE



VIOLAXANTHIN



LUTEIN



NEOXANTHIN

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FIGURE 15

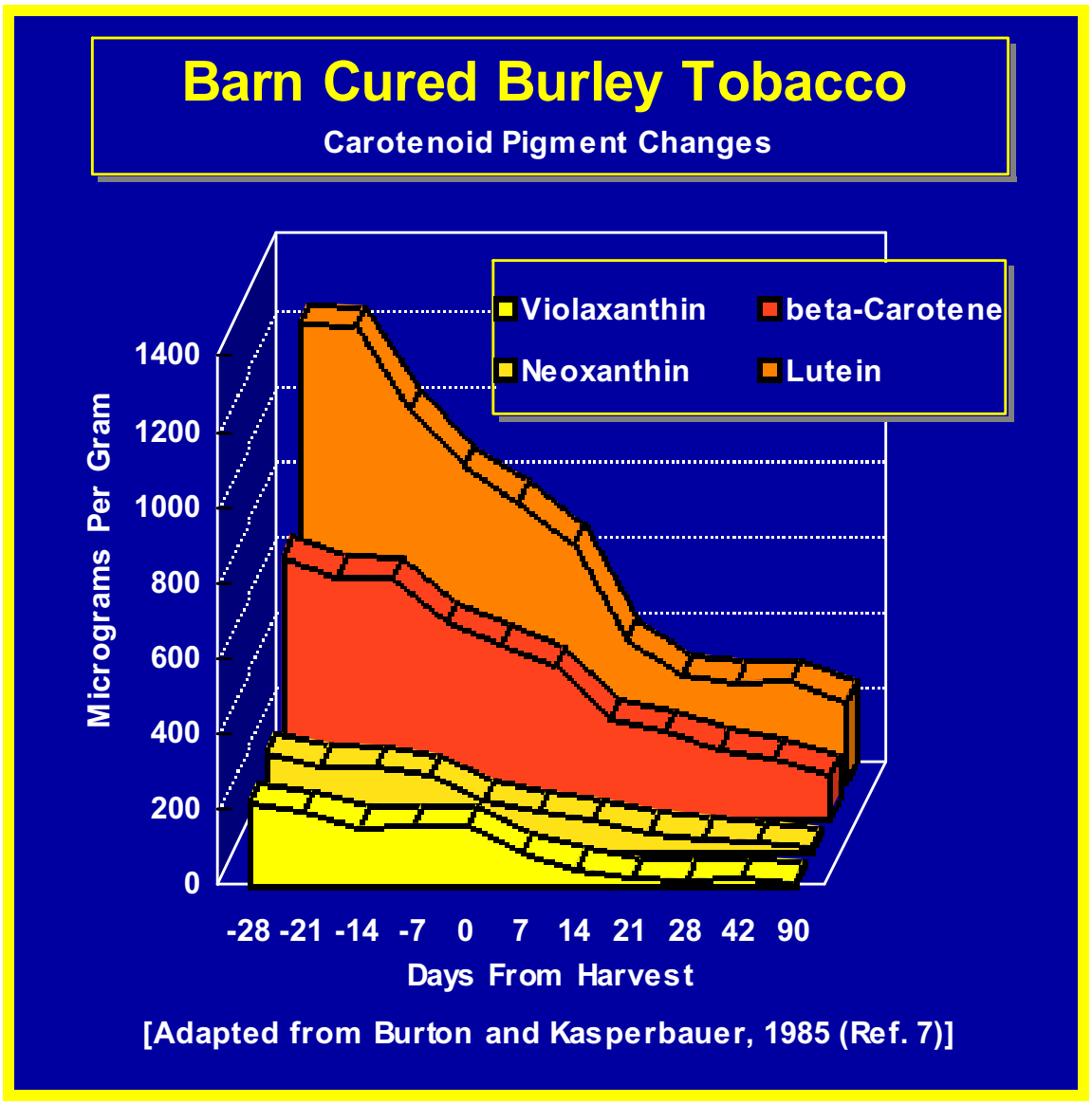


Figure 15 shows the decrease in carotenoids during maturation, senescence and curing for burley tobacco (7). Unlike the chloroplastid pigments, the carotenoids do not undergo as extensive degradation (metabolism). Lutein and carotene undergo about 65% degradation in burley. More extensive degradation of lutein has been reported for Virginia tobacco (11).

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Tobacco Isoprenoids

Wahlberg and Enzell (51) have comprehensively reviewed the subject of tobacco isoprenoid constituents and we will only provide comments on some of the more important materials.

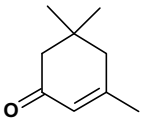
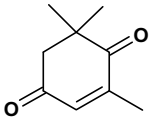
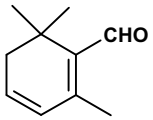
Degraded Carotenoids:

A large number of carotenoid metabolites are encountered in tobacco. The degradation process of the carotenoid pigments in tobacco has been hypothesized to probably be both enzyme and autooxidative in nature. As indicated in Figure 14 for carotene, the carotenoid chain may be cleaved in a number of locations resulting in a variety of C_9 to C_{13} chemicals, many of which possess important aroma properties. Table 8 (35,56) illustrates the quantitative amounts found for a number of important nor-carotenoids for various tobacco types.

TABLE 8

Major Tobacco Carotenoid Degradation Products

(Parts Per billion in Leaf)

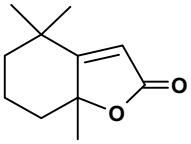
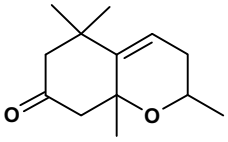
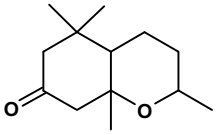
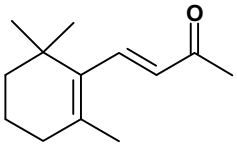
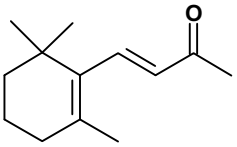
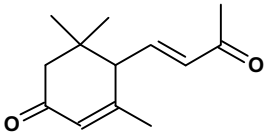
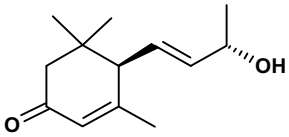
Compound	Virginia	Burley	Oriental
 Isophorone	112	29	33
 4-Ketoisophorone	47	17	42
 Safranal	32	4	43

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TABLE 8

Major Tobacco Carotenoid Degradation Products

(Parts Per billion in Leaf)

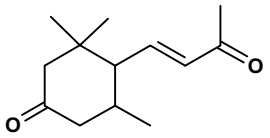
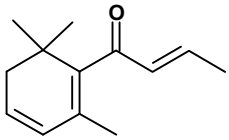
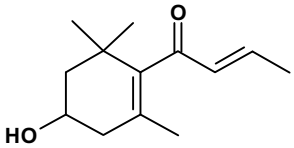
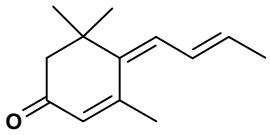
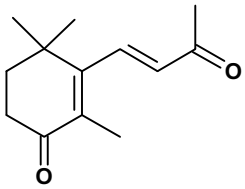
Compound	Virginia	Burley	Oriental
 Dihydractinodiolide	150	26	73
 Oxoeudalane	139	25	34
 Dihydro-oxoeudalane	47	27	73
 α-Ionone	+	+	+
 β-Ionone	+	+	+
 3-Keto-α-Ionone	+	NR	NR
 3-Keto-α-Ionol	+	4	+

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TABLE 8

Major Tobacco Carotenoid Degradation Products

(Parts Per billion in Leaf)

Compound	Virginia	Burley	Oriental
 3-Ketodihydroionone	51	31	23
 Damascenone	168	42	39
 3-Hydroxy-β-damascone	24	11	+
 Megastigmatrienones (5 Isomers)	1272	248	415
 4-Keto-β-ionone	NR	+	NR

NR = not reported; + = present, but quantitation not available
Table adapted from Wilson, et. al., 1982 (Ref. 56) and Leffingwell, 1988 (Ref. 35)

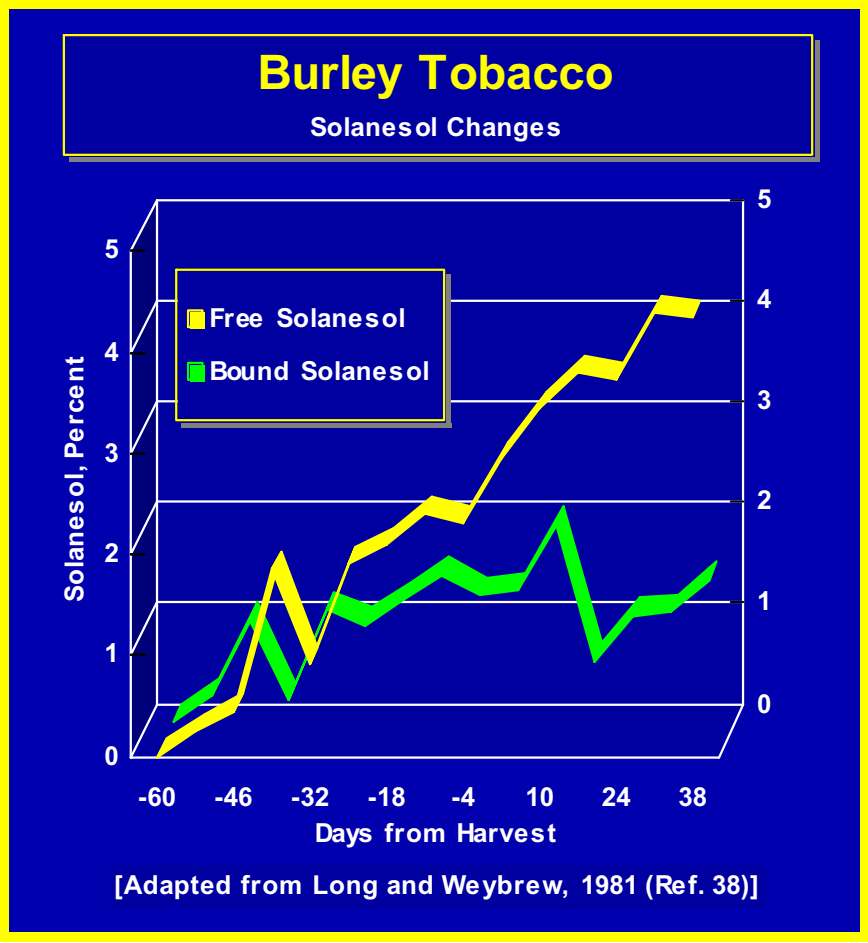
The degradation of both lutein and β-carotene by both photo-oxidation and high pressure oxidation (at elevated temperature) has recently been shown to generate many of the same nor-carotenoid components found in tobacco (28).

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Acyclic Isoprenoids and Nor-derivatives:

Solanesol (a C_{45} polyprenol) is a major component of tobaccos generally ranging in quantity from 0.4 to 4%. While this polyprenol was first isolated from tobacco, it is now considered to be a ubiquitous leaf component in the plant kingdom. The progression of solanesol change during growth and air curing for middle stalk burley tobacco is shown in Figure 16 (38).

FIGURE 16



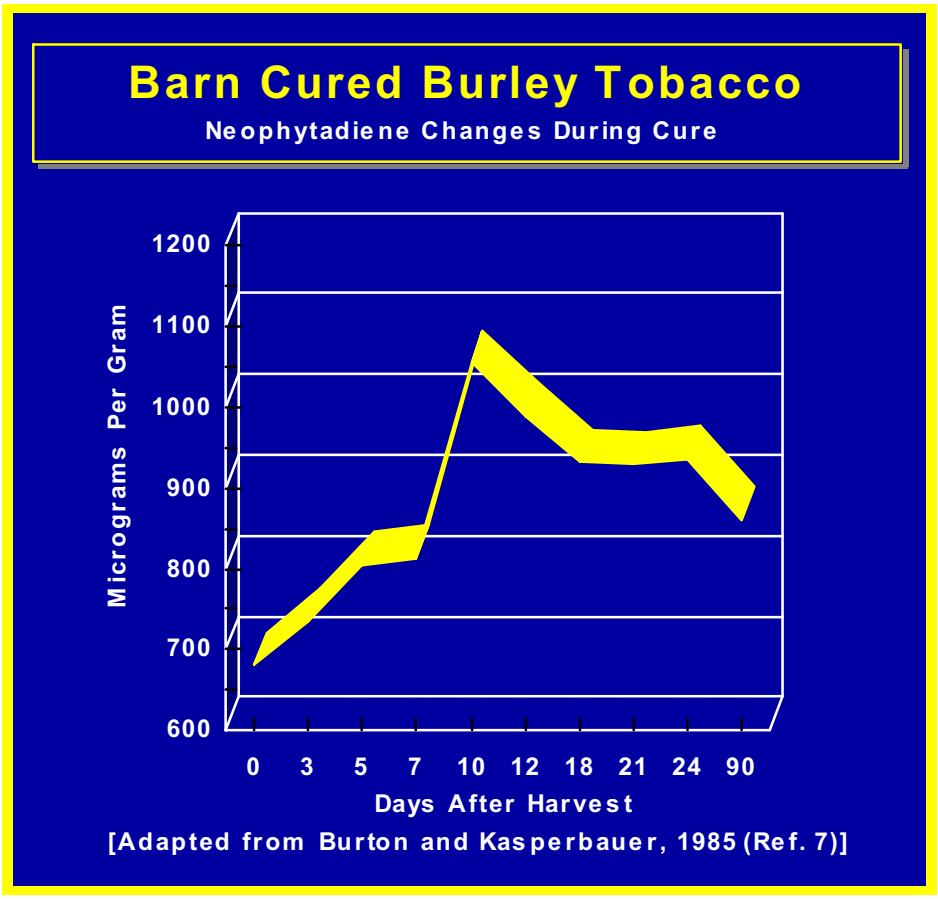
It should be noted that solanesol is present as both the free alcohol and in bound forms primarily as acetate, palmitate and linolate esters. Solanesol normally ranges from 1-2% in Virginia tobacco and from 1 % to as much as 4% in burley tobacco.

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The concentration of solanesol in burley tobacco has been shown to be effected by genetic lines and, more importantly, by production practices. Water stress, through decreased soil moisture leads to an increase in solanesol content as does the length of senescence from time of topping (9).

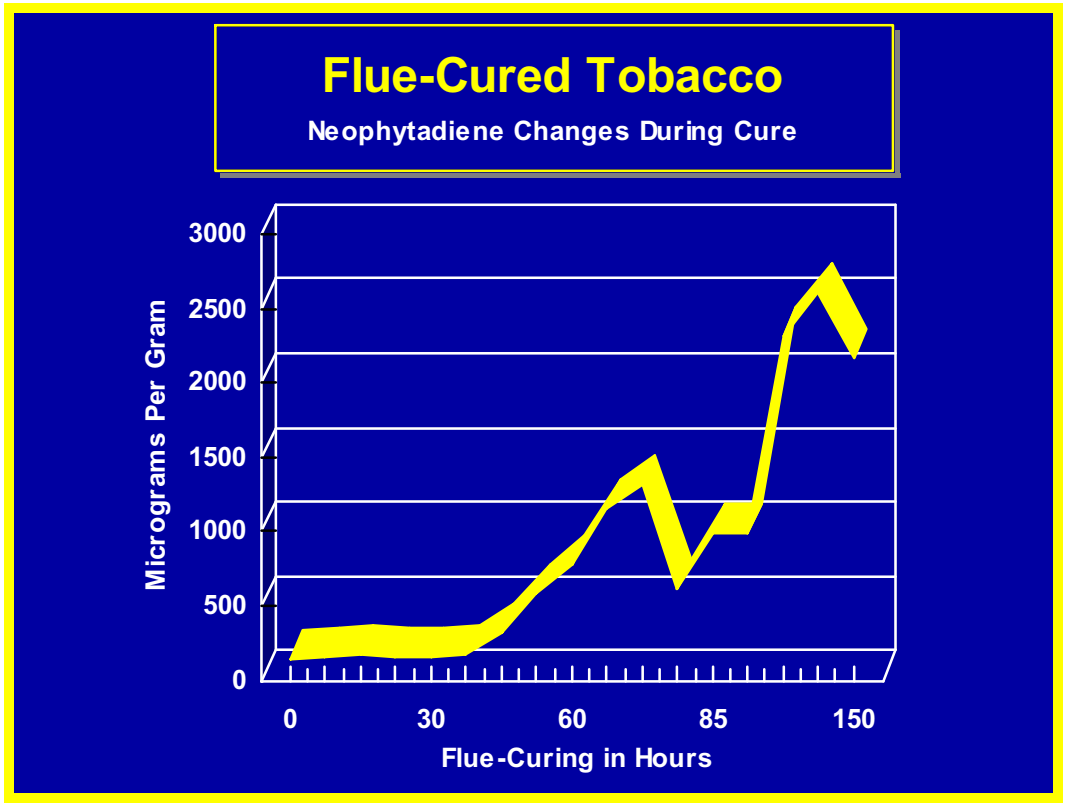
Neophytadiene (a C_{20} isoprenoid polyene) is a tobacco diterpene whose concentration increases significantly on curing and aging. Generally, low levels are present in green tobacco leaf, but neophytadiene increases during the yellowing and curing phases. Figure 17 shows the neophytadiene change in curing burley tobacco (7) and Figure 18 shows the change in flue-curing Virginia tobacco (38).

FIGURE 17



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FIGURE 18



It has been assumed that neophytadiene is produced by dehydration of phytol. Phytol is a metabolite from chlorophyll hydrolysis, but it has been concluded that only 15-30% of neophytadiene generated during flue-curing could be accounted for from this source in the case of flue-cured tobacco (1). Neophytadiene has been suggested to be a tobacco flavor enhancer in that it may act as a flavor carrier by entrapping volatiles in the tobacco smoke aerosol (35).

Among other major acyclic isoprenoids are linalool, linalool oxide, geranylacetone and farnesylacetone. Table 9 provides comparative data on some acyclic isoprenoids, nor-derivatives and neophytadiene obtained by the steam distillation of various tobacco types (56).

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TABLE 9

Some Major Acyclic Isoprenoids & nor-Isoprenoid Products Isolated from the Steam Distillation of Tobacco types (PPB in Leaf)

Compound	Virginia	Burley	Oriental
Linalool	281	160	--
Linalool oxide	118	--	--
Geranyl acetone	181	84	130
Farnesylacetone	16	22	trace
6-Methylhepta-3,5-dien-2-one	40	17	--
6-Methylhept-5-en-2-one	78	17	78

Carbocyclic Diterpenoids and their Degradation Products:

Two major classes of diterpenoids are found in tobacco, the monocyclic cembranoids and the bicyclic labdanoids (51). These diterpenoids are produced in the glandular heads of the trichomes on the leaf surface as well as in tobacco flowers and are part of the surface cuticular waxes where they occur along with the sucrose tetraesters, wax hydrocarbons, esters and alcohols.

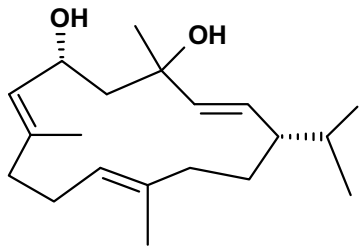
Virginia and burley tobaccos contain only the cembranoids, while Oriental and cigar tobaccos contain both labdanoids and cembranoids. The variety specific occurrence is due to the fact that Nicotiana tabacum L. is a hybrid of Nicotiana tomentosiformis (which produces labdanoids) and Nicotiana sylvestris (which produces cembranoids). It has been shown that the biosynthesis of the cembranoids is controlled by two dominant genes whereas labdanoid synthesis is controlled by a single dominant gene (51).

Cembranoids and Their Degradation Products:

More than 50 cembranoids have been isolated from tobacco. The cembra-2,7,11-triene-4,6-diols are the major cembranoids of tobacco (which, for example, have been found in Virginia to be present at 0.23% by weight) (11).

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FIGURE 19



CEMBRA-2,7,11-TRIENE-4,6-DIOLS (2 ISOMERS)

Perhaps more important to the quality of tobacco flavor are the cembranoid metabolite products produced by oxidative processes and retro-aldol reactions. More than 60 degradation products of cembranoids have been isolated from tobacco. Of these degradation products, some of the major metabolites are shown in Table 10.

TABLE 10

Some Major Cembranoid Degradation Products

Isolated from the Steam Distillation of Tobacco types

(PPB in Leaf)

Compound	Virginia	Burley	Oriental
Solanone	1725	493	1497
Solanol	545	84	177
Norsolanadione	156	26	137
Solanascone	24	33	trace
Solavetivone	18	15	trace
6- Methylhepta-2,5-dione	--	--	101

Solanone, a major cembranoid metabolite in tobacco, is considered to be an important tobacco flavor constituent.

Labdanoids and Their Degradation Products:

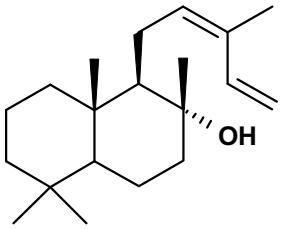
(Z)-Abienol is a major labdanoid in the green leaf of Oriental tobacco and has been shown to be a plant growth regulator. Upon the air and sun curing the concentration of abienol decreases significantly and numerous other labdanoids and labdanoid degradation products are

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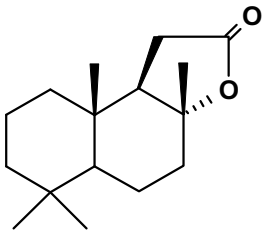
formed. In cured leaf about 20 labdanoids and an additional 25 or more degradation products are found. (Z)-Abienol has been shown to undergo various oxidative processes to many of these products (51). Figure 20 shows some important labdanoid derived products in Oriental tobacco.

FIGURE 20

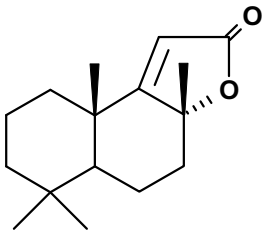
SOME LABDANOIDS IN ORIENTAL TOBACCO



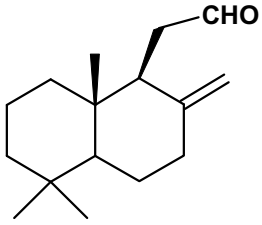
(Z)-ABIENOL



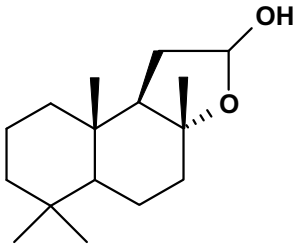
NORAMBREINOLIDE



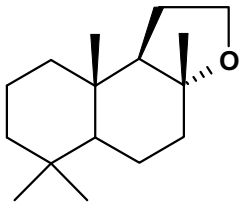
DEHYDRO-NORAMBREINOLIDE



γ-BICYCLOHOMOFARESAL



SCLARAL



AMBROX

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In 1959, norambreinolide (sclareolide), an important constituent of Oriental tobacco and cigar tobacco was patented (41) as possessing a cedar character on smoking, although isolation from tobacco (cigar leaf) was not reported until the early 1970's (33). It is now known that norambreinolide and related compounds, such as sclaral, are responsible for the characteristic cedar-amber notes of Havana leaf and Oriental tobacco. The important fragrance compound Ambrox® is found to a much lesser extent in Oriental tobacco, but this material is a key component in ambergris derived from whales; the exotic aroma and fixative value of ambergris is largely due to this material.

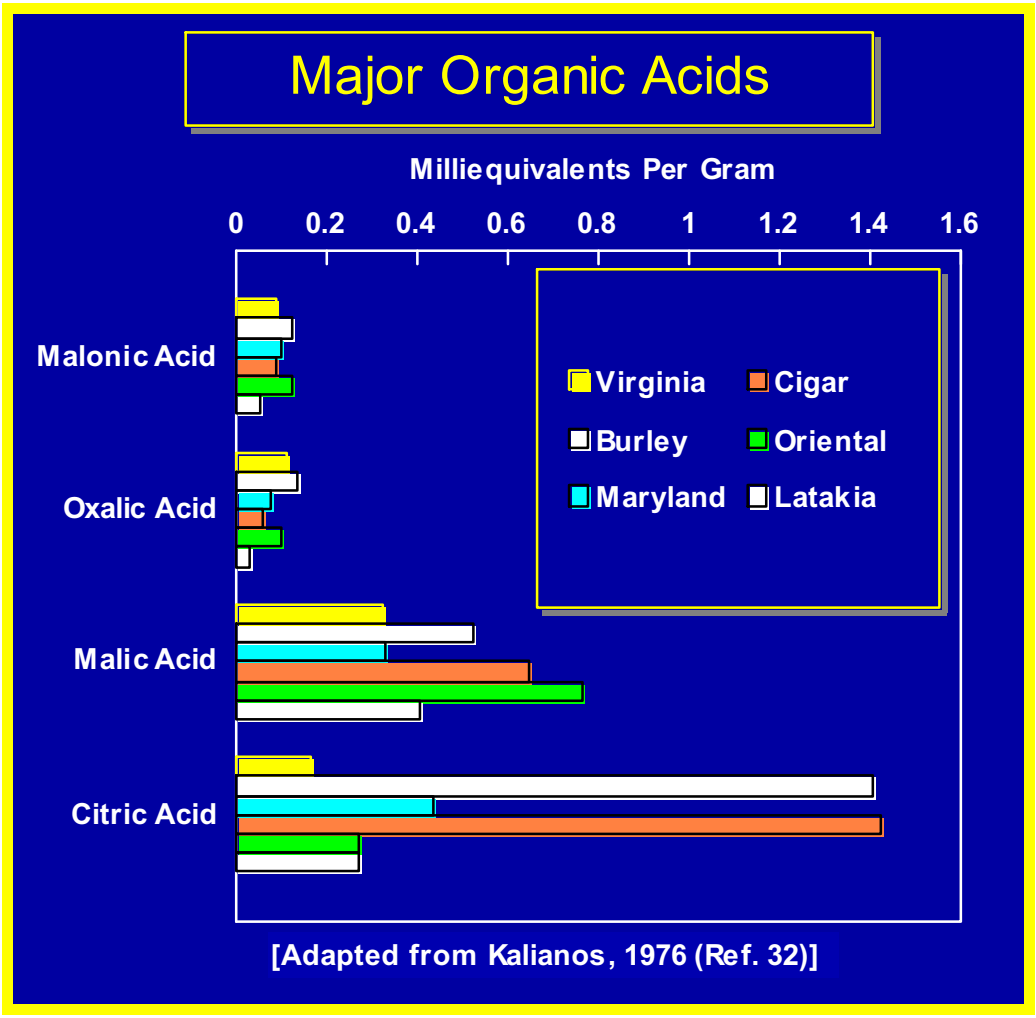
The combination of labdanoid derived flavorants and sucrose tetraesters present in Oriental comprise the major flavor forming components of this variety (35). Within the Oriental types, the Greek and Macedonian types tend to be higher in labdanoid derived compounds, while the Turkish Smyrna (Izmir) types are higher in sucrose tetraesters containing the 3-methylvaleric acid component.

Carboxylic Acids

The major carboxylic acids in tobacco are citric, malic, oxalic and malonic which in total can comprise 14-18% in burley and cigar tobacco, 7-10% in Maryland, 6-8% in Oriental and 5-10% in Virginia leaf, after curing. A substantial portion of such acids are complexed as salts with nicotine, ammonia and inorganic anions of calcium, potassium and sodium. The bar diagram (Figure 21) adapted from Kalianos (32) graphically shows the differing acidity profiles by tobacco type.

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FIGURE 21



Considerable effort has been expended trying to relate such acids to tobacco quality. Several studies indicate that there is an inverse relationship to the smoking quality of Virginia tobacco and the quantity of citric and oxalic acids (32), although this is probably just an indicator, and is not due to the absolute amounts of these acids present in leaf.

The volatile C_2-C_8 acids are known to be important aroma compounds in many fruits, foodstuffs and tobacco. Table 11 shows the relative amounts of the major volatile organic acids for various tobacco types (32).

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TABLE 11

Volatile Organic Acids in Tobacco

(micrograms per gram)

Acid	Virginia	Burley	Oriental	Threshold (in PPB)
Formic acid	597	288	587	45000
Acetic acid	877	372	688	22000
Propionic acid	13	12	24	20000
Isobutyric acid	32	29	72	8100
Butyric acid	2	0	0	240
2-Methylbutyric acid	247	26	313	1600
Isovaleric acid	116	20	202	120
2-Butenoic acid	12	1	3	NA
Valeric acid	1	0	4	3000
3-Methylvaleric acid	4	1	1372	300*
Hexanoic acid	5	3	5	3000
Heptanoic acid	5	5	6	3000
Octanoic acid	5	12	5	3000
Nonanoic acid	16	21	24	3000
2-Furoic acid	32	36	125	NA
Benzoic acid	22	14	25	85000
Phenylacetic acid	36	0	65	10000

*Authors estimate

It should be noted that the Oriental sample above is an Izmir type which should have a high 3-methylvaleric acid content and that the reported values must include the bound acids released by hydrolysis of sucrose tetraesters.

One of the ways to assess a compounds odor or flavor potency is to evaluate it's minimum detectable threshold level. In the table above, we have included the detection thresholds (36) for many of the tobacco acids. If one divides the relative concentration of a

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material by the threshold one can approximates the relative odor potency. For example, for acetic acid (688/2200 = 0.03) while for 3-methylvaleric acid (1372/300 = 4.57) indicating the aroma contribution of the latter would be about 152X that of the acetic acid.

Table 12 shows the concentrations of free acids in an Oriental tobacco blend and that of the corresponding cigarette smoke (27). This provides an indication of the increase in free acids due to thermolysis of the sucrose tetraesters (35).

TABLE 12
Increase in Aromatic Acids in Oriental Tobacco Smoke
Due to Thermolysis of Sucrose Tetraesters (27)

Acid	Tobacco(μg/g)	Smoke(μg/cig)	% Change
Propionic acid	17.0	66.8	+293
Butyric acid	2.5	9.7	+288
Valeric acid	4.3	3.6	- 16
2-Methylbutyric acid	2.1	12.4	+490
Isovaleric acid	5.1	19.7	+286
3-Methylvaleric acid	12.0	62.0	+417
Hexanoic acid	7.5	3.9	- 48
Octanoic acid	1.7	6.2	+265
Phenylacetic acid	48.9	19.9	- 59

(Heckman, et. al, 1981)

Without question, the volatile tobacco acids are some of the most important contributors to smoke quality and the powerful contribution from Oriental means that additions to cigarette blends at even low percentages can have a profound effect on product acceptability.

The higher fatty acids (Myristic, Palmitic, Stearic, Oleic, Linoleic and Linolenic) comprise about 0.75-1.1% in Virginia tobacco and about 0.5% in burley, with Palmitic being

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about 25% of these total acids. The following average amounts of fatty acids (Table 13) were found in a 2-year study on Virginia tobacco grown at Delphi, Ontario, Canada (11).

TABLE 13

% Fatty acids in Virginia Tobacco

Myristic acid	0.02%
Palmitic acid	0.28%
Stearic acid	0.05%
Oleic acid	0.11%
Linoleic acid	0.18%
Linolenic acid	0.35%

Tobacco Phenolics

Polyphenols:

The amount of total phenols varies considerably by tobacco type suggesting considerable genetic and cultural variability. In a two year study the following average polyphenol amounts were found (Table 14) (47).

TABLE 14

Percent Polyphenols by Tobacco Type (47)

	1980	1981
Virginia	3.13	3.75
Burley	1.78	2.05
Cigar Wrapper	2.13	2.65
Cigar Filler	2.03	3.40
Cigar Binder	2.27	3.54
Maryland	2.09	3.25
Dark Fire-Cured	2.78	3.64
Oriental	1.83	2.09

(Smeeton, 1987)

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Major polyphenolics found in tobacco include chlorogenic acid, rutin, scopoletin and scopolen along with materials such as quercetin and kaempferol. The biogenesis of plant phenolics with relationship to the pathways relevant to the major groups in tobacco for compounds formed via shikamic acid has been reviewed by Enzell (17). The amounts of polyphenols for Virginia NC 95 and Burley 21 have been reported (Table 15) (45).

TABLE 15

Typical Polyphenols in Tobacco (45)

(Milligrams per Gram)

	Virginia	Burley
Chlorogenic acids	34.71	12.83
Rutin	7.95	4.00
Scopoletin	0.13	0.06
Scopolin	0.94	0.35

(Sheen, et. al, 1979)

The chlorogenic acids are primarily 3-O-caffeoylquinic acid with lesser amounts of the 5- and 6- linked isomers. Scopolin and scopoletin are the major coumarin compounds in tobacco.

Lignan:

Lignan comprises as much as 4-5% of tobacco weight and is the most abundant natural organic aromatic polymer found in the vascular plant kingdom. With cellulose and hemicellulose , lignan is one of the major cell wall components in leaf. Lignan is composed of a chain of polymeric phenolic constituents, such as coniferyl alcohol, p-coumaryl- and synapyl alcohol. In many reviews on tobacco, lignan has been classified with “carbohydrates” due to its association with, and being bound to hemicellulose. In woody plants, lignan acts as a cementing agent to help bind the matrix of cellulose fibers (21).

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Lignan is known to produced high levels of phenolic constituents on thermolysis, such as 2-propylphenol, vanillin, eugenol, guaiacol, pyrogallol and cresols which are found in tobacco smoke.

Other phenolics:

Many more phenolics are found in tobacco smoke than in leaf, but the major isolated leaf phenols are shown in Table 16 (56), albeit they are present in low quantities in leaf.

TABLE 16

Major Phenolics Isolated from Steam Distillation of Tobacco Types (56)

(PPB in Leaf)

	Virginia	Burley	Oriental
Phenol	16	13	39
o-Cresol	9	4	11
m-Cresol	26	8	46
p-Cresol	31	4	18
Guaiacol	80	23	28
Dimethylphenols	9	3	15
4-Vinylphenol	54	2	161
2-Acetyl-3-methylphenol	--	2	2
Trimethylphenols	--	5	4
4-Ethylguaiacol	--	1	8
4-t-Butylphenol	1	1	8
Eugenol	0.5	--	--
4-Vinylguaiacol	14	3	8
4-Allyl-2,6-dimethylphenol	52	0.3	2

(Wilson, et. al., 1982)

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In addition, the vanilla-like phenolic, vanillin, has been isolated from the steam distillation of a tobacco blend (22). It is probable that *some* of the phenolics isolated from tobacco by steam distillation may arise from lignan hydrolysis, and, in fact, may be artifacts.

Phenolics are powerful aroma compounds and are considered to contribute significantly to smoke quality.

Tobacco Sterols

Sterols, steryl esters and esterified steryl glucosides comprise about 0.1-0.3% of tobacco weight. The distribution of sterols for both green and cured leaf has been studied and these can vary greatly within the tobacco plant. The major sterols are cholesterol, campesterol, stigmasterol and β-sistosterol. Table 17 provides the amounts of total individual sterols (after hydrolysis) reported in a sample of Virginia tobacco (15) and in two lines of burley tobacco (12).

TABLE 17

Major Sterols in Tobacco (12)

(Milligrams per Gram)

	Virginia	Burley Lines	
		L-1	L-7
Cholesterol	0.30	0.18	0.21
Campesterol	0.53	0.23	0.33
Stigmasterol	0.75	0.72	0.82
Sistosterol	0.88	0.38	0.94
Total Sterols	2.46	1.51	2.30

(Davis, 1976)

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Flue-cured, burley and Maryland tobaccos have been reported to have higher sterol contents than Oriental, Fire-Cured and cigar leaf tobaccos (31).

Sterols in tobacco are considered to be a potential health negative in that the parent polycyclic structure may form polynuclear hydrocarbons on pyrolysis (e.g., stigmasterol forms benzo[a]pyrene at 750° C) (31,48).

Inorganics

In Table 1, we have seen that a significant amount of calcium, potassium, magnesium, chlorine, phosphorus and sulfur are found in the ash of tobacco (as well as sodium). In addition, both free and complexed ammonia and nitrate are found in tobacco. The subject of ammonia in tobacco has previously been discussed above.

The tobacco content of these inorganics is in large part due to soil and fertilizer practices. Lesser amounts of many other inorganic anions (e.g., zinc, aluminum titanium, strontium, silicon, rubidium, nickel, manganese, lithium, lead, iron, copper, chromium, cesium, boron, barium and arsenic) have been found in tobacco (31,48). The presence of the major metallic anions certainly impact (for example, magnesium and potassium accelerate) the burning characteristics of tobacco leaf and the ash holding properties of cigarettes. Phosphorous and chloride tend to slow the burning process. In fact, high chloride levels provide both negative burning and taste characteristics.

Tobaccos with high nitrate content are assisted in the burning process as nitrate is itself a combustible material. In air-cured tobaccos, the nitrate tends to accumulate in the stem portion and is considered a major reason that the smoking properties of burley stems is inferior to Virginia stems. For many years, major cigarette manufacturers “washed” their burley stems to reduce the nitrate content. This served two purposes; (1) it improved the smoking properties and (2) removal of the nitrate oxidant lowers the level of nitrosamines in smoke.

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The trace elements (such as iron) in tobacco can have a profound effect on tobacco quality. For example, iron has been implicated in the speckling effects that develops in the so-called "Grey Tobacco" deficiency of Virginia tobacco (50).

Summary

Tobacco is a complex plant material from a chemical composition standpoint. No other plant material has been studied more extensively in the history of man. Yet even today, the quality of tobacco is judged largely by empirical experience and subjective sensorial evaluation. We are still unable to replicate the smoking properties of the tobacco leaf synthetically. Yet, through chemical knowledge and genetic advances, we now have a much better understanding of tobacco growth and important constituents that allows us to make improvements in quality using the best laboratory in the world - through optimization of the biology of tobacco itself in the tobacco growing field.

Finally, although tobacco consumption is controversial and considered to be a major health concern, it has been a fruitful field for natural product chemists because of the insight it has given into the formation of flavor ingredients found in many common foodstuffs and the fragrance materials found in flowers and other botanicals.

This publication is an update to an article published by the author in 1999 (60).

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Toxicological analysis of low-nicotine and nicotine-free cigarettes

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ABSTRACT

Low-nicotine and nicotine-free cigarettes are commercially available under the brand-name Quest®. Some consumers may believe that these are safer cigarettes, and they may smoke more cigarettes or inhale more smoke to compensate for low nicotine yields. Thus, we have studied the toxicological effects of these two cigarettes and compared them with the Kentucky reference cigarette 2R4F. Also, the availability of nicotine-free cigarettes allows for the assessing the role of nicotine in cigarette smoke. In addition to nicotine, some tobacco-specific nitrosamines, aldehydes, and volatile organic compounds were also reduced in the Quest® cigarettes compared to the 2R4F. However, aromatic amines were higher in the nicotine-free compared with low nicotine cigarettes. The Ames test revealed that cigarette smoke condensates from the nicotine-free (CSC-F), low nicotine (CSC-L) and 2R4F (CSC-R) cigarettes had a similar mutagenic potency. Exposure to any CSC caused a similar dose-dependent LDH leakage from normal human bronchial epithelial cells. However, CSC-F had more inhibitory effects on the cell growth than CSC-L and CSC-R. Adding nicotine to the CSC-F attenuated this inhibition. Both Quest® CSCs decreased gap junction intercellular communication and caused cell cycle arrest. CSC exposure increased cytoplasmic nucleosomes, sub-G1/G0 population and apoptotic comet tails. Proapoptotic protein Bax increased independent of p53 induction after exposure to CSC-F. In conclusion, these studies are not consistent with a perception that low-nicotine or nicotine-free cigarettes may have less toxicity in human cells. Nicotine, as it exists in CSC, attenuates cytotoxicity possibly in part through inhibition of apoptotic pathways.

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1. Introduction

Cigarette smoking causes approximately 80–90% of lung cancers in the US (Kabir et al., 2007). Currently, tobacco companies are developing and marketing cigarettes with the publicly stated intent to reduce health effects compared with conventional cigarette products. While there have been attempts in the past to manufacture safer cigarettes, the experience with "light" cigarettes, the best example of a failed attempt, has illustrated the dangers associated with such endeavors (Harris et al., 2004; Stratton et al., 2001). Newer technologies and promotion by the tobacco industry, therefore, require the development of a scientific basis to inform the current debates about whether the new products present hope or hazards. An example of novel tobacco products currently on the market are the cigarettes produced by Vector Tobacco under the trade names of Quest®-1, -2 and -3 which are low nicotine, extra-low nicotine and nicotine-free cigarettes yielding 0.6, 0.3 and <0.05 mg of nicotine, respectively. These products have been sold since 2003 and advertising leads the consumer to believe that they are less harmful, less carcinogenic and less addictive (O'Connor et al., 2005; Shadel et al., 2006). It is known that lower nicotine cigarettes can lead to compensatory smoking such that exposure to cigarette smoke may be increased (Strasser et al., 2007). Thus, a toxicological evaluation is needed to inform consumers and policy makers about actual similarities and differences of these products from conventional cigarettes.

Cigarette smoke (CS) is a complex chemical mixture containing more than 4000 different compounds, of which about 69 are known or probable human carcinogens (Fowles and Dybing, 2003; Hoffmann et al., 2001). While it is possible to study individual chemical constituent of tobacco smoke, this information does not provide a complete picture of the effects that these chemicals may have in the context of complex exposures. In laboratory animal

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studies, the shape of the dose-response curve (Smith and Hansch, 2000) and time- and tissue-gene expression response (Gebel et al., 2004) differ with different tobacco smoke constituents, and so the potency of a tobacco smoke constituent complex mixture cannot be predicted. Mainstream smoke consists of particulate and vapor phases of which the former can be collected with the use of smoking machines passing the smoke through a Cambridge filter pad. The material collected is known as cigarette smoke condensate (CSC) and contains most of the chemicals, and particularly the carcinogens in the smoke. Furthermore, the preparation of CSC can be standardized using a smoking machines, and so CSC rather than whole smoke or mixtures of the key constituent chemicals is what is most often used in smoke research (Foy et al., 2004; Hellermann et al., 2002). CSC is composed of tar, nicotine and water, and though the tar has often been the focus of toxicological studies, nicotine is one of the most physiologically active components of cigarette smoke. In addition to its addictive properties, some evidences support the hypothesis that nicotine may contribute directly to carcinogenesis through stimulation of nicotinic acetylcholine receptors (nAchR) in non-neuronal cells (Carlisle et al., 2004; Minna, 2003), thereby affecting cell proliferation and apoptosis (Chu et al., 2005; Mai et al., 2003), and promoting angiogenesis (Heeschen et al., 2001). Nicotine is, however, not itself tumorigenic in experimental animal studies (Martin et al., 1979), but there have been limited studies of the role of nicotine in CSC (Gullihorn et al., 2005). The availability of the nicotine-free Quest® cigarette offers a novel opportunity for toxicological evaluation of the role of nicotine in cigarette smoke and allows the direct assessment of the tar fraction of cigarette smoke independent of nicotine effects.

Most cigarette carcinogens are also strong mutagens which can form DNA adducts, and thereby activate proto-oncogenes, inactivate tumor suppressor genes (DeMarini, 2004), or disrupt critical regulatory pathways predisposing cells to carcinogenesis. To evaluate the mutagenic potential of CSC from the various products we made use of the *Salmonella*/microsome plate incorporation Assay, also known as the Ames test, which is the most widely used method to evaluate the mutagenicity of cigarette smoke and other mutagenic agents (Mortelmans and Zeiger, 2000). In addition to genetic insults, smoke constituents also activate biochemical pathways that are associated with apoptosis, cell cycle progression and cell growth (Tsurutani et al., 2005; Yang and Liu, 2004). The cytotoxicity of the CSC samples and their ability to alter proliferation, cell cycle progression and apoptotic index have been evaluated using primary cultures of human bronchial epithelial cells and standard methodologies. Inhibition of gap junction intercellular communication (GJIC) has been found to be associated with tumor promotion in most if not all tumor cells (Mesnil et al., 2005). Cultured human bronchial epithelial cells and endothelial cells derived from human coronary arteries were used to evaluate the inhibition of GJIC by CSC. The inhibition of GJIC by CSC fractions was considerably lower than that by un-fractionated CSC (Vang et al., 1995), indicating that compounds in the CSC act strongly synergistically. Change of cigarette components may also change its inhibitory effect on GJIC.

In order to more fully characterize the toxicological effects of Quest® cigarettes and to evaluate the impact that nicotine had on the end-points under study, we conducted smoke chemistry analysis and a comparative evaluation of the genotoxicity and cytotoxicity of the mainstream CSC from Quest® low-nicotine and nicotine-free cigarettes and 2R4F Kentucky Reference research cigarettes. Our studies did not show that low-nicotine or nicotine-free cigarettes would have less toxicity in human primary bronchial epithelial cells. Nicotine, as it exists in CSC, maybe afforded some protection from cytotoxicity.

2. Materials and methods

2.1. Materials

Quest® nicotine-free (<0.05 mg nicotine) and low-nicotine yield (0.6 mg nicotine) cigarettes, manufactured by Vector Tobacco Inc. (Timberlake, NC), were purchased from local commercial sources. The 2R4F Kentucky Reference Research Cigarette (0.8 mg nicotine yield) was obtained from the Smoking and Health Institute of the University of Kentucky (Lexington, KY). Nicotine hydrogen tartrate salt was purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade or better and bought from standard suppliers unless otherwise stated.

2.2. Smoke chemistry analysis

Smoke emission testing was conducted by Arista Laboratories (Richmond, VA). Five cigarettes were randomly selected from each of 50 packages for analysis. Smoking machines (Tri-City, Cerulean, Borgwaldt) were used for sample collection to a glass fiber pad (total particulate matter, TPM), a solvent based impinger (volatile constituents), or a combination of the two (semi-volatile constituents). Cigarette samples were conditioned prior to smoking by exposure to a controlled environment (60% relative humidity, 23.5 °C) for 48 h. Using smoking conditions specified by the Federal Trade Commission (FTC; 35 ml puffs with 2 second duration every 60 s, smoked down to a specified length), we analyzed 60 constituents in the mainstream smoke of two Quest® cigarettes and 2R4F cigarettes. The analytes selected were those whose abundance was sufficient to allow quantification of the analytes in a composite sample of 5–40 cigarettes, and which are of general interest as either direct toxicants or indicators of a class of compounds that may be toxicants (Roemer et al., 2004; Smith and Hansch, 2000).

2.3. Cigarette smoke condensate (CSC)

CSC was prepared using a custom 30-port rotary smoking machine (Baumgarten; Belmont, Switzerland) smoking the cigarettes using the standard FTC puffing regimen, as above. The total particulate matter (TPM), herein also referred to as CSC was collected onto Cambridge filter pads (Whatman, Maidstone, UK), and extracted (40 min with constant shaking) with dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO). The condensates were coded, aliquot and stored at −80 °C until assayed within 3 months after preparation. The condensates from 2R4F cigarettes, Quest® low-nicotines cigarette or nicotine-free cigarettes were designated as CSC-R, CSC-L and CSC-F, respectively.

2.4. Salmonella/microsome plate incorporation assay (Ames test)

This assay was conducted as previously described (Mortelmans and Zeiger, 2000). Briefly, the *Salmonella* TA98 or TA100 strain was inoculated into DIFCO nutrient broth (Becton Inc., France) and cultured at 37 °C overnight to a density of about 10^6 cells/ml. Top agar supplemented with biotin-histidine was melted and maintained at 45 °C. Test samples along with positive (2-anthramine, 1.0 μg/plate) and vehicle controls were mixed sequentially with Arochlor-induced Sprague–Dawley rat S9 mix (Moltox Inc., Boone, NC), then top agar and bacteria, and poured directly onto pre-prepared VBMG agar plates (Presque Isle Cultures, Erie, PA). After the top agar set, the plates were incubated at 37 °C for 72 h in the dark. Bacterial colonies were counted and analyzed using a AccuCount™ 1000 automatic counter (Biologics, Inc.). Five or six dose levels (0–0.25 mg/plate) were assayed in triplicate along with negative controls and used to identify the linear portion of the dose-response curve. The primary outcome is the number of revertant colonies per plate, corrected for the number of colonies in control plates. A plot of average revertant colonies against dosage was carried out to determine the dose-response curve slope of each testing product by linear regression method. A result is considered to be positive when there is a significant dose-related increase, where the number of revertant colonies is twice the spontaneous background.

2.5. Isolation and culture of normal human bronchial epithelial (NHBE) cells

Fresh human bronchi were immersed in cold DMEM/Ham's F12 medium immediately after resection. The use of these tissues was approved by the Georgetown University Institutional Review Board and with the informed consent of each patient. Histologically normal bronchial tissues were trimmed, cut, and incubated at 4 °C overnight in 0.1% protease media (Sigma) as described (Blouquit et al., 2002). After adding 10% fetal bovine serum, the cells were detached by gentle scraping, harvested by centrifugation, and resuspended in Clonetics® BEGM medium (Cambrex, Walkersville, MD). The cells were then plated on a type I collagen-coated flask and incubated at 37 °C in 5% CO_2/95% air. The media was changed after 24 h and then every other day thereafter. The cells were passaged when they reached 80% confluence. Immunocytochemical staining showed that more than 95% of cells prepared by this method were cytokeratin positive (clone AE-1/AE-3 pan, Signet, Dedham, MA). The cells were used between passages 2 and 6.

2.6. LDH release assay, WST-8 conversion assay and cell growth assay

The acute cytotoxicity of CSC or nicotine was examined using lactate dehydrogenase (LDH) release method. NHBE cells were placed into a 96-well plate at a density of 5000 cells/well overnight, and then treated with CSC and/or nicotine at 37 °C for 3 h. The plates were centrifuged and the supernatants transferred to a fresh 96-well plate. The LDH activity in the supernatant was determined as per the manufacturer's instruction (TOX-7; Sigma) (Putnam et al., 2002). All tests were performed in triplicate and the experiment was performed at least three times. Cell viability of treated NHBE cells in the plates was confirmed using the trypan blue dye exclusion method (Deng et al., 2000).

WST-8 was also used to determine cytotoxic activity of CSC or nicotine on NHBE cells. The detection sensitivity of CCK-8 is higher than other tetrazolium salts such as MTT, XTT, MTS or WST-1. NHBE cells were seeded into 96-well plate at a density of 1×10^4 cells/well and treated with CSC with or without nicotine overnight. WST-8 conversion was then assessed using a one-step Cell Counting Kit-8 (CCK-8), according to the manufacture's instructions (Dojindo Molecular Technologies Inc. Gaithersburg, MD). The WST-8 method only generates a signal from the viable cells. All tests were carried out in triplicate.

To determine the inhibitory effects of CSC and nicotine on cell growth, NHBE cells were seeded into 24-well plate at a density of 5×10^4 cells/well and treated with sub-lethal dosage of CSC (as determined by LDH release assay) in the absence or presence of nicotine for 3 days. The floating and adherent cells were then harvested with 10 mM EDTA and counted by a Coulter Counter. The cell growth inhibition was expressed as percentage of untreated control wells. All treatments are performed in triplicate. The experiment was performed at least three times.

2.7. Gap junction intercellular communication (GJIC)

GJIC was determined by a 'flow cytometry' technique adapted from the methods of Czyz et al. (2000) and Rudkin et al. (2002). Normal bronchial epithelial cells were grown to 70–80% confluence in T-25 flasks. Cells, designated as "donor cells" were exposed to 40 μg/ml of CSC or nicotine overnight and then sequentially labeled with the gap junction–permeable dye calcein AM (0.05 μM, Molecular Probes) for 30 min and PKH26 (2 μM, Sigma), a nontransferable membrane dye for 7 min. The double-stained donor cells were then plated onto monolayers of unstained "recipient" cells at ratio of 1:10 (donor:acceptor) and incubated for 2 h at 37 °C. The co-cultures were then harvested, washed, resuspended and subjected to FACS analysis (Becton Dickinson FACStar Plus dual laser system, Heidelberg, Germany). GJIC results in the transfer of calcein from donor cells to recipient cells. The results are presented as a coupling ratio C_r, which is given by the number of calcein-transferred recipient cells which are labeled green, *per* donor cell (labeled with red and green). Heptanol (Sigma), a rapid inhibitor of GJIC, was used as a positive control (3 mM) for the inhibition of GJIC.

2.8. Cytoplasmic nucleosome detection

NHBE cells were plated in 6-well plate at 50% confluence and treated with CSC and/or nicotine at 37 °C overnight. The cells were then harvested by trypsinization, washed, resuspended and assayed for apoptosis as indicated by the amount of cytoplasmic nucleosome which was determined with Cell Death Detection ELISA kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. As a positive control for the induction of apoptosis, etoposide (VP-16, Calbiochem, La Jolla, CA) was used.

2.9. Cell cycle analysis

NHBE cells were placed in T-25 flasks at 50% confluence and incubated with CSC and/or nicotine at 37 °C for different times. After treatment, $1–2 \times 10^6$ of floating and adherent cells were harvested by trypsinization, fixed in ice-cold 75% ethanol at −20 °C overnight. The cells were then treated with DNase-free RNase A (0.15 mg/ml, Sigma) at 37 °C for 30 min followed by incubation with propidium iodide (10 μg/ml, Sigma) at 37 °C for 10 min. The DNA content of individual cells was analyzed with a fluorescence-activated cell sorter. Cells with less DNA than that of G1/G0 cells (sub-diploid DNA peak) were considered to be apoptotic (Liu et al., 2005).

2.10. Neutral Comet assay

The Comet assay was conducted using a CometAssay kit (R&D Systems Inc., Minneapolis, MN) under neutral conditions, which detects mainly double strand DNA breaks and can be useful for assessing the DNA fragmentation associated with apoptosis. Briefly, NHBE cells were cultured in 6-well plates and treated with 50 μg/ml CSC or VP-16 (1 mM for 18 h). Attached and floating cells were then harvested and mixed with molten LMAgarose. The agarose/cells were pipetted over the sample area of CometSlides and after hardening the slides were immersed in Lysis solution for 30 min followed by horizontal electrophoresis in TBE buffer at 1 V/cm for 15 min. After fixing with 70% ethanol for 5 min, the slides were air-dried and stained with SYBR Golden. The cells were viewed with an epifluorescence microscope and photographed. For scoring the comet pattern, 100 nuclei from each slide were counted. The comet was scored from 0 to 4 according to Collins criteria by measuring tail length, head size, tail intensity and head intensity (Collins, 2002; Yasuhara et al., 2003). Typical apoptotic cells are defined as those with diffuse fan-like tails and very small heads (Liu et al., 2005; Yasuhara et al., 2003). Scores more than 2 (i.e. relative tail intensity >50%) are attributed to apoptosis (Collins, 2002; Yasuhara et al., 2003).

2.11. Western blotting

Protein levels were assayed by Western blot analysis, as previously reported (Chen et al., 2005). NHBE cells were plated in 6-well plates at 80% confluence and treated with CSC and/or nicotine at 37 °C overnight. After treatment, the cells were harvested with lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.5 mg/ml leupeptin, 1 mM EDTA, 1 mg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride) and the protein concentration determined by BCA assay (Pierce, Rockford, IL). Samples (30 μg) of protein were loaded onto a Novex® 10% tri-glycine gels (Invitrogen, Carlsbad, CA), separated by PAGE, and transferred to a sheets of hydrophilic polyvinylidene fluoride (PVDF). The membranes were blocked with 5% nonfat milk for 30 min and then immuno-stained with primary antibodies against either p53, PARP, Bcl2, Bax, or Caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight, followed by a peroxidase-conjugated secondary antibody. The immunopositive bands were visualized with Super Signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) system.

2.12. Statistical analysis

Evaluation was carried out using SAS software. Data were expressed as mean ± S.D. Each experiment was repeated at least two times. Concentration-dependent differences of testing products in Ames test and cell cycle with flow cytomery were analyzed by analysis of covariance (ANCOVA). The unpaired Student's *t*-test was used to evaluate the significance of difference in chemistry analysis, cell growth and proliferation assays and cytotoxic experiment at equal concentrations of CSC from different cigarettes. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Smoke chemistry analysis of Quest® and reference cigarettes

We compared the cigarette smoke from the nicotine-free (we confirmed as 0.032 ± 0.001 mg/cig), 0.6 mg for the low-nicotine cigarette (we confirmed as 0.53 ± 0.01 mg/cig), and the 2R4F cigarette (we confirmed as 0.80 mg/cig) generated using the FTC smoking protocol. Table 1 showed statistically significant differences and similarities for only 20 smoke constituents (because of space limit) which are well investigated. The tar yields were 7.87 ± 0.16, 8.99 ± 0.26 and 9.62 ± 0.38 mg/cig, respectively, although the puff counts were 30% lower for the Quest® cigarettes. Compared with reference 2R4F cigarette, the levels of tobacco-specific nitrosamines (TSNAs) were substantially lower for the two Quest® cigarettes. NNK [4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone] and NNN (*N*-nitrosonornicotine) are the two carcinogenic TSNAs primarily formed during the curing process from nicotine and nornicotine (Hayes et al., 2007). The levels of NNK were 10.3 ± 1.9, 24.8 ± 4.1 and 132.0 ± 4.0 ng/cig for the nicotine-free, low-nicotine and reference cigarettes, respectively ($p < 0.001$). Other TSNAs (NNN, NAT, NAB) were also lower, but not to the same degree, and the pattern was different, with the yield of NNN being higher in nicotine-free cigarettes (67.9 ng/cig) than in low-nicotine cigarettes (49.2 ng/cig) ($p < 0.01$). The yield of other carcinogens, such as benzo(*a*)pyrene were a little bit but significantly lower in the low-nicotine or nicotine-free cigarettes compared to 2R4F cigarettes. A similar reduction in volatile organics was seen compared to the reference cigarettes, in general about 50–60% for 1,3-butadiene and benzene ($p < 0.01$), although the semi-volatile constituents were not different. Formaldehyde levels were similar for the 2R4F and the low-nicotine Quest®, but reduced by almost half for the nicotine-free Quest®. Interestingly, though the levels for aromatic amines were similar for the low-nicotine Quest® and 2R4F,

Table 1
Yields of smoke constituents under FTC condition (mean ± S.D.) of reference cigarette, Quest® low-nicotine and nicotine-free cigarettes

Constituents	Unit per cig	Reference 2R4F	Quest® Low-nicotine	Quest® Nicotine-free
FTC parameters				
TPM	mg	11.13 ± 0.41	10.32 ± 0.34	8.50 ± 0.22*
Tar	mg	9.62 ± 0.38	8.99 ± 0.26	7.87 ± 0.16*
Nicotine	mg	0.80 ± 0.03	0.53 ± 0.01*	0.032 ± 0.001*
Water	mg	0.71 ± 0.00	0.80 ± 0.13*	0.60 ± 0.06*
Carbon-monoxide	mg	12.05 ± 0.17	10.69 ± 0.21*	9.97 ± 0.57*
Puff count	puff	9.2 ± 0.1	6.6 ± 0.1*	6.0 ± 0.0*
Aldehydes(carbonyls)				
Acrolein	μg	57.2 ± 6.6	22.0 ± 3.5*	19.8 ± 3.0*
Acetaldehyde	μg	617 ± 75	337 ± 45*	363 ± 53*
Formaldehyde	μg	20.3 ± 0.6	21.0 ± 1.7	9.5 ± 2.1*
Aromatic amines				
2-Naphthylamine	ng	6.30 ± 0.07	5.51 ± 0.71	8.32 ± 0.86*
4-Aminobiphenyl	ng	1.25 ± 0.01	1.11 ± 0.08	1.93 ± 0.14*
N-Nitrosamines				
NNK	ng	132.0 ± 4.0	24.8 ± 4.1*	10.3 ± 1.9*
NNN	ng	140.0 ± 12	49.2 ± 2.4*	67.9 ± 3.3*
Phenols				
Catechol	μg	43.7 ± 2.7	35.3 ± 1.9*	20.0 ± 0.6*
Phenol	μg	8.33 ± 0.62	11.6 ± 0.7*	9.47 ± 0.43
Resorcinol	μg	0.67 ± 0.01	0.74 ± 0.05	0.37 ± 0.02*
PAH				
Benzo(*a*)anthracene	ng	13.9 ± 0.8	10.5 ± 0.4*	8.66 ± 0.29*
Benzo(*a*)pyrene	ng	5.82 ± 0.25	4.51 ± 0.27*	3.75 ± 0.21*
Volatiles				
Benzene	μg	49.4 ± 1.6	15.0 ± 1.0*	14.8 ± 1.5*
Isoprene	μg	371 ± 3	149 ± 8*	130 ± 7*
1,3-Butadiene	μg	32.7 ± 1.1	18.1 ± 1.5*	15.7 ± 0.9*

* $p < 0.05$ compared with Reference 2R4F.

it was higher for the nicotine-free Quest®. For example, the levels of 4-aminobiphenyl, were increased 42% over the low-nicotine Quest® ($p < 0.05$) and 35% over the 2R4F ($p < 0.05$).

3.2. In vitro mutagenicity of CSC and nicotine

The mutagenic activity of CSC and nicotine was measured using the Ames test. CSC from 2R4F reference (CSC-R), Quest® low nicotine (CSC-L) and nicotine-free (CSC-F) cigarettes tested against *Salmonella* TA98 with activation by S9 all exhibited a dose-dependent revertant response ($p < 0.01$) with slopes of 0.388, 0.356 and 0.364, respectively. The revertant colonies at a dose of 0.15 mg CSC/plate were more than 10 times the spontaneous background for the three CSC ($p < 0.001$). When compared on a revertants per mg TPM basis, there was no statistical difference ($p > 0.05$) in the dose response slopes of the three CSCs (Fig. 1) by ANCOVA test. The Ames test with TA100 bacteria produced similar data (not shown). Nicotine alone had no mutagenic activity and when nicotine was added back to Quest® CSC-F at levels similar to conventional "low tar" yield cigarettes, the slope of the dose response curve was not altered.

3.3. In vitro cytotoxic and anti-proliferative effects of CSC and nicotine in NHBE cells

To determine the cytotoxic and antiproliferative effects of the CSCs, four *in vitro* assays with different endpoints were selected and performed using NHBE cells according to methods previously published (Matsuoka et al., 2000; Putnam et al., 2002). Acute toxicity of the CSCs was assessed using an LDH release assay (Fig. 2A) and trypan blue exclusion test, the toxic effects of an overnight exposure to the condensates was determined by evaluating total cell viability with a CCK-8 assay (Fig. 2B), and effects on cell proliferation and growth were measured by determining the change in cell number after three days of exposure (Fig. 2C).

LDL release reflects direct damage to the cell plasma membrane and is the most sensitive cytotoxic method for short-time exposures (Putnam et al., 2002). Quest® and 2R4F CSCs all caused a dose-dependent elevation in LDH release at doses > 60–80 μg/ml, but there was no significant difference among them at any dose level ($p > 0.05$) (Fig. 2A), indicating they produce similar acute toxicity. The studies assessing cell viability by trypan blue dye exclusion produced similar data again showing dose-dependent toxicity, but no difference among the three CSCs (data not sown). A viability > 90%

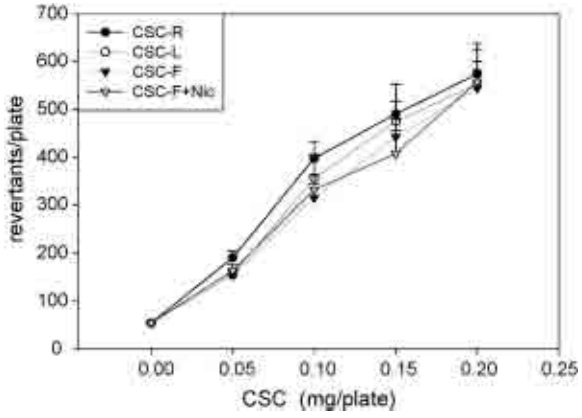


Fig. 1. Mutagenic activity of different CSCs by Ames test. Evaluation was performed in bacterial strain TA98 in the presence of S9 solution. Mutagenicity was expressed as revertants/plate/mg CSC. All testing was conducted with triplicate plates at each concentration. Ames test in TA100 (data not shown here) revealed a similar pattern. Shown is one example of three or more independent experiments, all with similar results.

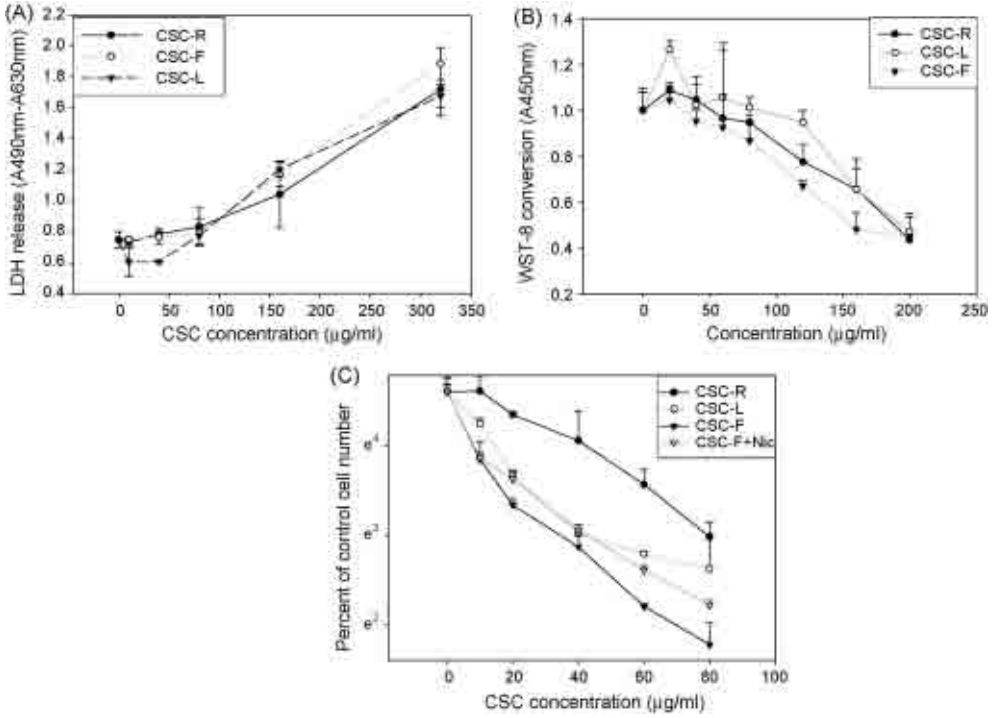


Fig. 2. Cytotoxic and anti-proliferative effects of CSC and nicotine in normal human bronchial epithelial (NHBE) cells. NHBE cells were exposed to the indicated concentration of CSC from Kentucky 2R4F reference cigarettes (CSC-R), or Quest® low-nicotine cigarette (CSC-L) or nicotine-free cigarette (CSC-F) in the absence or presence of nicotine (10 μM): (A) the level of LDH released from cytoplasm into the culture media after exposure for 3 h was determined with Sigma TOX-7 kit; (B) after overnight exposure, cell viability was assayed with a CCK-8 kit, which measures mitochondrial dehydrogenase activities of viable cells by reducing WST-8 to produce a yellow color formazan dye; (C) cell number after exposure for 3 days. The inhibition of cell growth was expressed as percentage of untreated control wells. All tests were performed in triplicate. Shown is one example of three or more independent experiments, all with similar results.

of NHBC was observed when exposure to CSC at dose < 60 μg/ml which was used as a sublethal dose threshold for other assays.

The CCK-8 assay (Fig. 2B), quantifies total cell number and viability by assaying mitochondrial dehydrogenase activity and revealed that although all three CSC treatments suppressed WST-8 conversion, CSC-F at doses of 80, 120 and 160 μg/ml was apparently more toxic than CSC-L or CSC-R ($p < 0.05$). That meant that besides the similar acute cell killing mechanism evidenced by LDH release assay, other toxic mechanisms existed and they were different for Quest® and reference cigarette smokes.

In addition to their cytotoxicity, all three CSCs exhibited a dose-dependent suppression of NHBE cell proliferation and growth (Fig. 2C) even the concentration was as low as 10 μg/ml which did not kill the cells (Fig. 2A and B). However, the reference cigarette CSC produced less inhibition than the two Quest® cigarette CSCs, in spite of possessing higher levels of some of the carcinogens (Table 1). Furthermore, the CSC-F inhibited the cell growth to a greater extent than CSC-L ($p < 0.05$). When 10 μM of nicotine (equivalent to the nicotine content in 80 μg/ml of CSC-L) was added to the CSC-F, growth suppression was significantly reduced ($p < 0.05$) yielding cell numbers close to those with CSC-L treatment, supporting a possible role for nicotine in cell survival.

3.4. Effects of CSC on gap junction intercellular communication (GJIC)

To evaluate the effects on gap junction formation, a GJIC was conducted at noncytoxic concentrations of CSC, as determined by the LDH release assay. Calcein, which can travel from cell to cell through gap junctions, was transferred from donor bronchial epithelial cells to unstained recipient cells after co-culture for 2 h with a coupling ratio (C_r) of 2.05 (Fig. 3). Treatment of donor cells with 40 μg/ml of CSC-R, or Quest® CSC-L or CSC-F overnight did not affect the staining of the donor cells with PKH26 or calcein the retention of which is dependent on cell viability (data not shown), but remarkably impaired the ability of the cells to transfer the calcein to the recipient cells (Fig. 3). The C_r decreased to 1.21, 0.85 and 0.62 for CSC-R, or Quest® CSC-L or CSC-F, respectively. This indicates that the Quest® CSCs inhibited GJIC significantly more than CSC from the reference cigarettes. There was, however, no statistical difference between the two Quest® CSCs according to three independent experiments ($p > 0.05$). The addition of nicotine at 10–50 μM did not significantly affect the GJIC (data not shown).

3.5. Effects of CSC and nicotine on apoptosis

To completely elucidate the mechanisms responsible for the NHBE cell loss by exposure to CSCs, we investigated their effects on apoptosis in addition to acute necrotic effect and antiproliferative effect. NHBE cells were exposed overnight to the apoptosis-inducer VP-16 or CSCs at either low dose (50 μg/ml) or high dose (100 μg/ml). Cytoplasmic nucleosome levels were then determined using the sensitive ELISA method. CSC-R and both Quest® CSCs exposure at sub-lethal doses of 50 μg/ml did not trigger a significant apoptotic response (Fig. 4A). In contrast, at higher doses, the cytoplasmic nucleosome level was significantly increased ($p < 0.05$) after exposure to CSC-F (100 μg/ml), but not to CSC-L or CSC-R, indicating that the nicotine-free CSC could induce an apoptotic effect. When the equivalent amount of nicotine (10 μM) was added to the CSC-F, this increasing effect was abolished, indicating that nicotine in the CSC suppressed the CSC-induced apoptosis.

To further explore if CSC induces apoptosis, the DNA content of populated cells was analyzed by flow cytometry, and DNA fragmentation and chromosomal condensation in individual cell was

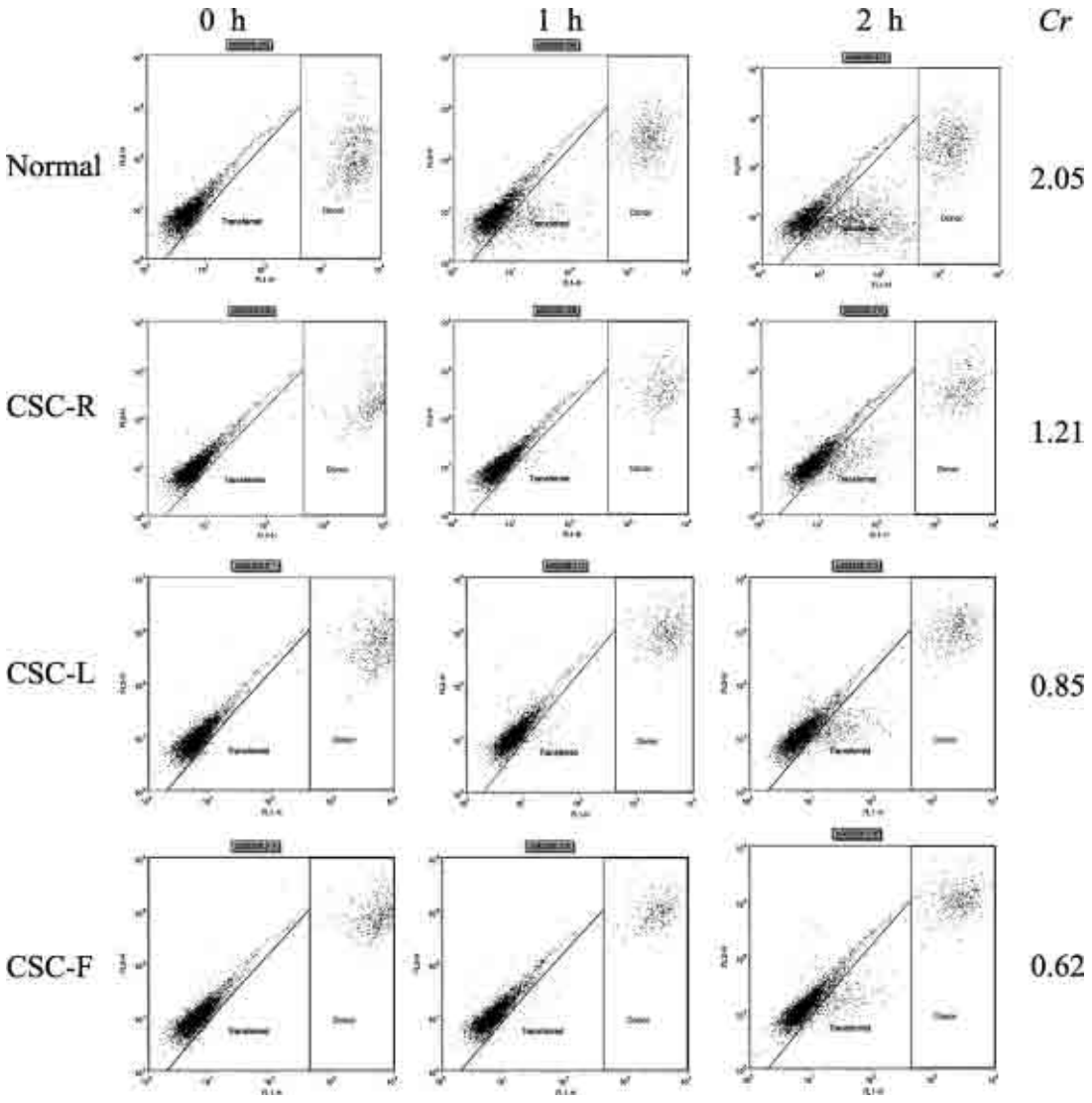


Fig. 3. Effects of CSC on gap junction intercellular communication (GJIC) analyzed by fluorescent dye transfer between NHBE cells. Double-stained (Calcein AM and PKH26) normal or CSC treated donor cells were mixed with non-dyed recipient cells at a ratio of 1:10. Calcein transfer occurred after contact between donor and recipient cells. The number of coupled recipient (calcein-dyed) cells per donor cell (coupling ratio C_r) was determined by flow cytometry after co-culture for 0, 1 and 2 h. Shown is one example of two or more independent experiments, all with similar results.

evaluated by the single-cell electrophoresis assay (neutral Comet assay). After exposure of NHBE cells to CSC-L or CSC-F, there was a gradual increase of the number of cells with hypodiploid DNA content with concentrations of up to 60 μg/ml (Fig. 4B and Table 2). The cell numbers in sub-G1/G0 peaks were significantly increased (>5%) after exposure to 40–80 μg/ml of CSC-F or CSC-L for 48 h (Table 2) compared with background numbers (spontaneous apoptosis-related), it was even higher for CSC-F compared to CSC-L ($p < 0.05$). When NHBE cells exposed to 50 μg/ml of CSCs for 18 h were electrophoresed under neutral conditions, there were more frequent and longer comet tails (oligonucleosomal DNA fragmentation) with small comet heads (chromosomal condensation) compared with control cells (Fig. 4C). The percentage of nuclei that yielded comet scores >2 were 1%, 43%, 15%, 12%, and 17% for medium control, VP-16, CSC-R, CSC-L, and CSC-F, respectively.

To investigate what molecules were involved in the CSC-induced apoptosis, we first tested if p53 can be induced in NHBE cells. As showed in Fig. 4D, VP-16 induces p53 expression. This was confirmed in four NHBE cell strains from four subjects. But none of three CSC treatments upregulated the expression of p53 protein or its phosphorylation at Ser 15 residue in NHBE cells. The PARP protein was not detected in three NHBE cell strains before or after exposure to CSC-F (data not shown). Apoptosis can be divided into three interdependent phases: induction, decision and execution (Xin and Deng, 2005). The decision phases is largely regulated by the Bcl-2 family of apoptotic regulators, which includes Bcl-2 and Bax (Oltvai et al., 1993). CSC-F increased the Bax expression even at the low dosage (the band intensity at 40 μg/ml increased 3.26-fold compared to control), but it did not change the Bcl-2 expression (Fig 4D). Therefore the ratio of Bax/Bcl-2 increased after exposure to CSC-F. We did not find the expression of caspase-3 in NHBE cells before and after CSC treatment (data not shown).

3.6. Effects of CSC and nicotine on cell cycle

To further explore how CSC inhibited the cell proliferation and growth (Fig. 2C), CSC effect on the cell cycle was tested using FACS analysis of the NHBE cells. As showed in Table 2, both Quest® CSCs caused a dose-dependent cell cycle arrest early (16 h) at G1 checkpoint and later (48 h) at the G2/M checkpoint when CSC

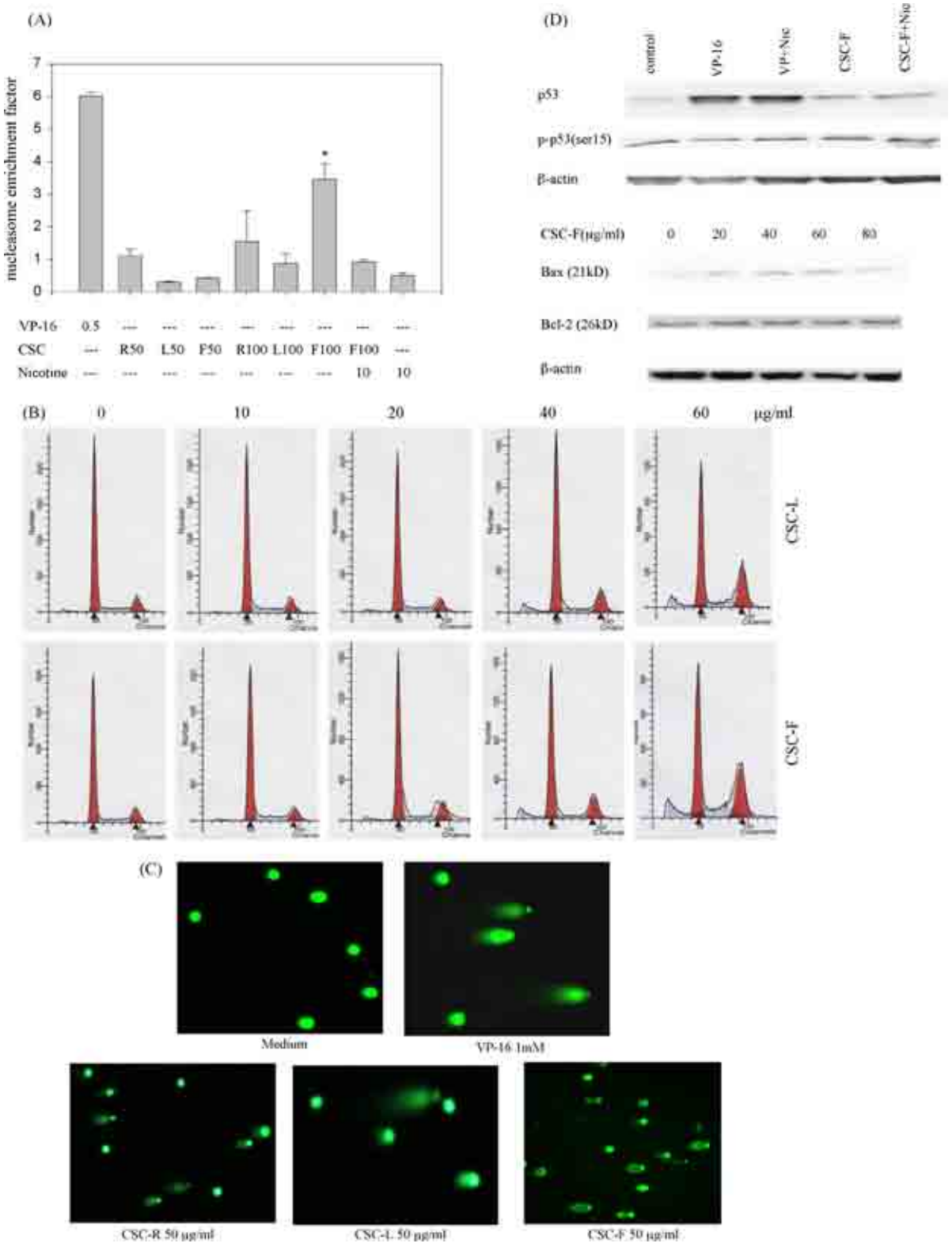


Fig. 4. Effects of CSC and nicotine on the induction of apoptosis. For all assays for apoptosis, NHBE cells were placed in 6-well plate and treated with different dose of CSC and/or nicotine or apoptosis-inducers at 37 °C overnight. The attached and floating cells were then harvested, washed and resuspended. For DNA fragmentation, the amount of cytoplasmic nucleosome (A) was determined with Cell Death Detection ELISA kit. For DNA content (B), the cells were collected and fixed with 75% ethanol. After stained with propidium iodine, cell cycle analysis was performed by flow cytometry. Sub-diploid DNA peaks were considered apoptotic cells. For neutral Comet assay (C), the cells were mixed with molten LMAgarose and placed in CometSlide. Cells in the slides were then lyzed and subjected to electrophoresis in TBE buffer horizontally at 1 V/cm for 15 min. After fixing with 70% ethanol and staining with SYBR Green I, cells were observed and photographed under an epifluorescence microscopy. The comet was scored from 0 to 4 according to Collins criteria by measuring tail length, head size, tail intensity and head intensity. (D) Effects of CSC and nicotine on the induction of apoptosis-related protein expression. NHBE cells were exposed to CSC-F and/or nicotine (10 μM), or apoptosis-inducer VP-16 (0.5 mM) at 37 °C overnight. Cell lysates (30 μg) were then immunoblotted with indicated antibodies.

Table 2
Effects of Quest® CSCs on the cell cycle progress in NHBE cells

CSC	Concentration (μg/ml)	G1/G0	S	G2/M	Sub G1/G0
CSC-F 16 h	0	74.7	11.7	13.6	6.60
	20	77.6	12.2	10.2	5.62
	40	80.8	9.5	9.7	4.29
	60	81.4	7.6	11.0	6.09
	80	78.6	9.7	11.7	10.35
CSC-L 16 h	0	71.5	13.6	14.9	3.12
	20	76.9	12.5	10.6	4.17
	40	79.8	10.3	9.9	7.81
	60	81.5	7.6	10.9	8.68
	80	77.8	8.4	13.8	10.83
CSC-F 48 h	0	70.8	16.1	13.1	1.33
	20	66.8	17.3	15.9	3.88
	40	68.0	11.6	20.5	9.75
	60	46.3	21.4	32.3	13.15
	80	34.5	28.9	36.6	22.95
CSC-L 48 h	0	72.4	15.7	11.8	1.65
	20	70.9	18.4	10.6	2.20
	40	70.5	12.6	16.9	6.86
	60	53.0	16.0	31.0	9.77
	80	40.4	29.6	30.1	13.01

concentrations were greater than 40 μg/ml, and the CSC-F did not show significantly different effects from CSC-L ($t = 2.14$, $P > 0.05$). When the nicotine (10 μM) was added to the CSC-F, it did not change the cell cycle progression. Furthermore, nicotine itself at the range from 1 to 100 μM did not affect the cell cycle (data not shown). This indicates that components other than nicotine from Quest® CSC resulted in cell cycle arrest effects. The data also showed that both CSC-F and CSC-L treatment resulted in more sub-G1/G0 cells than control (Table 2), which is usually recognized as apoptotic cells. This is consistent with the previous apoptosis data in Fig. 4.

4. Discussion

Quest® cigarettes are commercially available as low nicotine and denicotinized products. Some smokers might have the perception that lower nicotine yields equate with a safer cigarette, particularly in light of the marketing that has been done around these products. However, the data described herein does not support the perception that low-nicotine and nicotine-free Quest® cigarettes might be less harmful than conventional cigarettes. While the TSNAs and some volatile organics in Quest® cigarette smoke were variably decreased, aromatic amines were increased. Smokers using Quest® might reduce their exposure to some toxicants, their exposure to others would be increased. This situation would likely be exacerbated by the users compensating for the lower nicotine yield and inhaling more (Strasser et al., 2007), thereby further altering the smoke chemistry. In addition, Quest® CSCs were shown to have a similar mutagenic potential in Ames assay to 2R4F, and were slightly more potent with respect to the tumor promotion-relevant GJIC effect than 2R4F. Quest® CSCs as well as 2R4F CSC had strong cytotoxic and anti-proliferative effects on the primary NHBE cell cultures while CSC-F exhibited more anti-proliferative activity. Nicotine in cigarette smokes played some role in increased cell survival, possibly in part by anti-apoptosis. To our knowledge, this is the first demonstration that nicotine would have these effects in CSC, rather than experimental models using only nicotine.

CSC contains a variety of chemical constituents that exhibit a wide range of toxicities. For the Ames test conducted with the TA98/TA100 *Salmonella* strains, the primary sources of mutagenic activity in the CSC are aromatic amines and heterocyclic amine protein pyrolysate products (DeMarini, 2004), and mutagenicity is closely linked to the TPM yield of the cigarette (Roemer et al., 2004). Our data confirms that TA98/TA100 responsiveness is not limited to these chemical constituents since although there were differing levels of aromatic amines in the CSCs, there was no significant difference in the TPM and mutagenic activity between the nicotine-free and low nicotine Quest® CSCs. This study also showed that nicotine alone had no mutagenic activity and did not affect the slope of CSC-F-induced mutation in the Ames test when added back to mirror the levels found in CSC-L. This is in agreement with prior reports (Mizusaki et al., 1977).

Airway epithelial cells are directly exposed to cigarette smoke, and are susceptible to its toxic and carcinogenic properties. To investigate the cytotoxicity of Quest® cigarette smoke, we established a primary cell culture system for normal human bronchial epithelial cells derived from large airways, which are the precursor cells for squamous cell carcinomas. NHBE cells have been used in modeling the effect of chemicals on cell growth and morphology (Fields et al., 2005). For example, CSC has been proven to alter NHBE cells growth, the induction of terminal squamous differentiation, gap junction intercellular communication, and result in the formation of single strand DNA breaks (McKarns et al., 2000). Interestingly, compared to primary NHBE cell cultures, the immortalized NHBE cell line BEAS-2B exhibited different responses to CSC in some experiments. For example, BEAS-2B cells have high background expression levels of p53 (although the SV40 T antigen in these cells can bind to p53 and block its function) and PARP, compared with NHBE cells, and were sensitive to gamma irradiation or CSC stimulation (data not shown). In addition, overnight CSC treatment caused a G1cell cycle arrest in NHBE cells, but stimulated cell cycle transition through G1 in the BEAS-2B cells (data not shown). The BEAS-2B cell line has been studied extensively to elucidate carcinogenic mechanisms (Fields et al., 2005; Liu et al., 2005; Minna, 2003; West et al., 2003). However, it is different from NHBE cell in some ways, and only the NHBE cells are closer to physiological conditions.

The presence of functional gap junctions has been hypothesized to play a role in the normal physiological regulation of cell growth, differentiation and function, and it has been known for many years that loss of functional gap junctions is a common feature of cancer. It has been postulated that the loss of gap junctions may play a role in carcinogenesis and tumor progression (Mesnil et al., 2005). The GJIC assay tests the ability of agents to disrupt gap junction function in short term exposures since this ability has been suggested to relate to the tumor promotional effects of an agent (Tai et al., 2007). Our data showed that Quest® cigarette CSCs impaired the GJIC in NHBE cells, and that this inhibition was even slightly greater than that produced by CSC from the "low tar" reference cigarette. The tar yield of the cigarettes did not closely reflect the effects seen in the GJIC assay and nicotine had essentially no effect with CSC from the two Quest® cigarettes having similar GJIC disrupting activity independent of the nicotine content. The CSC constituents responsible for this effect are unknown but may relate to components such as anthracene and methylated anthracenes (Tai et al., 2007).

Our data showed that Quest® CSCs killed the primary NHBE cells through both acute necrotic cell death and apoptosis mechanisms. The former is consistent with the literatures that cigarette smokes can induce necrosis in a variety of cells, including NHBE cells (Liu et al., 2005) and the alveolar epithelial cell line A549 (Wickenden et al., 2003). However, there is conflicting data about whether CS damage leads to apoptosis. While some previous studies indicated that CSC induced apoptosis (Hellermann et al., 2002; Yang and Liu, 2004), Liu et al. (2005) cannot confirm this in NHBE cells. Wickenden et al. (2003) even found that CSC inhibited apoptosis induced by staurosporine or Fas ligation and the activation

of caspase 9 and 3, an effect that they postulated was caused by CSC mediated inhibition of caspase activation. There are three possible limitations of these studies that may account for their disparities. First, some detection techniques alone used for apoptosis may be providing misleading results. Intrinsic changes in the time courses of the apoptosis make it possible that an apoptotic signal are missed at detection time point, or the apoptotic effect is somewhat obscured by other more acute toxic death mechanisms. For example, the assays for DNA-strand breaks, such as TUNEL and ISEL (Hellermann et al., 2002), may have inadvertently identified cells with CSC-induced DNA strand breaks (Liu et al., 2005) as apoptotic. In our study, three assays (cytoplasmic nucleosome, DNA content and neutral Comet assays) were used to make it less likely that CSC is affecting the techniques themselves producing artifactual data. Another possibility for the discrepant results in the literature may relate to the preparation or handling of the CSC, resulting in different chemical constituent yields. While some investigators used gravimetric methods for tar collection onto Cambridge filters (Fields et al., 2005; Hellermann et al., 2002; Putnam et al., 2002) (the method used in this study), others have used "cold trap" methods for collecting compounds from the vapor phase as well as the particulate phase (Liu et al., 2005; Wickenden et al., 2003; Yang and Liu, 2004). These different methods might lead to collection of different chemical constituents, for example more free radicals and oxidants in vapor phase (Lee and Shacter, 1999). The final reason is that the preparations used in the literature used conventional CSC that contains high levels of nicotine. Nicotine and its nitrosated carcinogenic derivative NNK play an important role in the attenuation of apoptosis (West et al., 2003). This study is the first to demonstrate that nicotine-free CSC triggered a significant apoptotic response in NHBE cells and nicotine protected NHBE from CSC-induced injury at least in part by inhibition of apoptosis.

The ratio of Bcl-2 to Bax is thought to determine survival or death after apoptotic stimulation (Oltvai et al., 1993; Xin and Deng, 2005). Our data showed that CSC-F up-regulated the expression of Bax protein, but did not alter the Bcl-2. The decline in the Bcl-2/Bax ratio prompts cells to undergo apoptosis. This may be the important mechanism of CSC-F-induced apoptosis. On the other hand, nicotine can inactivate the Bax function through phosphorylation at ser-184 site (Xin and Deng, 2005). Bax is possibly the common target for CSC and nicotine for inducing apoptosis. Nicotine attenuated CSC-F-induced apoptosis maybe by inactivating the function of increased Bax proteins. This is worth further investigating because lung cancer cells express high levels of endogenous Bax not the Bcl-2. The increase of Bax protein in lung cancer cells may be caused by CSC and inactivated by nicotine.

In summary, a toxicological analysis does not indicate that low-nicotine and nicotine-free Quest® cigarettes have less adverse toxicological effects in the laboratory than conventional cigarettes. This should draw the attention of the consumers and policy makers. A more comprehensive evaluation in smokers is expected for these new products.

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Potentially Reduced Exposure Cigarettes Accelerate Atherosclerosis: Evidence for the Role of Nicotine

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Abstract The tobacco industry markets potentially reduced exposure products (PREPs) as less harmful or addictive alternatives to conventional cigarettes. This study compared the effects of mainstream smoke from Quest, Eclipse, and 2R4F reference cigarettes on the development of atherosclerosis in apolipoprotein E-deficient (apoE –/–) mice. Mice were exposed to smoke from four cigarette types for 12 weeks beginning at age of 12 weeks, and in a separate study for 8 weeks, beginning at age of 8 weeks. In both studies, mice exposed to smoke from high-nicotine, high-tar Quest 1, and 2R4F cigarettes developed greater areas of lipid-rich aortic lesions than did non-smoking controls. Exposure to smoke from the lower-nicotine products, Eclipse, and Quest 3, was associated with smaller lesion areas, but animals exposed to smoke from all of the tested types of cigarette had larger lesions than did control animals not exposed to smoke. Urinary levels of isoprostane F2 alpha VI, increased proportionally to cigarette nicotine yield, whereas induction of pulmonary cytochrome P4501A1 was proportional to tar yield. Lesion area was associated with both nicotine and tar yields, although in multiple regression analysis only nicotine was a significant predictor of lesion area. Smoke exposure did not alter systolic blood pressure (SBP), heart rate (HR), blood cholesterol, or leukocyte count. Taken together, these observations suggest that smoking may accelerate atherosclerosis by increasing oxidative stress mediated at least in part via the actions of nicotine.

Keywords Isoprostane · Oxidative stress · cytochrome P450 · Sympathetic nervous system · Complete blood count

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Introduction

The tobacco industry promotes potentially reduced exposure products (PREPs) with claims that they are less harmful or less addictive than standard cigarettes because they deliver lower doses of toxic, carcinogenic, and/or addictive agents. Among the currently marketed PREPs are the cigarettes Eclipse and Quest. Eclipse heats the air inhaled through the cigarette to volatilize its contents without burning the tobacco resulting in lower levels of nicotine and tar delivery than from conventional cigarettes. A recent study showed lower tumorigenicity of smoke condensate from Eclipse compared to a conventional reference cigarette (1R4F) [1]. Quests 1–3 are a group of conventionally burned cigarettes in which genetically modified low-nicotine tobacco is blended with normal tobacco to provide three cigarette types containing different nicotine levels ranging between 0.6 mg and 0.05 mg/cigarette. Quest cigarettes are marketed as

providing smokers the opportunity to gradually reduce their level of nicotine intake. The health risks associated with the use of these products have not been adequately investigated.

The cardiovascular risks associated with cigarette smoking include atherosclerosis, thrombosis, and vascular dysfunction [2]. Cigarette smoke may promote atherosclerosis by inducing inflammation, vasomotor dysfunction and proatherogenic changes in lipid profile [2, 3]. In humans, smoking causes a proatherogenic lipid profile in which serum triglycerides, cholesterol, and LDL-cholesterol are elevated, while HDL-cholesterol decreases [2, 4, 5]. Smoking may also affect the levels of circulating monocytes, and induce expression of adhesion molecules that accelerate the initiation and progression of atherosclerosis [6].

Despite the well-known association of tobacco smoke exposure with cardiovascular disease, few studies have sought to identify the components of tobacco smoke that promote atherosclerosis, in part because of the lack of suitable animal models [7–12]. Several recent studies have examined the effects of smoke and smoke components on atherosclerosis in apolipoprotein E-deficient (apoE –/–) mice, which develop lesions similar to those of humans over a relatively short time frame (8–16 weeks of age) [13]. Exposure to cigarette sidestream smoke has been shown to stimulate atherosclerosis in apoE –/– mice [14] and oral administration of either nicotine or benzo[a]pyrene, two principal smoke components, can stimulate the growth of atherosclerotic lesions in apoE –/– mice [12, 15]. However, neither the effects of mainstream smoke nor the relative contributions of inhaled nicotine and tar to the atherogenicity of tobacco smoke have been investigated.

The objectives of the present study were to determine if exposure to mainstream tobacco smoke affects aortic atherosclerosis in apoE–/– mice and if lesion areas differ after exposure to smoke from conventional cigarettes compared to smoke from the PREPs, Quest, and Eclipse.

Materials and Methods

All animal experimental procedures were approved by the Weill Medical College Institutional Animal Care and Use Committee. Male apoE –/– mice obtained from Jackson Laboratories were maintained on a normal laboratory diet containing 4.5% fat (Labdiet 5053). In Study 1, groups of 10 mice were exposed to smoke from each cigarette type for 12 weeks beginning at age of 12 weeks. In Study 2, groups of 20 mice were exposed to smoke from each cigarette type for 8 weeks beginning at age of 8 weeks.

Smoke Exposure

Nicotine and tar yields of the cigarettes used in these studies are shown in Table 1. Nicotine and tar yields are as stated by the manufacturer based on their implementation of Federal Trade Commission (FTC) protocols. 2R4F research cigarettes were obtained from the Kentucky Tobacco Research Institute, and were stored at 4°C. Eclipse, Quests 1 and 3 cigarettes were obtained freshly from retail sources every 1–2 weeks and stored at room temperature. All cigarettes were equilibrated at ambient temperature and humidity for at least 18 h before use.

Smoke exposure was carried out using a 24-port smoke delivery manifold (CH Technologies, Westwood NJ). Mice were placed in Plexiglas holders fitted with an aluminum end cap through which they directly breathed the tobacco smoke from the manifold. Mice were smoke-exposed for 1 h each day from Monday through Friday of each week, except for holidays. Animals in Study 1, the age of 12–24 weeks exposure group, were smoke-exposed for exactly 1 h/session, during which 10–12 cigarettes were burned. Animals in Study 2, the age of 8–16 weeks exposure group, were exposed to smoke from exactly 12 cigarettes per session, which took approximately 1 h. Smoke delivery was controlled by a computer-driven pump/valve mechanism that smoked cigarettes serially one at a time, delivering the smoke directly to the manifold chamber. Cigarettes were smoked according to a modified FTC protocol at the rate of 2 puffs/min, 2 s/puff, and 35 mL/puff. Airflow through the smoke manifold was 4 L/min, which diluted the smoke stream and filled the manifold with fresh air between puffs. Total smoke particulate (TSP) was quantified by aspirating smoke at a fixed rate through a glass micro fiber filter attached to one of the manifold ports, and the weight of smoke product collected on the filter divided by the total aspirated volume was calculated as the TSP in mg/m^3. Control animals were placed in the smoking chambers for 1 h/day on a similar schedule, but were not exposed to cigarette smoke during that time.

Physiological Measurements

Animals were weighed weekly. Systolic blood pressure (SBP) and heart rate (HR) were measured by non-invasive tail-cuff plethysmography using a Visitech 2000 automated device as described previously [16]. SBP was measured in the mornings, before animals were smoke exposed. Blood was collected by retrorbital puncture after light anesthesia induced with isoflurane. For Study 1, hematocrit was measured by centrifugation of whole blood collected in heparinized hematocrit tubes. For Study 2, blood was diluted 4-fold in 10 mmol/L EDTA/5% BSA and a complete blood count (CBC) carried out using a Bayer ADVIA 120 analyzer. At the midpoint and last week of each study, urine was collected from animals housed in metabolic cages. Urine was collected after the mice had been smoke

Table 1 Cigarette nicotine and tar. FTC yields and total smoke particulate exposure

Length of exposure (weeks)	FTC nicotine yield (mg)	FTC tar yield (mg)	TSP Study 1 (mg/m^3) 8	TSP Study 2 (mg/m^3) 12
Quest 1	0.6	10	395 ± 16	475 ± 2
Quest 3	0.05	10	383 ± 15	446 ± 2
2R4F	1.0	10	334 ± 12	459 ± 2
Eclipse	0.2	5	187 ± 12	272 ± 1
Control	NA	NA	NA	NA

NA, not applicable

exposed for four consecutive days. Urinary total nicotine and cotinine were analyzed as described previously [17]. Total and HDL cholesterol were measured using kits from WAKO diagnostics following the manufacturer's instructions. Isoprostane F2 alpha VI, a sensitive and specific marker of lipid peroxidation, was measured in urine as described previously [18].

Tissue Collection and Measurements

After the final smoke exposure, animals were deeply anesthetized with isoflurane and exsanguinated. The heart and vasculature were perfused for 10 min with 4% paraformaldehyde in 0.15 M NaCl, delivered at 100 mmHg pressure via a needle placed in the left ventricle, removed *en bloc*, and then immersed in the same fixative for 24 h before further processing. The aorta was divided into three sections: aortic arch (aortic sinus to the brachiocephalic artery), suprarenal aorta (brachiocephalic artery to the left renal artery), and infrarenal aorta (left renal artery to the iliac bifurcation). A circular cross section approximately 2 mm long was taken from the distal end of each of these fixed segments and paraffin-embedded for histology. The remaining aortic segments were opened longitudinally and stained *en face* with Oil Red O to visualize lipid deposits corresponding to lesions. Atherosclerotic lesion area was quantified by digital microscopy and expressed as the percentage of the lumenal area covered by lipid-rich lesions. All measurements were carried out by an observer blinded to the treatment group of the animals. Measurements were repeated by a second independent observer also blinded to treatment. Lungs were stored frozen in liquid nitrogen. Lung cytochrome P450 1A1 (CYP1A1) content was determined by western blotting as described previously [19].

Statistical Analysis

Data were analyzed using Dunnet's test for multiple comparison to single control. For multiple comparisons between groups Student's *t*-test was used, applying the Bonferroni adjustment as appropriate. $p < 0.05$ was considered significant. Statistical analysis was carried out using Statview software Version 5.0.1 (SAS Institute, Cary, NC).

Results

Smoke Exposure

In Study 1 (12–24 weeks exposure), cigarettes burned according to the modified FTC protocol over 1 h produced a TSP of 300–400 mg/m^3 except for Eclipse, which produced a TSP of ~190 mg/m^3, proportional to the lower tar content of this product (Table 1). There were no significant differences in TSP among Quests 1 and 3 and 2R4F, over the 12 weeks of the study as evaluated on a daily basis. In Study 2 (8–16 weeks exposure), TSP for each cigarette type was 20–40% higher than in Study 1, although the variability in TSP for each type of cigarette was much lower because a fixed number of cigarettes was burned in each session.

Total nicotine and cotinine measured in the urines of Study 1 mice were proportional to the FTC nicotine yield of each cigarette, although the slope of the relationship tended to be lower at high FTC nicotine yield (Fig. 1).

Atherosclerotic Lesion Area

Atherosclerotic lesion area was measured *en face* in Oil Red O stained whole mounts of aortic arch, suprarenal aorta, and infrarenal aorta (Fig. 2). Comparisons were made between each smoke exposed group and control, and each smoke exposed group and 2R4F (Fig. 2). In Study 1, lesion areas were approximately double the area of lesions in controls in the aortic arch, infrarenal aorta, and all aortic segments combined (total) of animals exposed to high-nicotine 2R4F and Quest 1 smoke. Among animals exposed to smoke, only in the Eclipse group was the measurement of total lesion area significantly lower than in the 2R4F group.

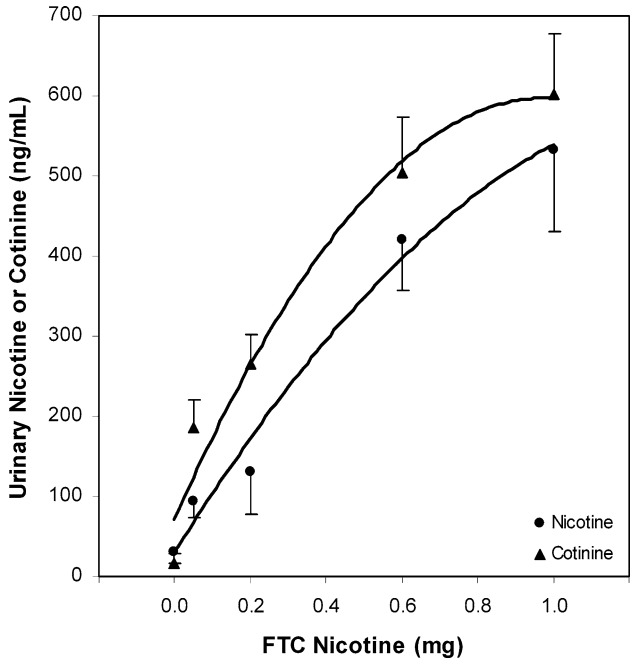


Fig. 1 Relationship of urinary nicotine and cotinine excretion after 12 weeks smoke exposure in Study 1 mice to FTC nicotine yield stated by the manufacturer. Two or three mice were placed in each metabolic cage overnight. Each point represents the mean and SEM of four separate group collections

In Study 2 animals, lesion areas were generally smaller than in Study 1 animals, presumably reflecting both younger age and the shorter period of smoke exposure. Lesion areas in mice varied with type of cigarette: animals exposed to high-nicotine 2R4F and Quest 1 smoke had lesions 0.5 to 3-fold larger than in control mice. Although lesion areas in mice exposed to the lower nicotine Quest 3 and Eclipse smoke were smaller than those in mice exposed to 2R4F or Quest 1 smoke, they were still significantly larger than controls. Moreover, suprarenal and total aortic lesion areas in Quest 3 and Eclipse exposed mice were significantly smaller than in 2R4F exposed mice. Similarly, Quest 1 exposed mice had significantly smaller lesion areas in the total aorta than did 2R4F exposed mice. These conclusions were supported by measurements made by two independent observers.

Relationship between Lesion Area, Nicotine, and Tar

Plots of total lesion areas against the FTC nicotine and tar yields of the cigarettes tested revealed dose–response relationships between both nicotine and tar with total lesion area for both studies (Fig. 3). Multiple regression analyses indicated that only nicotine was a significant predictor of lesion area in the 12–24 weeks study ($r^2 = 0.289$, $p < 0.002$), whereas in the 8–16 weeks study, both nicotine ($p < 0.001$) and tar ($p < 0.02$) were significant predictors

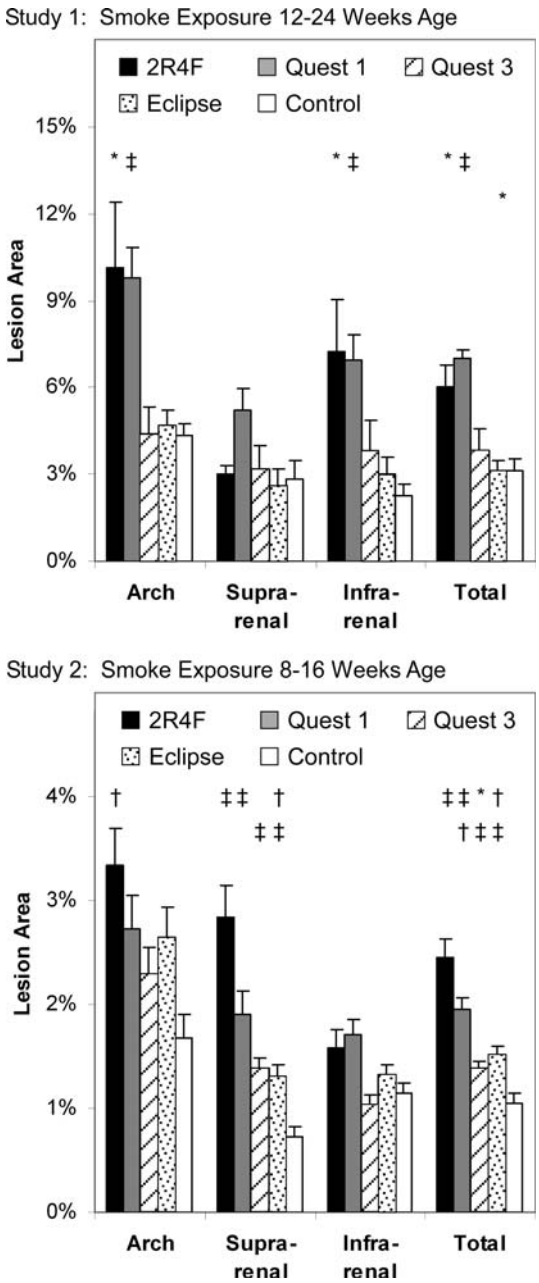


Fig. 2 Lesion areas measured *en face* in aortic segments (mean and SEM). Study 1 utilized 10 animals per group; Study 2 had 20 animals per group. A cube root transformation was applied to the data to normalize the variance between groups (raw data are shown in Fig. 3). Lesion areas were compared between each smoke exposure group and control (p values, upper row of symbols), and between each smoke exposure group and 2R4F (p values, lower row of symbols). p values are adjusted for seven comparisons. *$p < 0.05$, †$p < 0.005$, ‡$p < 0.001$

of lesion area that together accounted for almost half of the variance ($r^2 = 0.47$). A large part of the effect of tar observed in the 8–16 weeks study may reflect the smaller lesion area in the control group. When the smoke exposed groups were compared to one another, nicotine content alone remained a significant predictor of lesion area. Both

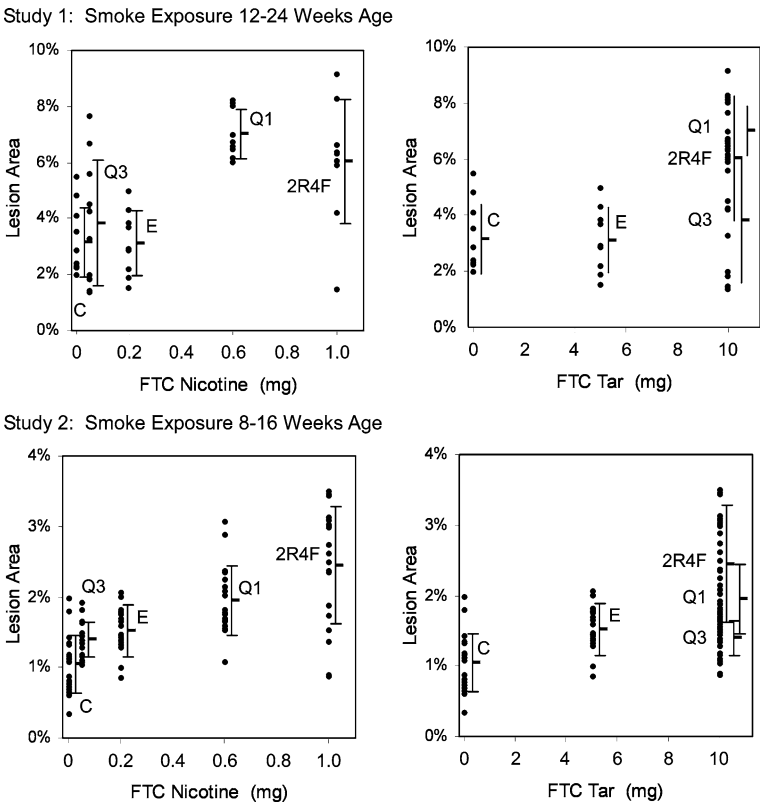


Fig. 3 Relation between total lesion area and FTC nicotine and tar yield among treatment groups (C, control; E, Eclipse; Q1, Quest 1; Q3, Quest 3; 2R, 2R4F). Raw data points are shown above nicotine or tar yield for each product. Means±SD are shown immediately to the right. Multiple regression analyses were carried out using data subjected to transformation in order to normalize the variances. See text for details

the co-linearity between the independent variables, nicotine, and tar ($r = 0.60$) and the small number of different tar levels in the cigarettes tested limit our ability to determine their relative contributions whether total aorta or subregions were evaluated.

Relationship between Nicotine and Oxidative Stress

Isoprostane F2 alpha VI, a sensitive and specific marker of lipid peroxidation and oxidative stress in vivo, was measured in urine collected overnight commencing immediately after smoke exposure for the 8–16 weeks study. Isoprostane F2 alpha VI levels were elevated in each of the smoke-exposed groups except for Quest 3, which has the lowest nicotine yield of all products tested (Fig. 4A). There was also a clear dose–response relationship between nicotine yield and urinary levels of isoprostane F2 alpha VI that tended to flatten with higher nicotine exposures (Fig. 4B).

Relationship between Tar and CYP1A1 Induction

CYP1A1 levels were measured by Western blot in lung tissue from animals in the 12–24 weeks study. CYP1A1 levels in the lungs increased in all smoke-exposed animals relative to controls (Fig. 5). Compared to levels in mice

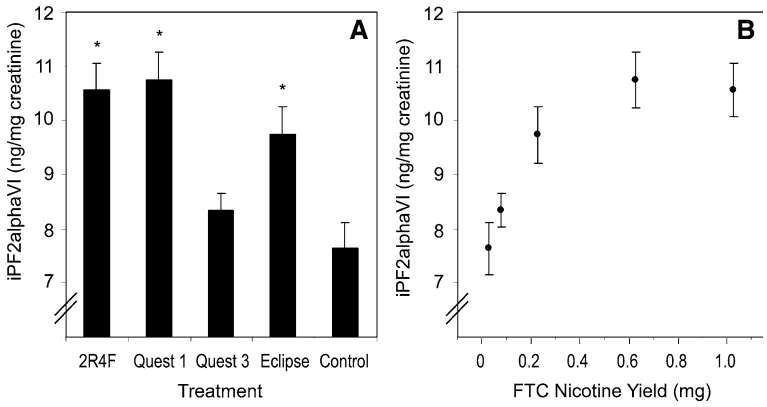


Fig. 4 Isoprostane F2 alpha VI levels (mean and SEM) measured in urine samples normalized to urine creatinine content plotted by treatment (**A**) and against the corresponding FTC nicotine yield (**B**). *$p < 0.05$, Dunnet's test for comparisons to control

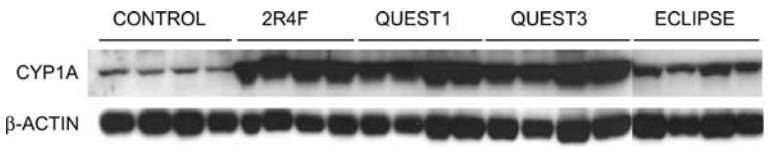


Fig. 5 CYP1A1 levels in lungs of selected mice from Study 2. Lungs were obtained from four mice in each group. Tissue lysate proteins (100 μg) were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and sequentially probed with antibodies specific for CYP1A1 and β-actin

exposed to 2R4F or Quest, CYP1A1 levels were less elevated in mice exposed to Eclipse cigarette smoke.

Physiological Measurements

All animals gained weight during the course of the study (Table 2), without differences between control and smoke-exposed groups except for the Eclipse group in Study 1. Mice grow most rapidly up to 8 weeks age after which growth slows, so that the greater weight gain of animals in Study 2 was most likely due to their younger age.

In Study 1, hematocrit was significantly higher at the end of the study in all smoke-exposed animals compared to control mice, except for the Quest 1 group, in which the hematocrit increase did not reach significance (Table 2). In the shorter 8–16 weeks study, there was a trend to increased hematocrit in all smoke-exposed mice compared to controls. The differences in hematocrit between the two studies are most likely due to the different analytic methods used. Elevated hematocrit is consistent with exposure to CO in cigarette smoke. A CBC on all mice from Study 2 showed no significant differences in leukocyte counts with smoke exposure, although mean platelet volume was slightly (1–3%), but significantly decreased in all smoke-exposed mice compared to controls (data not shown).

SBP and HR were measured before treatment, at midpoint and at the end of both studies. Generally it was difficult to detect a tail pulse immediately after smoke exposure, suggesting peripheral vasoconstriction. Therefore, SBP was routinely measured before smoke exposure, usually >20 h after the previous smoke exposure. SBP increased with time in all groups in both studies (Table 2). However, there were no significant differences in SBP among treatment groups at each time point compared to the control group. HR tended to decrease with age in all groups. However, there were no significant differences in HR among treatment groups at each time point compared to the control group with the exception of a small (11%) reduction in HR observed in the 2R4F group at 12 weeks compared to controls.

Total cholesterol increased with age (Table 2). Although total cholesterol at the end of both studies had increased by 4–16% in most smoke exposed groups compared to control, none of these differences were statistically significant. Conversely, HDL levels tended to fall with age, but not significantly with the exception of the Eclipse group in which HDL levels were slightly, although significantly increased compared to controls after 6 weeks smoke exposure in the 12–24 weeks study but not at the end.

Histology and Morphometry

Oil Red O staining of thoracic aortas showed that lipid-rich lesions occurred primarily at the branch points of side arteries, but also occurred in regions of the arterial wall in which there were no branch arteries (Fig. 6A). Treatment groups with greater lesion areas had larger lesions both at branch points and on uninterrupted sections of wall. Similar patterns were observed in other aortic segments.

Histological examination revealed early plaque development consisting mainly of intimal infiltration by macrophages and foam cells (Fig. 6B). Significantly increased numbers of breaks in the elastic laminae were observed at each level of the aortas of mice from the 2R4F, Quests 1 and 3 groups compared to controls (Figs. 6B and 7). There were also small increases in the number of elastic laminae present in the aortic arch of mice in the 2R4F, Quest 1, and Eclipse groups (Fig. 7). Morphometry revealed that lumenal and medial cross-sectional areas were similar between controls and each treatment group (data not shown).

Discussion

In two separate studies, we exposed atherosclerosis-prone apoE –/– mice to a variety of cigarettes for either 12 weeks beginning at 12 weeks age (Study 1), or for 8 weeks beginning at 8 weeks age (Study 2). The findings from both studies indicate that nicotine exposure is important in stimulating arterial lesion development, although tar content may also play a role. In both studies, we observed larger lipid rich lesion areas in aortas of mice exposed to the high-nicotine 2R4F and Quest 1 cigarettes (1 and 0.6 mg nicotine yield per cigarette, respectively). In the shorter Study 2, we also observed that lesion areas in the mice exposed to smoke from lower nicotine Eclipse and

Table 2 Physiological parameters for mice exposed to cigarette smoke between 12–24 weeks age (Study 1) and 8–16 weeks age (Study 2)

	Weight gain (g)	Hct (%)	Total cholesterol (mg/dL)			HDL cholesterol (mg/dL)			SBP (mmHg)			HR (bpm)		
Length of Exposure (weeks)	12	12	0	6	12	0	6	12	0	6	12	0	6	12
Study 1: 12–24 weeks age														
Quest 1	0.6 ± 0.3	43.4 ± 0.8	413 ± 23	555 ± 38	515 ± 26	28 ± 1	25 ± 1	20 ± 2	106 ± 1	107 ± 2	106 ± 3	625 ± 10	622 ± 13	617 ± 13
Quest 3	0.8 ± 0.3	45.6 ± 1.5*	377 ± 25	564 ± 43	546 ± 39	28 ± 2	27 ± 1	23 ± 2	103 ± 2	105 ± 2	105 ± 2	640 ± 12	617 ± 13	631 ± 10
2R4F	1.3 ± 0.3	46.3 ± 0.6*	446 ± 23	577 ± 28	542 ± 34	25 ± 1	23 ± 1	19 ± 1	106 ± 1	105 ± 1	110 ± 1	629 ± 9	624 ± 5	582 ± 18*
Eclipse	0.2 ± 0.3*	45.6 ± 1.2*	445 ± 32	532 ± 51	515 ± 17	27 ± 1	30* ± 2	19 ± 2	102 ± 1	102 ± 1	108 ± 3	629 ± 12	629 ± 10	621 ± 17
Control	1.3 ± 0.3	42.4 ± 0.6	421 ± 23	451 ± 31	470 ± 34	26 ± 1	24 ± 1	22 ± 1	102 ± 2	104 ± 2	110 ± 3	625 ± 11	624 ± 9	648 ± 9
Length of exposure (weeks)	8	8	4	8		4	8		0	4	8	0	4	8
Study 2: 8–16 Weeks Age														
Quest 1	2.2 ± 0.2	57.4 ± 1.0	543 ± 22	622 ± 15		20 ± 1	16 ± 1		102 ± 1	102 ± 2	107 ± 2	672 ± 9	600 ± 8	612 ± 10
Quest 3	3.5 ± 0.8	56.3 ± 0.9	521 ± 20	605 ± 20		21 ± 1	17 ± 1		98 ± 2	100 ± 1	104 ± 2	672 ± 8	590 ± 10	626 ± 13
2R4F	2.5 ± 0.1	57.0 ± 1.0	523 ± 19	621 ± 23		19 ± 1	16 ± 1		98 ± 1	103 ± 2	107 ± 2	660 ± 8	602 ± 8	633 ± 11
Eclipse	2.9 ± 0.1	56.3 ± 2.3	514 ± 20	579 ± 19		20 ± 1	18 ± 1		101 ± 1	106 ± 2	106 ± 1	675 ± 10	636 ± 11	619 ± 11
Control	2.6 ± 0.2	54.0 ± 0.5	516 ± 16	583 ± 22		19 ± 1	17 ± 1		102 ± 1	100 ± 2	103 ± 1	668 ± 8	631 ± 11	632 ± 8

*$p < 0.05$ compared to control (Dunnet's test)

Quest 3 cigarettes (0.2 and 0.05 mg nicotine yield per cigarette, respectively) were significantly smaller than in mice exposed to 2R4F and Quest 1 cigarettes, yet were significantly larger than lesion areas in controls. Although Quests 1 and 3 and 2R4F cigarettes all have the same tar yield (10 mg/cigarette) and produced similar TSPs under identical smoking conditions, mice exposed to smoke from the high-nicotine 2R4F and Quest 1 cigarettes developed larger aortic lesion areas than did mice exposed to smoke from Quest 3, which has the lowest nicotine content of all the products tested. Lesion areas in mice exposed to smoke from Eclipse cigarettes, which yield an intermediate amount of nicotine per cigarette (0.2 mg) and 50% less tar than Quest and 2R4F, were smaller than lesion areas in mice exposed to cigarettes with high tar and high nicotine, but were nonetheless significantly larger than in control mice.

Relationships between Smoke Components and Lesion Area

Regression analyses revealed a strong dose dependency between nicotine yield and lesion area in both studies, suggesting that nicotine plays an important role in stimulating lesion development. Our data suggested that lesion area also may be related to tar yield, but the limited range of tar yields in the products tested, and the close correlation between tar and nicotine yields limited our ability to establish an association between tar content and lesion size.

Our ability to detect significant differences in lesion areas between the lower nicotine Eclipse and Quest 3 products versus controls in the 8–16 weeks study may relate to the younger age of animals in that study, so that lesion areas in the smoke treated groups were compared to smaller lesions in the control group, giving a larger relative difference. In contrast, in the 12–24 weeks study lesion areas in the smoke treated groups were compared to relatively more evolved lesion areas in the control group so that the increase in lesion size in smoke treated groups appeared relatively less marked. The greater number of animals in the 8–16 weeks study also increased the power to detect differences. It should be noted, however, that under our study conditions in which animals were fed a normal low fat (4.5%) laboratory diet, lesion areas were relatively small both in smoke exposed animals and in controls, and there was no significant hypertrophy of the vascular walls during the observation period.

Possible Involvement of Oxidative Mechanisms

Increased oxidative stress has been widely implicated in promoting atherogenesis, and levels of isoprostanes, a measure of oxidative stress, are increased both in human

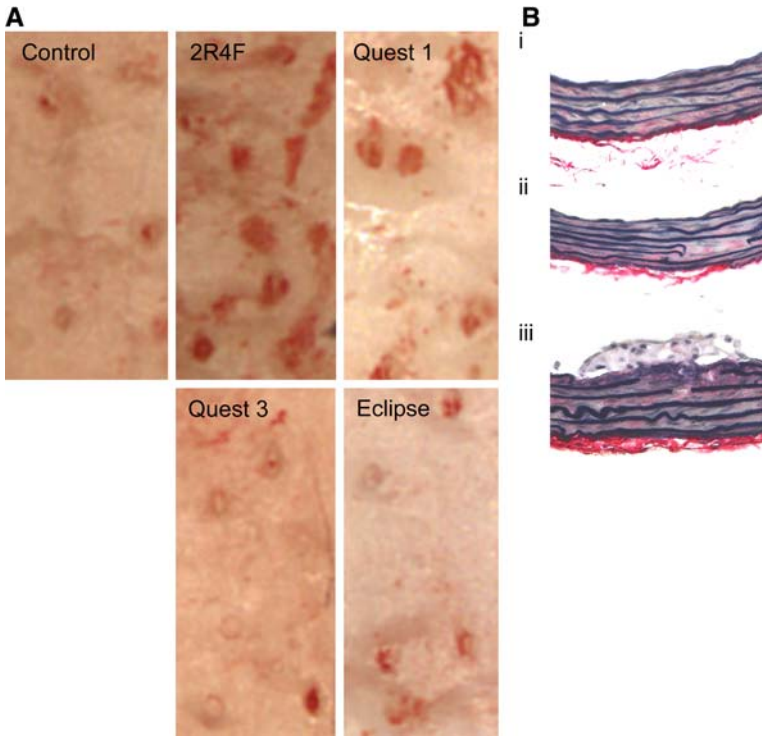


Fig. 6 (**A**) Oil Red O staining *en face* of thoracic aortas representative of each treatment group. (**B**) Aortic cross sections showing (i) normal aorta; (ii) breaks in elastic laminae under normal intima; and (iii) a typical early intimal lesion containing macrophages and breaks in the underlying media

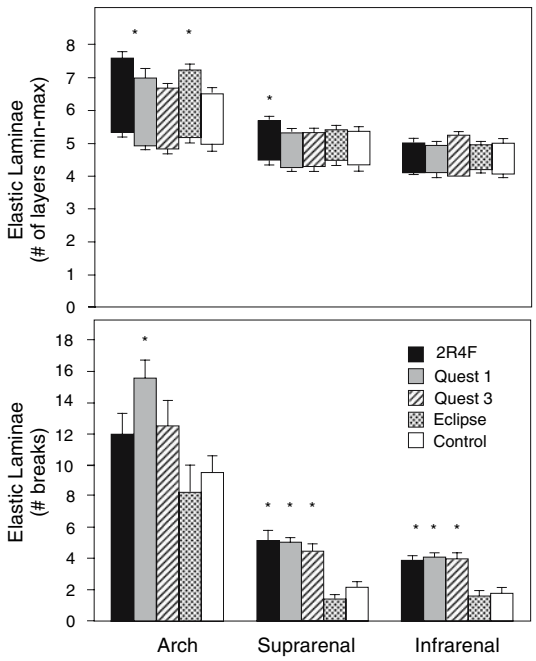


Fig. 7 Morphometric analysis of aortic cross sections. *$p < 0.05$, Dunnet's test for comparisons to control

and animal models of atherosclerosis [20]. Urinary excretion of isoprostane F2 alpha VI was significantly increased in mice exposed to smoke from 2R4F, Quest 1, and Eclipse cigarettes, but not in those exposed to Quest 3, suggesting that nicotine content is key in the production or augmentation of oxidative stress.

Cigarette smoke contains thousands of compounds that may foster development of atherosclerosis and cardiovascular disease [7–12]. The atherogenic potential of the few compounds that have been studied appears to be independent of their carcinogenicity, suggesting different effector mechanisms [9]. Recent studies suggesting that oxidative stress may play a role in atherosclerosis have focused on the involvement of plasma membrane-associated NAD(P)H oxidases [21], although other sources of reactive oxygen species have also been implicated [22]. The ability of various cigarette tar components to stimulate atherogenesis may be related to reactive oxygen species generated during their metabolism by cytochrome P450 enzymes [23]. Depletion of the cellular antioxidant, glutathione, by cigarette smoke components may further exacerbate these effects [24, 25]. In the present study, we found that mice exposed to smoke from high tar cigarettes exhibited higher levels of CYP1A1 than mice exposed to Eclipse smoke, in which enzyme levels were intermediate between the high tar groups and controls. This observation suggests that microsomal enzyme induction in response to cigarette smoke exposure may represent a source of oxidative stress stimulating atherogenesis. However, there was no direct relationship between cigarette tar yield and urinary excretion of isoprostane F2 alpha VI, a marker of oxidative stress (see below), suggesting the involvement of additional or alternative mechanisms.

Oxidative mechanisms also represent a target for interactions with other processes mediated by nicotine. Nicotine blocks nitric oxide production [26], and studies in humans

clearly demonstrate that nicotine can inhibit the protective functions of endothelium normally mediated by nitric oxide [27, 28]. Thus, while vascular nitric oxide generally counteracts proatherogenic effects of oxidative stress, if that oxidative stress is induced by cigarette smoking the nicotine absorbed from the smoke may suppress this vasculoprotective mechanism. The finding that isoprostane F2 alpha VI was increased in mice exposed to smoke from all cigarettes tested except Quest 3, which delivers minimal nicotine, is consistent with this hypothesis.

Other Physiological Parameters Involved in Atherogenesis

Many cardiovascular effects of cigarette smoke have been attributed to activation of the sympathetic nervous system by nicotine [29]. While we did not detect overall increases in SBP or HR among the different groups of mice exposed to smoking, which might indicate a sustained increase in sympathetic activity, our measurements were made >20 h after smoke exposure, by which time any short-term hemodynamic effects of smoke exposure due to acute sympathetic stimulation could have resolved. The observation that tail pulse was blunted immediately after smoke exposure suggests that there may be peripheral vasoconstriction, which could result in an increase in central aortic blood pressure. Histological studies of the aortas showed significant increases in the number of sharp breaks in the elastic laminae of smoke-exposed mice characterized by retraction of the broken elastic laminae. This type of break is associated with hemodynamic stress, in contrast with the more diffuse breaks associated with proteolytic degradation of the elastic laminae by macrophage proteases [30]. However, increases in breaks in elastic laminae were associated with exposure to smoke from cigarettes with high tar yield, rather than high nicotine yield, and therefore the evidence does not support the involvement of sympathetic activation by nicotine leading to sustained increased hemodynamic stress. Nicotine alone has also been shown to increase plaque vascularization in chronic models of mouse atherosclerosis in which lesions were more advanced than in the present study which focused on early vascular changes [15].

In human smokers, serum triglycerides, cholesterol, and LDL-cholesterol are elevated while HDL-cholesterol decreases [2]. In the present study, total cholesterol increased with age, as expected in this genetically hypercholesterolemic mouse strain, while HDL cholesterol decreased. However, despite a trend to increased LDL, there were no significant differences between smoking groups and controls, suggesting that the limited smoke exposure used in our model does not accelerate hypercholesterolemia. Similarly, we observed no differences in blood leukocyte counts that might contribute to increased atherogenesis.

In this study, total nicotine and cotinine values in urine were generally in proportion to the FTC nicotine yield of the cigarettes, with the dose–response relationship tending to flatten out with higher nicotine yield. These observations are consistent with the relatively flat dose response relationship between cigarette consumption and urinary nicotine and cotinine content reported in human studies [31]. Notably, the cotinine excretion in mice exposed to 2R4F cigarettes was only modestly lower than that of human smokers [32], suggesting that the nicotine exposures in this study were representative of the typical exposure in human smokers.

Study Limitations

A limitation of this animal model is that it does not reproduce the typical human temporal smoking pattern. While the mice were smoke exposed for only 1 h/day, humans tend to smoke through the course of the day to maintain plasma nicotine levels. Additionally, humans using low nicotine cigarettes tend to alter their smoking behaviors to maintain constant nicotine levels [33]. However, even with this limited duration of smoke exposure, effects of smoking on promoting lesion formation were clearly discernable in our model. Additional studies are required to determine whether a more typical 'human' pattern of smoke exposure would further accelerate atherosclerosis in this mouse model.

The regression modeling analysis performed in this study was also limited by the small range of tar exposures (0, 5, and 10 mg). To accurately ascertain the relative contributions of nicotine and tar to atherosclerosis would require the independent manipulation of tar yield in smaller increments within the range of available tobacco products.

Conclusions

Although nicotine is the addictive component of cigarette smoke, its contribution to the cardiovascular risk associated with cigarette smoking remains controversial. Our present findings suggest that nicotine is an important contributor to atherogenesis in this in vivo smoking model. This finding suggests that lower nicotine cigarettes might be "safer". However, smokers adjust their tobacco consumption to maintain constant nicotine levels even when they select low nicotine cigarettes [33]. Therefore, the cardiovascular risks of smoking are very difficult to diminish or eliminate in habitual smokers.

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Tobacco-specific nitrosamines in new tobacco products

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New tobacco products, designed to attract consumers who are concerned about the health effects of tobacco, have been appearing on the market. Objective evaluation of these products requires, as a first step, data on their potentially toxic constituents. Tobacco-specific nitrosamines (TSNAs) are an important class of carcinogens in tobacco products, but virtually no data were available on their levels in these products. In the present study, we analyzed several new products—Ariva, Stonewall, Exalt, Revel, Smokey Mountain, and Quest—for TSNAs and compared their TSNA levels with those in nicotine replacement products and conventional smokeless tobacco and cigarette brands. TSNAs were not detected in Smokey Mountain, which is a tobacco-free snuff product. The lowest levels among the new products containing tobacco were in Ariva and Stonewall (0.26–0.28 μg/g wet weight of product). The highest levels in the new products were found in Exalt (3.3 μg/g tobacco), whereas Revel and Quest had intermediate amounts. Only trace amounts were found in nicotine replacement products, and conventional brands had levels consistent with those reported in the literature. These results demonstrate that TSNA levels in new tobacco products range from relatively low to comparable with those found in some conventional brands.

Introduction

Tobacco-specific nitrosamines (TSNAs) are widely considered to be among the most important carcinogens in smokeless tobacco products and cigarette smoke (Bartsch & Spiegelhalder, 1996; Hecht, 1998; Hecht & Hoffmann, 1988; Magee, 1996; Preston-Martin & Correa, 1989). Two of these tobacco-alkaloid derived compounds, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN), are consistently carcinogenic in laboratory animals, with NNK showing higher activity (Hecht, 1998). The other two commonly measured TSNAs are *N*′-nitrosoanabasine (NAB) and *N*′-nitrosoanatabine (NAT). NAB is a weak carcinogen and NAT apparently lacks activity (Hecht, 1998). NNK and NNN have recently been evaluated by the International Agency for Research on Cancer (2005) as carcinogenic to humans (Group 1).

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In recent years, some new types of tobacco products have been appearing on the market. Ariva is a lozenge that contains compressed powdered tobacco and is advertised as "the alternative for smokers in a smoke-free environment." Stonewall Hard Snuff is another compressed tobacco lozenge. Both products are made with tobacco cured by a process that minimizes nitrosamine formation. General and Exalt are types of Swedish snus in which the GothiaTek process, designed to "continuously reduce or eliminate alleged harmful components in tobacco," is used. Revel is an American smokeless tobacco product designed for "tobacco satisfaction without smoking." Smokey Mountain is a tobacco-free and nicotine-free herbal snuff, designed as a snuff substitute. Quest is a cigarette available in three varieties: 1, low nicotine; 2, extra low nicotine; and 3, nicotine free. Quest cigarettes are "the first that gradually step you to nicotine free smoking."

With the exception of one review that includes data for Ariva, Exalt, and Revel, but without experimental details, we are aware of no published studies on TSNA levels in these products (Rodu & Jansson, 2004). These analyses were carried out here, in parallel with analyses of TSNAs in nicotine replacement products, and in conventional

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smokeless tobacco products and cigarette tobacco, for comparison.

Method

Tobacco samples

The 19 brands collected for analysis represent smokeless spit-free tobacco products, compressed tobacco lozenges, tobacco-free herbal snuff, new cigarettes with reduced nicotine content, nicotine replacement therapy products, and conventional smokeless tobacco products and cigarettes. The products were purchased between June 2003 and January 2005. General was ordered online from Snus Worldwide, Sweden. Exalt was purchased from retail stores in Washington, D.C., and Golden Valley, Minnesota; it was also ordered online from Snus Worldwide, Sweden (for comparison). Revel was ordered online (houseoxford.com) and also purchased in a convenience store in Fort Worth, Texas. Ariva was also obtained from different locations: A pharmacy in Belleville, Illinois; a pharmacy in Richmond, Idaho; and a Virginia cigarette factory outlet. Stonewall was purchased from a tobacco shop in Minneapolis, Minnesota. Quest cigarettes were ordered online through smokes-spirits.com and 001Cigarettes.com; cigarettes purchased in Richmond, Idaho, were analyzed for comparison. Conventional moist snuff, tobacco-free herbal snuff (Smokey Mountain), and commercial premium cigarettes were obtained from retailers in Minneapolis. Nicotine replacement therapy products were purchased from a pharmacy in Minneapolis. For 24 h prior to analysis, the tobacco was conditioned at room temperature in a chamber at a relative humidity of 60%.

Apparatus

Tobacco-specific nitrosamines were analyzed on a model 5890 gas chromatograph (GC; Hewlett Packard, Palo Alto, California) interfaced with a model 610 thermal energy analyzer (TEA; Orion Research, Beverly, Massachusetts). The gas chromatography conditions were as follows: DB-1301 capillary column (30 m × 0.32 mm × 0.25 μm; 6% [cyanopropylphenyl]methylpolysiloxane; J&W Scientific, Folsom, California) and a 2 m × 0.53 mm deactivated fused silica pre-column; flow rate 2.6 mL/min He; splitless injection port temperature 225°C. The following oven temperature program was used: 80°C for 2 min, then 12°C/min to 150°C, then 7 min at 150°C, then 12°C/min to 200°C, then 10 min at 200°C.

Reagents

Reference NNN, NNK, NAB, 5-methyl-*N*′-nitrosonornicotine (5-McNNN), and 5-(methylnitrosamino)-1-(3-pyridyl)-l-pentanone (C5-NNK) were synthesized as described previously (Amin, Desai, Hecht, & Hoffmann, 1996; Carmella, McIntee, Chen, & Hecht, 2000; Stepanov, Carmella, Hecht, & Duca, 2002). NAT was purchased from Toronto Research Chemicals Inc., Toronto, Ontario, Canada.

Tobacco-specific nitrosamine analyses

Moist snuff, cigarette tobacco, and lozenges. We used a slight modification of a method described previously (Adams, Brunnemann, & Hoffmann, 1983; Stepanov, Hecht, Ramakrishnan, & Gupta, 2005). Humidity-conditioned tobacco (200–500 mg) and 10 mL of citrate-phosphate buffer (pH = 4.5) containing ascorbic acid were added to a 30 mL Nalgene centrifuge tube (Nalge Nunc International, Rochester, New York) to which 200 ng each of 5-MeNNN and C5-NNK internal standards were added. The samples were homogenized for 30 s with a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, New York) and sonicated for 1 hr. The buffer extracts were separated from the particles of tobacco by high-speed centrifugation (15,000 rpm, 10 min). The extracts were filtered into 50-mL glass screw-top centrifuge tubes (Kimble, Vineland, New Jersey), and the pH was adjusted to 7 by adding 100 μL of 10 N NaOH. Each sample was applied to a 20-mL ChemElut cartridge (Varian, Harbor City, California), eluted with 3 × 20 mL CH_2Cl_2, and the eluants were combined and concentrated to dryness with a model SVT200H Speedvac concentrator (Thermo Savant, Farmingdale, New York). Residues were dissolved in 0.5 mL of CH_2Cl_2 and further purified by solid-phase extraction using Sep-Pak Plus silica cartridges (Waters Corp., Milford, Massachusetts), pre-equilibrated with CH_2Cl_2. The cartridges were washed with 5 mL of CH_2Cl_2/ethyl acetate: 50/50, and the TSNAs were eluted with 10 mL of ethyl acetate. The ethyl acetate eluants were concentrated to dryness (Speedvac). The dry residues were transferred into gas chromatography microvials with 3 × 50 μL methanol, concentrated to dryness, and redissolved in 100 μL of acetonitrile. Then 3 μL of the prepared sample was injected into the GC-TEA.

Patch and gum. The patches were cut in three pieces and, after removal of the backing, rolled into 30 mL Nalgene centrifuge tubes containing 10 mL of citrate-phosphate buffer (pH = 4.5). The gum was cut into small pieces, added to Nalgene tubes containing 10 mL of citrate phosphate buffer, and homogenized for 30 s with a Polytron tissue homogenizer. Then 100 ng each of 5-MeNNN and C5-NNK internal standards were added to the samples. The samples were incubated overnight at 37°C and the next day

were sonicated for 1 hr. The buffer extracts were separated from the particles of patch or gum and transferred into 50-mL glass screw-top centrifuge tubes. The pH was adjusted to 7 by adding 100 µL of 10 N NaOH, and each sample was extracted three times with equal volumes of ethyl acetate. The extracts were combined, dried with approximately 10 g of Na_2SO_4 and concentrated to dryness with a Speedvac concentrator. Residues were dissolved in 0.5 mL of CH_2Cl_2 and further purified by solid-phase extraction and analyzed as described for tobacco products.

Results

Table 1 summarizes levels of TSNAs in the products. TSNAs were not detected in Smokey Mountain, a tobacco-free snuff product. The lowest TSNA levels among the new products that contain tobacco were found in the compressed tobacco lozenges Ariva and Stonewall, which had totals of 0.19 and 0.28 µg/g wet weight of product, most of which was due to NAT. The highest nitrosamine levels among the new tobacco products were found in Exalt, and these were independent of the place of purchase. TSNA levels were relatively low in Quest cigarette tobacco, as compared with Marlboro, Camel, Winston, and Newport. The amounts of NNN were similar in Quest 1, 2, and 3; the levels of the other TSNAs were similar in Quest 1 and 2 and lower in the nicotine-free Quest 3.

Nicotine replacement therapy products did not contain detectable levels of NAT or NAB. Traces of NNN were found in three out of six gum pieces taken for analysis (0.002 µg/piece), and traces of NNK were found in six patches analyzed (0.008 µg/patch). The levels of TSNAs in the conventional products represent means for two to six analyses, carried out at different times.

We observed substantial variation in nitrosamine content measured in the same brand at different times (see Table 2 for some examples). The Swedish snus General, produced in 2003, was found to contain lower levels of TSNAs compared with that produced in 2002. The tobacco of Marlboro and Camel had variable levels of individual TSNAs, with the total amount in Marlboro cigarettes generally lower in 2004 than in 2001 (Table 2).

Discussion

Tobacco companies are trying to create alternative smokeless tobacco products and safer cigarettes, which would potentially reduce the risk of tobacco-attributable cancers. A number of new brands are being test marketed in the United States. These products are targeted to smokers and smokeless tobacco users who wish to reduce or quit tobacco use or who want to use "safer" products. Manufacturers' claims include statements of reduced toxin content and implied reduced risk, but it may take years before the real health effects of these new tobacco products are known. TSNAs are among the most important carcinogens in tobacco, and it is imperative that objective data on levels of these compounds be available.

The lowest TSNA levels in the tobacco-containing products we analyzed were found in the compressed tobacco lozenges Ariva and Stonewall. Levels of the strongly carcinogenic NNN and NNK were only 56–99 ng/g, with most of the TSNA content comprised of NAT, which is apparently noncarcinogenic. These products use Star Scientific specially cured tobacco known to be low in TSNAs. The emergence of these new products with relatively low levels of carcinogenic TSNAs is an encouraging sign.

The Swedish snus General, which is manufactured using the GothiaTek process and quality standard designed to minimize nitrosamine contamination, contained relatively low levels of TSNAs, compared with conventional smokeless tobacco products. The variation in TSNA content observed in General in 2002 and 2003 (Table 2) is consistent with a study by the Swedish National Food Administration (Österdahl, Jansson, & Paccou, 2004) that demonstrated a noticeable decrease in TSNA content in moist snuff on the Swedish market. However, TSNA levels in Exalt, which is supposedly produced by the same technology, were comparable with those in some conventional commercial brands of smokeless tobacco such as Copenhagen and Kodiak, which have had relatively high amounts of these compounds for many years (Hecht & Hoffman, 1988; Hoffmann et al., 1995; Rodu et al., 2004). Lower levels were found in Revel; however, these levels were still considerably higher than nitrosamine levels in other products such as food and beer (Bartsch & Spiegelhalder, 1996). Based on comprehensive dietary surveys, exposure to carcinogenic volatile nitrosamines such as *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine is estimated to be about 1 µg/day (Bartsch & Spiegelhalder, 1996). The same exposure to carcinogenic TSNAs would be reached by using just 1–2 g of Revel. One packet of Revel contains approximately 27 g tobacco.

Quest cigarette tobacco contained substantial amounts of NNN, which did not decrease with reduced nicotine content. Levels of NNN and other TSNAs in tobacco are a major determinant of smoke levels (Fischer, Spiegelhalder, Eisenbarth, & Preussmann, 1990). Therefore, cigarettes lacking nicotine will still expose smokers to significant amounts of known carcinogens. However, NNN

Table 1. Tobacco-specific nitrosamine levels.

	Tobacco-specific nitrosamine level (μg/g product wet weight)				
Product	NNN	NNK	NAT	NAB	Total
New tobacco products					
Hard snuff					
Ariva	0.019	0.037	0.12	0.008	0.19[a]
Stonewall	0.056	0.043	0.17	0.007	0.28[b]
Swedish snus					
General	0.98	0.18	0.79	0.06	2.0[c]
Spit-free tobacco packets					
Exalt					
Purchased in Sweden	2.3	0.27	0.98	0.13	3.7[d]
Purchased in the United States	2.1	0.24	0.68	0.05	3.1[b]
Revel					
Mint flavored	0.62	0.033	0.32	0.018	0.99[b]
Wintergreen flavored	0.64	0.032	0.31	0.017	1.0[b]
Tobacco-free snuff					
Smokey Mountain	nd	nd	nd	nd	nd[b]
Nicotine-reduced cigarettes					
Quest 1 (low nicotine)	0.93	0.17	0.31	0.013	1.4[d]
Quest 2 (extra-low nicotine)	0.82	0.19	0.19	0.01	1.2[d]
Quest 3 (nicotine free)	0.83	0.054	0.045	0.003	0.93[d]
Nicotine replacement therapy products					
NicoDerm CQ (patch, 4-mg nicotine)[f]	nd	0.008	nd	nd	0.008[b]
Nicorette (gum, 4-mg nicotine)[f]	0.002	nd	nd	nd	0.002[b]
Commit (lozenge, 2-mg nicotine)[f]	nd	nd	nd	nd	nd[b]
Conventional tobacco products					
Smokeless tobacco					
Copenhagen					
Snuff	2.2	0.75	1.8	0.12	4.8[b]
Long cut	3.9	1.6	1.9	0.13	7.5[b]
Skoal					
Long cut straight	4.5	0.47	4.1	0.22	9.2[b]
Bandits	0.9	0.17	0.24	0.014	1.3[b]
Kodiak					
Ice	2.0	0.29	0.72	0.063	3.1[b]
Wintergreen	2.2	0.41	1.8	0.15	4.5[b]
Cigarette tobacco					
Marlboro					
Full flavor	2.9	0.96	2.3	0.1	6.3[e]
Light	2.8	0.68	1.1	0.051	4.6[b]
Ultra-light	2.9	0.75	1.1	0.058	4.8[b]
Camel					
Full flavor	2.5	0.90	1.7	0.091	5.2[e]
Light	2.7	0.55	1.3	0.061	4.6[b]
Ultra-light	2.8	0.77	1.2	0.055	4.8[b]
Winston (full flavor)	2.2	0.58	0.56	0.025	3.4[b]
Newport (full flavor)	1.1	0.83	1.9	0.055	3.9[b]

Note. NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; nd, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine. [a]Mean of five analyses, each performed in duplicate. [b]Single analysis performed in duplicate. [c]Mean of two analyses, each performed in duplicate. [d]Mean of three analyses, each performed in duplicate. [e]Mean of four analyses, each performed in duplicate. [f]Values are expressed per piece.

Table 2. Variation of tobacco-specific nitrosamine levels in some products.

			Tobacco-specific nitrosamine level (μg/g tobacco)				
Product	Date of purchase	Number of samples	NNN	NNK	NAT	NAB	Total
General	2002	2	1.2	0.28	0.93	0.076	2.5
	2003	2	0.78	0.075	0.65	0.049	1.6
Marlboro (full flavor)	July 2001	2	4.3	1.8	2.7	0.14	8.9
	Oct. 2003	2	3.0	1.2	4.9	0.19	9.3
	Oct. 2004	2	2.0	0.37	0.71	0.03	3.1
	Jan. 2005	2	2.5	0.49	1.0	0.046	4.0
Camel (full flavor)	July 2001	2	3.1	1.4	1.6	0.11	6.2
	Oct. 2003	2	1.9	1.2	2.8	0.15	6.1
	Oct. 2004	2	2.1	0.40	0.75	0.032	3.3
	Jan. 2005	2	3.0	0.56	1.5	0.074	5.2

Note. NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine.

and NNK levels in Quest were substantially lower than in currently available conventional brands.

Overall, the results of the present study demonstrate that TSNA levels in new tobacco products range from relatively low to comparable with those found in some conventional brands.

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Activation of platelets exposed to shear stress in the presence of smoke extracts of low-nicotine and zero-nicotine cigarettes: The protective effect of nicotine

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Mainstream and sidestream smoke extracts of both high-tar and low-tar cigarettes have been shown to increase platelet activation directly and to sensitize them to further activation by exposure to mechanical stimuli such as shear stress. However, nicotine has an inhibitory effect on platelet activation, opposite to that of whole cigarette smoke extracts. To distinguish between the nicotine-dependent and non-nicotine-dependent effects of smoke, platelets were exposed to mainstream and sidestream smoke extracts of low-nicotine and zero-nicotine cigarettes in vitro under flow conditions comparable with the normal circulation, and their activation state was measured using a modified prothrombinase-based assay. Both low-nicotine and zero-nicotine extracts caused increased platelet activation upon exposure to shear stress, and they caused significantly greater activation than extracts from ordinary high-tar, high-nicotine cigarettes. That nicotine was crucial was confirmed by adding 50 nM nicotine (comparable with the nicotine level in smokers' plasma) to zero-nicotine smoke extracts and demonstrating a reduction in the shear-dependent rate of platelet activation of more than 75%.

Introduction

Cigarette smoke is a primary risk factor for cardiovascular disease, including the development of atherosclerosis and, in particular, coronary heart disease and peripheral vascular disease (Kritz & Sinzinger, 1996). Enhanced function of the hemostatic system, in which platelets play a major role, is a significant underlying mechanism in cardiovascular disease and its progression. The problems associated with tobacco smoke are not confined to smokers but also apply to nonsmokers exposed to environmental, or secondhand, smoke, which is derived mostly from the smoldering end of the cigarette. Like mainstream smoke, secondhand smoke adversely affects platelet function, causing high states of activation, increased response to physiological and mechanical agonists, and high thrombogenic potential (Glantz & Parmley, 1991; Rubenstein, Jesty, & Bluestein, 2004).

The general role of platelets in cardiovascular disease associated with smoking is reasonably well established, but the role of nicotine in smoking-related cardiovascular disease has not been demonstrated (Benowitz & Gourlay, 1997). It has sometimes been assumed tacitly that the various deleterious effects of tobacco smoke are related mainly to nicotine, but in the case of platelets, no evidence supports this assumption, and the effects of nicotine on platelets remain unclear. The key focus of the present study was to discriminate between the nicotine-dependent and non-nicotine-dependent effects of tobacco smoke on platelets.

Enhanced thrombin generation is an important factor in the pathogenesis of cardiovascular diseases. Platelet-dependent thrombin generation is elevated in smokers, even after abstaining from smoking for several hours (Hioki et al., 2001). Nicotine, however, which is the major psychoactive constituent of cigarette smoke, does not appear to be directly

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responsible for these changes (Blann, Steele, & McCollum, 1997; Ludviksdottir, Blondal, Franzon, Gudmundsson, & Sawe, 1999). Nicotine replacement therapies, such as nicotine gum and transdermal patches, deliver doses of nicotine comparable with those obtained through cigarette smoke but do not entail the inhalation of smoke. These therapies are reported not to influence platelets (Benowitz, Fitzgerald, Wilson, & Zhang, 1993; Wennmalm et al., 1991). Rubenstein et al. (2004) demonstrated that small concentrations of pure nicotine, in the absence of smoke, desensitize platelets to activation, under both static and dynamic flow conditions.

In the present study, we measured the thrombogenic properties of platelets exposed to mainstream and sidestream smoke extracts from low-nicotine and zero-nicotine cigarettes under flow conditions that approximately mimicked normal vascular blood flow, and we determined the effect of adding nicotine to the zero-nicotine smoke extracts.

Method

Smoke extracts

Three cigarette brands were used to prepare smoke extracts (tar and nicotine contents refer to mainstream smoke contents as determined by industry-standard smoking-machine protocols): (a) High nicotine (high tar)—Marlboro 100's (16 mg tar, 1.2 mg nicotine), Philip Morris, Richmond, Virginia; (b) low nicotine (medium tar)—Quest 1 (10 mg tar, .6 mg nicotine), Vector Tobacco, Timberlake, North Carolina; and (c) zero nicotine (medium tar)—Quest 3 (10 mg tar, $\leq$.05 mg nicotine), Vector Tobacco. None of these cigarettes has filter bypass ventilation holes of the type found in light cigarettes (Rubenstein et al., 2004).

Aqueous smoke extraction was carried out using a modification of Su, Han, Giraldo, De Li, and Block's (1998) method. In the mainstream extraction, all smoke passes through the cigarette and the filter; whereas in the sidestream extraction, smoke is collected only from the smoldering end of the cigarette, and none passes through the filter (Rubenstein et al., 2004). In addition to these extracts, one mainstream extract was prepared from zero-nicotine cigarettes from which the filters had been removed. A standard extract of each type was prepared by extracting the smoke from two cigarettes into 100 ml of Hepes-buffered saline pH 7.4 (depth $\sim$4.5 cm). In the case of the cigarettes with filters removed, mounting in the smoking apparatus prevented complete burning, and instead four half-cigarettes were burned to make an equivalent extract. To minimize the effects of variations in combustion and extraction, each standard extract was pooled from four separate extractions. Extracts were stored in 30-ml aliquots at -20°C.

(−)-Nicotine hydrogen tartrate (>98% by thin-layer chromatography) was obtained from Sigma-Aldrich and prepared as a stock solution of 1 mM in water. It was stored at -20°C and used at a final concentration of 50 nM.

Platelets

After we obtained informed consent from participants (approved by the Stony Brook University institutional review board), blood was drawn by venipuncture from healthy adult volunteers who had never smoked or who had quit smoking more than a year previously, and who had abstained from aspirin or ibuprofen for at least 2 weeks. Gel-filtered platelets were prepared as described by Jesty, Yin, Perrotta, and Bluestein (2003) and were used within 4 hr of gel filtration.

Measurement of platelet activation state

Platelet activation state (PAS) of timed samples was measured using a prothrombinase-based assay that measures acetylated thrombin generation from acetylated prothrombin by factor X-a (Jesty & Bluestein, 1999; Jesty et al., 2003). Activated platelets contributed two essential cofactors in this reaction: Negative phospholipids and factor Va. Hence, the rate of thrombin generation reflects the activation state and thrombogenic potential of platelets. To account for initial PAS variability in individual platelet preparations, which vary significantly in base activation state and maximum attainable activity, PAS data values from individual experiments were normalized to full activation of the same platelets with 5 μM calcium ionophore A23187 (Jesty & Bluestein, 1999; Jesty et al., 2003). Hence, PAS data are dimensionless with a maximum value of 1. PAS values reported by this assay have been shown to correlate with those reported by measurement of annexin V binding by flow cytometry (Jesty et al., 2003).

Circulation system

Calcium ions (final concentration, 3 mM) and smoke extracts (final concentration, 1% of the standard extracts—see above) were added to platelets (100,000/μl) 15 min before each circulation experiment. The platelets were then subjected to intermittent shear stress in a flow loop containing a 1-m section of PTFE .86-mm capillary tubing, as described previously (Jesty et al., 2003). Conditions were such that the shear stress in the capillary section was 12 dyne/cm^2, and the time of exposure to shear was 25% of the overall 21-min circulation time. Experiments were carried out at $37 \pm 2^{\circ}$C, and samples were taken every 3 min for PAS assay.

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Statistical analysis

The final result of each individual experiment is given as a platelet activation rate (PAR, min^{-1}), which is the rate of increase in normalized PAS value per unit time. PAR values for each individual experiment were determined by linear regression. Because platelets are subject to substantial daily variation in baseline PAS and susceptibility to shear, and because all platelets slowly activate between preparation and use, experimental comparisons were designed rigorously to minimize the effects of conditions. Statistical comparison of PAR values was restricted to paired datasets collected together on the same days and with the same platelet preparations. To account for the slowly rising base activation state over the course of a day, palindromic arrangements were used, as follows: Daily sets of pairwise comparisons between two conditions, e.g., A and B (Figures 1 and 2), were done in the order ABBABA in the first day's series, followed by the order BAABAB in the next daily series. For triple comparisons of conditions, e.g., A, B, and C (Figure 3), the daily order of experiments was ABCCBA throughout.

For each pairwise comparison, a total of six determinations of PAR was made for each condition, generating a mean PAR and standard deviation for each. These pairs were then analyzed by Student's paired *t*-test.

Results

Mainstream smoke extracts

A major purpose of the present study was to determine whether low-nicotine and zero-nicotine cigarette smoke extracts render platelets more susceptible to activation than extracts from ordinary (high-nicotine) cigarettes. Platelets were circulated for 21 min in the presence of smoke extracts at an intermittent shear stress of 12 dyne/cm^2, which roughly approximates normal shear exposure in the vasculature. Figure 1 shows the effect of exposing platelets to extracts of zero-nicotine, low-nicotine, and high-nicotine cigarettes. The results of pairwise comparisons (Table 1) demonstrate that platelet activation is inversely related to nicotine content;

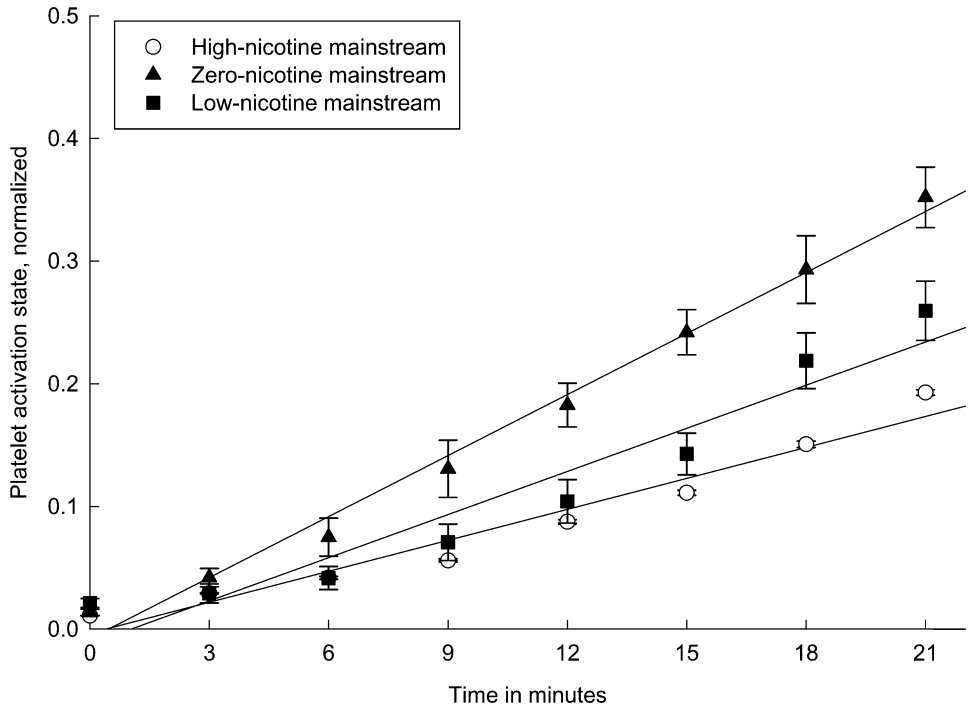


Figure 1. Platelet activation under intermittent shear stress of 12 dyne/cm^2 in the presence of mainstream smoke extracts of zero-nicotine, low-nicotine, and high-nicotine cigarettes. Platelets were circulated at 37°C for 21 min through a capillary loop. Samples were removed every 3 min for the determination of platelet activation state (PAS). Plots of PAS vs. time for each circulation run were fitted by linear regression to obtain a platelet activation rate (PAR). The data shown represent the sum of three separate pairwise experimental setups, organized as described in the Methods section. The pairs are as follows: (A) zero-nicotine ($n=6$) vs. high-nicotine ($n=6$), (B) zero-nicotine ($n=6$) vs. low-nicotine ($n=6$), and (C) low-nicotine ($n=6$) vs. high-nicotine ($n=6$). Statistical comparisons on the collected PAR values from each pair (6 vs. 6) were carried using Student's paired *t*-test (Table 1). Each condition appears in two pairwise comparisons, and for visual brevity, the points and lines shown represent the sum of all data (12 PAR values for each condition), even though each pairwise analysis only used the strictly paired data.

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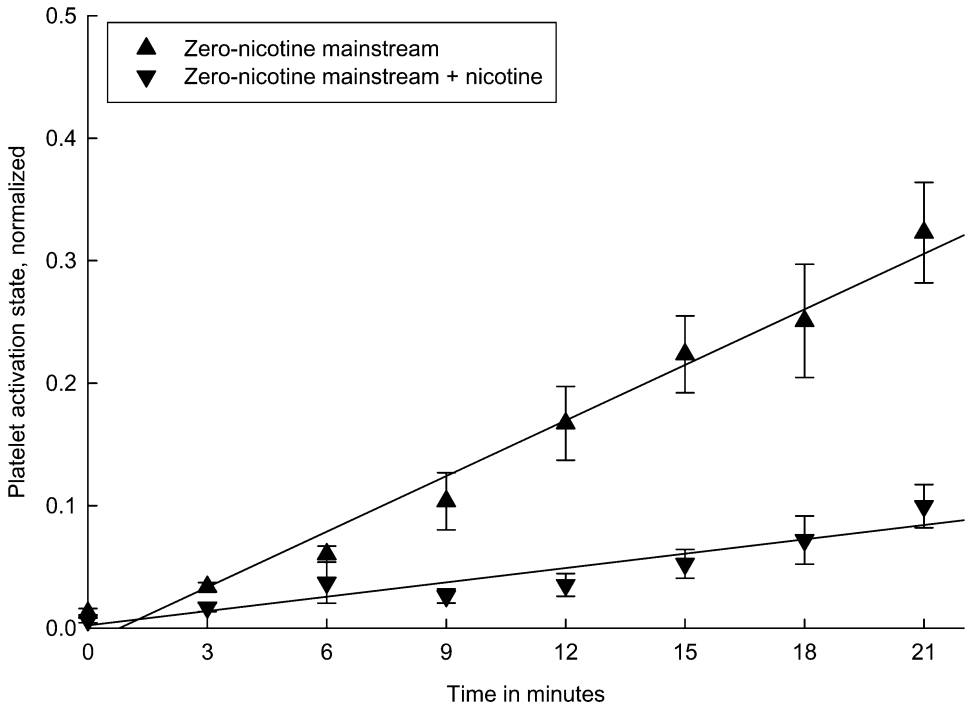


Figure 2. Effect on platelet activation of adding nicotine to the zero-nicotine mainstream smoke extract. Platelets were circulated, as described for Figure 1, in the presence of (A) zero-nicotine extract ($n=6$) and (B) zero-nicotine extract + 50 nM nicotine ($n=6$); and platelet activation rates (PAR) were determined, using a palindromic organization (see Methods section). Statistical comparison of the collected PAR values for the two conditions was carried out using Student's paired *t*-test ($p<.01$; Table 1).

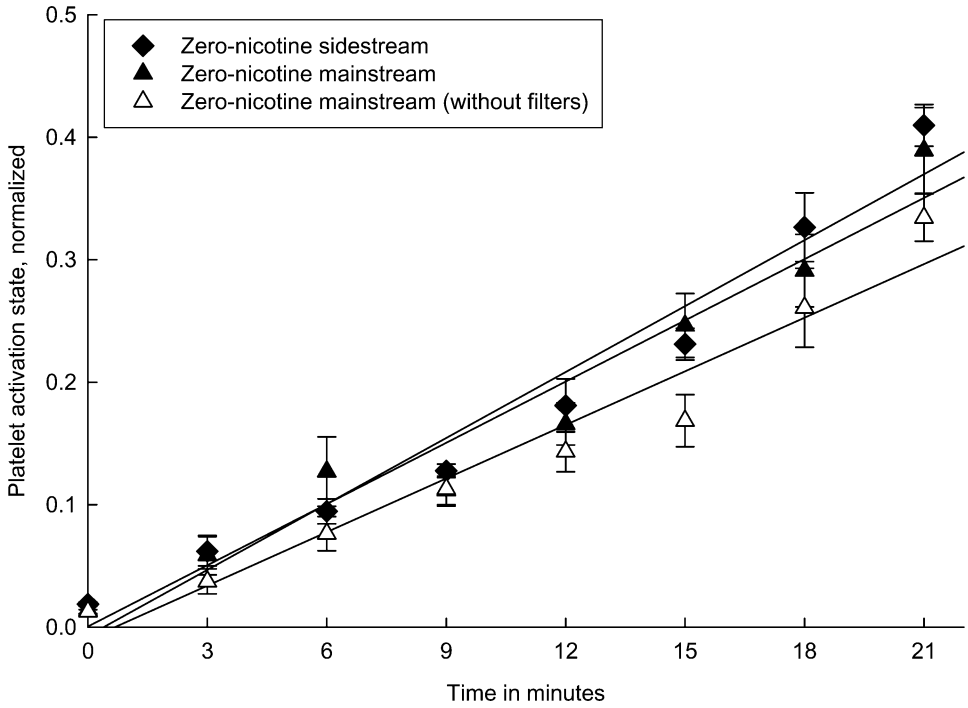


Figure 3. The effect of the zero-nicotine cigarette filter on platelet activation rate (PAR), and comparison with sidestream extract. Platelets were circulated, as described for Figure 1, under three extract conditions: (A) zero-nicotine mainstream, (B) zero-nicotine mainstream without cigarette filter, and (C) zero-nicotine sidestream. The relevant comparisons here are A vs. B and A vs. C. All three conditions were tested in each experimental set, in balanced order (see Methods section). Three such experimental sets provided six PAR estimates for each condition. Statistical pairwise comparisons were done using Student's paired *t*-test ($p>.2$ for both A vs. B and A vs. C; Table 1).

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Table 1. Mean platelet activation rates (PARs) under intermittent shear stress of 12 dyne/cm^2 in the presence of smoke extracts.

Extract	High-nicotine mainstream	Low-nicotine mainstream	Zero-nicotine mainstream	Zero-nicotine sidestream	Zero-nicotine mainstream + nicotine	Zero-nicotine mainstream without filters
PAR (min^{-1})	.0084 ($n=12$)	.0117 ($n=12$)	.0159 ($n=24$)	.0179 ($n=6$)	.0039 ($n=6$)	.0146 ($n=6$)
Zero-nicotine mainstream	$p=.005$	$p=.2$	—	$p>.2$	$p=.007$	$p>.2$
Low-nicotine mainstream	$p=.09$	—				

Probabilities (p) for the null hypothesis that activation rates were identical were determined from the individual experimental pair comparisons ($n=6+6$), as described in the Methods section. The platelet activation rates (PARs) are the combined means of PAR values for each extract condition in all its experimental pairings (Figures 1–3). Thus, for extract conditions that were measured in more than one experimental pairing, total $n>6$.

mainstream extracts of zero-nicotine cigarettes render platelets more susceptible to activation than do extracts from low-nicotine or high-nicotine cigarettes.

Nicotine. To confirm the direct inhibitory effect of nicotine in flow-induced platelet activation, we added 50 nM nicotine (the approximate plasma concentration in smokers immediately after smoking one light cigarette) to the mainstream smoke extract of zero-nicotine cigarettes. The effect was a highly significant reduction in the rate of platelet activation, of more than 75% ($p<.01$; Figure 2 and Table 1). At this nicotine concentration, the rate of platelet activation was significantly lower than with the smoke extract of high-nicotine cigarettes. The cause for this finding is unknown but likely reflects the aqueous extraction procedure producing fairly dilute extracts, when compared with the acute concentrations of smoke components that may be found in smokers' plasma.

Sidestream smoke extracts

Sidestream smoke, which issues directly from the tip of a burning cigarette, is by far the major constituent of secondhand smoke. Whereas the effects of mainstream smoke are relevant for smokers, the effects of secondhand smoke are critical for nonsmokers. Sidestream extracts were prepared from zero-nicotine cigarettes and examined in the same way as mainstream extracts under conditions of intermittent shear. The results demonstrate that for the cigarettes used here, which did not incorporate filter bypass ventilation holes of the type found in light cigarettes, sidestream and mainstream extracts were equally potent in their sensitization of platelets to shear stress (Figure 3).

We noted in preparing the extracts that the two Quest cigarette brands, in addition to being manufactured with zero-nicotine or low-nicotine tobacco, also incorporate carbon in their filters. Although the equivalence of mainstream and sidestream extracts suggested that these filters had no effect on the platelet-sensitizing potency of the smoke, we decided to address the question directly by preparing smoke extracts from zero-nicotine cigarettes from which the filters were removed. The rates of platelet activation in the presence of this extract were slightly lower than those seen with the regular mainstream and sidestream smoke extracts of these cigarettes, but the differences were not statistically significant (Figure 3 and Table 1). Although the filters of the zero-nicotine cigarettes might reduce slightly the levels of putative inhibitors of platelet activation (perhaps traces of nicotine still present in these cigarettes), the effect is not large.

Discussion

PAS

The modified prothrombinase-based assay has been used successfully in previous studies to determine the thrombogenic state of platelets without discriminating between the activation related to whole platelets and microparticles, which may be released upon activation or mechanical damage. PAS is therefore a global measure. The results of Rubenstein et al. (2004) suggest that microparticle generation is not a major confounding parameter, given that platelet loss in this circulation system is fairly small (approximately 11%).

Mainstream smoke extracts

We hypothesized that reduced-nicotine cigarettes might enhance thrombogenic risk by rendering platelets more susceptible to activation, compared with ordinary high-nicotine cigarettes. We tested this hypothesis by using the mainstream smoke extracts of zero-nicotine and low-nicotine cigarettes (Figures 1 and 2, Table 1). These were both Quest cigarettes, and they had identical tar ratings. Smoke extracts prepared from these cigarettes were more potent in sensitizing platelets to flow-induced activation than were extracts from even high-tar, high-nicotine cigarettes (we used the most common such cigarette, Marlboro red pack). Quest cigarettes are manufactured from genetically modified tobacco, in which the

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gene of an essential enzyme in nicotine biosynthesis, quinolinate phosphoribosyl transferase, is knocked out (Fogarty, 2003).

Although the experimental results are clear, they do not prove that the low nicotine content is the direct cause. However, there is no reason a priori to suspect that genetically modified tobacco is significantly different in other respects from ordinary tobacco, and this is borne out partly by the fact that the tar content of smoke from these cigarettes (10 mg tar in a standard machine-smoking protocol) is not a great deal less than that from ordinary high-tar cigarettes (approximately 15 mg). Nonetheless, the possibility that components other than nicotine are different in these cigarettes cannot be discounted. For example, higher-than-normal levels of a platelet-activating component in the genetically modified tobacco would produce the same results that we observed.

That possibility notwithstanding, the major platelet-desensitizing effect of nicotine remains clear (Figure 2). The addition of pure nicotine, at a level in the range of its plasma concentration after a smoker smokes just one light cigarette, reduced the rate of platelet activation by more than 75%. Notably, the PAR with added nicotine was less than that observed with the ordinary high-nicotine extract. In this regard, it is relevant that our aqueous extraction procedure involved only brief exposure of the smoke to the extraction buffer and was therefore likely quite inefficient compared with the extraction efficiency of a smoker's lungs.

Because low-nicotine cigarettes are a new product, no information exists about whether smokers might compensate for the lower nicotine content by increasing puff frequency and puff volume, as they do with the more common light cigarettes (U.S. Department of Health and Human Services, 2001). Should such cigarettes become popular, the question is an important one. In the present study, we used the same extraction procedure for the three cigarette types, the concentrations of smoke extracts were held constant, and zero-nicotine and low-nicotine cigarettes were still more thrombogenic to platelets. If smokers were to compensate for the nicotine reduction by increasing their smoke intake, the increased thrombogenic effects would be expected to be even greater. Although the increases in platelet thrombogenicity observed in vitro have not been proven to bear direct relation or relevance to the situation when people smoke or are exposed to secondhand smoke, the possibility should be kept in mind.

Sidestream smoke extracts

Secondhand smoke is mainly sidestream smoke from the smoldering end of a cigarette. Exposure to secondhand smoke remains a major worldwide health risk, despite major headway made in various cities, states, and countries. As expected for cigarettes that incorporate no filter bypass (Rubenstein et al., 2004), the platelet-activating properties of Quest variable-nicotine cigarettes did not differ significantly between mainstream and sidestream smoke at the same dose; thus, the highly potent nature of the zero-nicotine extract was essentially the same for both mainstream and sidestream extracts.

Summary

We assessed the thrombogenic activation of platelets subjected to mainstream and sidestream smoke extracts of cigarettes with varying nicotine content under flow conditions approximating those in the vasculature. Smoke extracts from low-nicotine and zero-nicotine cigarettes caused substantially greater activation of platelets than extracts from ordinary high-tar, high-nicotine cigarettes. In support of the conclusion that this is a direct result of nicotine reduction, the addition of a low concentration of nicotine to zero-nicotine smoke extracts was shown to substantially reduce the platelet-activating potential of the extracts. Although the in vitro experiments described here do not constitute definite proof, we conclude that even though low-nicotine and zero-nicotine cigarettes may be useful in weaning smokers from cigarettes, they also might cause a significantly increased risk of thrombotic disease in smokers and others exposed to the secondhand smoke from such cigarettes.

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In Vitro Model of Platelet-Endothelial Activation Due to Cigarette Smoke Under Cardiovascular Circulation Conditions

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Abstract—Cigarette smoke has been shown to increase platelet activation and endothelial cell (EC) adhesion molecule expression. In the present study, we utilized a hemodynamic shearing device (HSD) to investigate the above effects *in vitro* in a combined system of platelets and cultured HUVECs (Human Umblical Vein ECs) under physiological shear stress. We investigated the alteration of E-selectin expression on ECs upon exposure to: (1) platelets and nicotine-free smoke extract (NFE), (2) platelets alone, (3) NFE alone, under physiological shear stress. We additionally confirmed the protective effect of nicotine on platelet activation. We found that: (i) surface expression of E-selectin on ECs was significantly increased upon simultaneous exposure of ECs and platelets to NFE relative to exposure of ECs to either platelets or NFE alone ($p < 0.05$). (ii) Platelet activation was significantly increased in the presence of NFE ($p < 0.05$). (iii) Nicotine (200 nM) when added to NFE, significantly reduced platelet activation due to NFE ($p < 0.05$), an effect additionally confirmed by conventional cigarette extracts which contain nicotine ($p < 0.05$). We therefore conclude that: (a) NFE and platelets additively increase EC E-selectin surface expression, and (b) nicotine modulates platelet activation regardless of ECs.

Keywords—Cardiovascular diseases, Inflammation, Nicotine, Couette, Cone and plate, Viscometers.

INTRODUCTION

Cigarette smoking has been well established as a risk factor for development of cardiovascular disease.[1,9] A major part of this risk involves increased platelet activation due to cigarette smoke, as has been investigated both *in vivo*[18] and *in vitro*.[23] In two studies involving platelets alone, we have shown that this smoke-induced platelet activation is largely due to the non-nicotine smoke components and their effects can be modulated in the presence of nicotine.[22,23] While the direct desensitizing effect of nicotine on platelet activation is likely associated with reduced cardiovascular disease risk, such investigations need to be extended to a more physiologically relevant scenario to include the presence of the vasculature (endothelial cells (ECs), smooth muscle cells, and extracellular matrix). Additionally, this should be investigated under physiological shear stresses, as those play an important role in the effects of cigarette smoke and nicotine on platelets.

Prior *in vitro* studies have shown that high concentrations of cigarette smoke extract[6,28,29] and nicotine[32–34] increase adhesion molecule expression on ECs. These studies preclude the involvement of physiological shear stresses. Whether inflammatory effects due to smoke extract exposure may be attributed to its nicotine or nicotine-free components also remains unknown. Thrombin-activated platelets when introduced over cultured ECs bind to the cultured cells by several adhesion (receptor–ligand pairs) molecules[4,5,16,31] and may promote increase in EC adhesion molecule expression by downstream signaling. A link has been established between platelet factor-4 and endothelial E-selectin expression,[35] resulting from activation of nuclear factor-kappa B. The same mechanism has been reported for E-selectin expression on ECs due to cigarette smoke.[28] It is likely that if smoke extract and activated platelets both increase E-selectin expression on ECs by similar or distinct mechanisms, the levels of E-selectin surface expression on ECs may be significantly enhanced by their combined activity. Following such hypothesis, ECs simultaneously exposed to smoke extracts and smoke-activated platelets will be expected to increase EC adhesion molecule expression as a result of their combined activity.

The mechanical stress conditions (both fluid shear stress and cyclic strain) largely dictate the development of either pro- or anti-inflammatory phenotype of ECs.[7,12] Investigation of dynamic flow-dependent effects on cultured cells *in vitro* involves the use of devices that impart minimal inertial effects to the fluid

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and avoid re-circulation of fluid. The cone and plate viscometer and the annular Couette viscometer have been extensively used to study spatio-temporal effects of fluid shear stresses on cultured and circulating cells, respectively. Of note is the unique ability of the cone-plate viscometer to study both physiological and pathophysiological flow regimes within the same device,[8] while controlling any secondary flow effects.[27] If the two devices can be combined, both cultured cells and cell suspensions can be subjected to the same shear stress and the effects of interaction between them can be investigated by periodic sampling and subsequently analyzed.

In the present study, we utilize a hemodynamic shear device (HSD)[19] that combines features of the cone-plate with those of the annular Couette viscometer (cross-sectional view, Fig. 1a). The basic advantage of this device is that uniform and identical shear stress levels are maintained in both the cone-plate and Couette regions, and it facilitates convenient periodic cell sampling from the Couette region. We have recently used this device to study fluid shear-induced platelet activation under programmed shear stress.[19] In the present study, we test the hypothesis that platelets and the nicotine-free extract would confer an additive or enhanced inflammatory effect on ECs, by investigating the effects of cigarette smoke extract and platelets together on ECs under physiological shear stresses within the HSD. We additionally hypothesize that in contrast to conventional cigarette extracts, nicotine-free smoke extract would increase platelet activation more significantly, and that this effect may be independent of the presence of ECs.

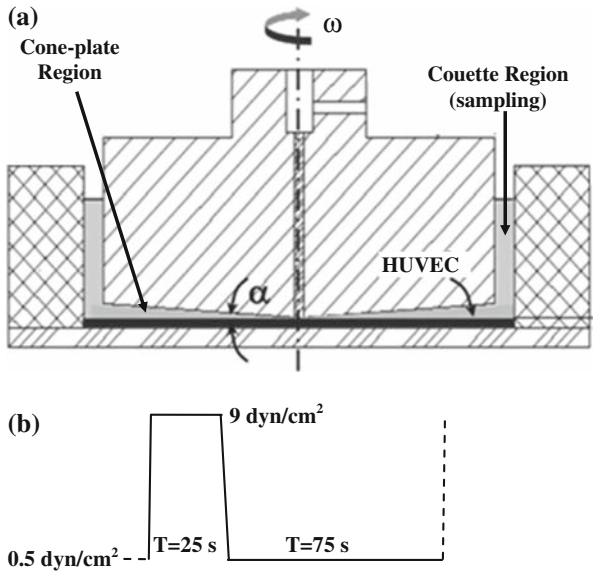


FIGURE 1. (a) (Adapted and modified from Nobili et al (2008) [19]) Cross-sectional view of the hemodynamic shear device (HSD). Platelets ± ECs are exposed to identical shear stress in the HSD. ECs are cultured on disks on top of the base-plate, platelets are sampled from the Couette region for PAS. The cone angle is represented by α, and rotational speed of the cone by ω. (b) Shear-stress waveform applied repeatedly for 33 cycles (100 s each) to the ECs and platelets in HSD.

METHODS

Reagents

Thrombin Receptor Activator Peptide-6 (TRAP-6), nicotine hydrogen tartrate salt, human fibronectin, EDTA, bovine serum albumin (BSA), phosphate buffered saline (PBS, pH 7.4), 10% paraformaldehyde (PFA, EM grade), and Trypsin-EDTA from Sigma (St. Louis, MO); FITC-conjugated mouse anti-human-E-selectin antibody from BD Biosciences (Franklin Lanes, NJ); Human Umbilical Vein Endothelial Cells (HUVECs) from Lonza (Walkersville, MD); Cigarettes—Quest 1 and 3 (Vector Tobacco, Mebane, NC) and Marlboro Red (Philip Morris, Richmond, VA) from a local pharmacy, glycol-modified polyethylene terepthalate (PETG) sheets from McMaster Carr (Robbinsville, NJ), Tumor Necrosis Factor-α (TNF-α; R&D Systems, Minneapolis, MN).

Smoke Extracts

Mainstream smoke extracts from two cigarettes of the same type were prepared in 100 mL of Hepes Buffered Saline (HBS, pH 7.4) as described previously,[23] with a small modification—the smoke was being bubbled through HBS in a vertical column (100 mL measuring cylinder) instead of a beaker used earlier, to facilitate a more efficient transport by increasing smoke-HBS contact time. The following three types of cigarettes corresponding to zero, medium, or high nicotine were used to prepare the three extract solutions: Quest-3 (nicotine-free extract or NFE, 10 mg tar, 0.05 mg nicotine), Quest-1 (medium-nicotine extract, 10 mg tar, 0.6 mg nicotine), Marlboro Red (high-nicotine extract, 16 mg tar, 1.2 mg nicotine). A final concentration of 10% extract was used in all studies.

Platelets

Thirty mL of citrated whole blood was obtained from adult human volunteers after informed consent. Platelet-rich plasma (PRP) was obtained after centrifugation at $300 \times g$ for 6 min, and loaded onto a Sepharose 2B-CL column to separate platelets from plasma proteins. Platelets at a concentration of 2×10^8/mL were eluted in citrated buffer as described previously[23] and used within 3 h of purification. The

order of experiments involving platelets from the same batch on a given day was randomly chosen.

Endothelial Cell Culture

HUVECs (passages 5–10) were cultured on human fibronectin (5 μg/mL)-coated PETG disks (diameter: 3 mm) until complete confluence was obtained as indicated by cobblestone morphology and absence of intracellular gaps. Functionality of HUVECs was confirmed by release of von-Willebrand factor (vWf) in culture and under shear stress. For this purpose, matched antibody-pair (primary and secondary, anti-human vWf) was purchased from Affinity Biologicals (Ontario, Canada) for analysis in an ELISA assay. HUVECs did not detach upon exposure to a constant shear stress of 9 dynes/cm^2 and remained confluent throughout the duration of experiments.

Hemodynamic Shear Device (HSD)

A combined annular Couette and cone-plate viscometer setup (Fig. 1a) was utilized to study the interaction between cultured HUVECs and circulating platelets under shear stress. HUVECs, cultured on PETG disks, occupy the base of the cone and the base of the annular Couette regions. The construction and characterization of this device has been described in our recent study.[19] The platelet suspension occupies the space (cone and annular Couette regions) above the HUVECs. The annular gap (740 μm) was determined to maintain identical shear stress levels under the cone and in the Couette regions. Sampling of platelets was performed from this annular Couette region. Capabilities of the cone-plate viscometer as a compact *in vitro* device to investigate a wide range of dynamic flow regimes experienced *in vivo* have been extensively discussed.[8,10] A repeated shear stress cycle of 100 s as indicated in Fig. 1b was used in the study, for a total of about 33 cycles or 54 min per experiment. All experiments were conducted within the HSD at the same shear stress. The objectives were to determine: (1) Expression of E-selectin on ECs upon exposure to platelets and nicotine-free smoke extract, and (2) Change in platelet activation upon addition of nicotine-free smoke extract and modulation of this effect by nicotine. TRAP-6 was used as a positive control in place of nicotine-free smoke extract, for objective 1. The parameters measured are further explained below, and summarized in Tables 1 and 2.

Platelet Activation State (PAS)

In experiments involving determination of platelet activation (Table 2), sampling was performed from the Couette region every 6 min and platelet activity (PAS or platelet activation state) was determined over the duration of the experiment (54 min), by a modified prothrombinase assay as described elsewhere.[13] This measure of platelet activation reflects primarily the release of factor Va from the α granules to the surface, plus the exposure of anionic phospholipid upon activation.[25] This method has been demonstrated to correlate well with platelet Annexin-5 expression,[14] as determined by flow cytometry. The results were normalized to maximum platelet activity (PAS determined after sonication of the platelet suspension for 10 W at 10 s) for the particular batch of platelets. Additionally, another parameter (Platelet Activation Rate or PAR) was determined to investigate whether PAS changes

TABLE 1. Summary of experiments for investigation of E-selectin expression on: (A) ECs with platelets and nicotine-free smoke extract and (B) ECs with platelets and TRAP-6.

Objective: Investigation of E-selectin expression on ECs by flow cytometry

Cells in HSD	Nicotine-free extract (10%)	Parameter
(A)		
EC	+[a]	E-selectin
EC	–[a]	E-selectin
EC + platelets	+	E-selectin
EC + platelets	–	E-selectin
Cells in HSD	**TRAP-6 (100 μM)**	**Parameter**
(B)		
EC	+[b]	E-selectin
EC	–[b]	E-selectin
EC + platelets	+	E-selectin
EC + platelets	–	E-selectin

[a]The signs (+/–) indicate the presence or absence of extract in the experiment.
[b]The signs (+/–) indicate the presence or absence of TRAP in the experiment.

TABLE 2. Determination of platelet activation state (PAS) for platelets exposed to the nicotine-free extract, two commercial cigarette extracts (Quest-1 and Marlboro), control and pure nicotine added to the nicotine-free extract.

Objective: Effect of nicotine on platelet activation state (PAS)

Cells in HSD	Type of smoke extract (10%)	Parameter
Platelets ± EC	NFE (Nicotine-free/Quest-3)	PAS, PAR
Platelets ± EC	NFE + 200 nM nicotine	PAS
Platelets ± EC	Medium-nicotine (Quest-1)	PAS
Platelets ± EC	High-nicotine (Marlboro)	PAS
Platelets ± EC	–	PAS, PAR

Platelet activity rate (PAR) was determined for selected conditions to exclusively determine the effect due to nicotine-free extract on platelets. Note that identical studies were performed for platelets alone and platelets + ECs, as indicated by ± sign.

more rapidly with time upon addition of nicotine-free extract.

E-Selectin Expression on ECs

At the end of studies outlined in Table 1, HUVECs were gently washed with pre-warmed (37 °C) PBS to remove any bound platelets, trypsinized, washed with FACS buffer (5 mM EDTA, 1% BSA, 4 °C, pH 7.4) and fixed with 4% PFA on ice. Prior to flow cytometry, the HUVEC samples were washed and resuspended (1×10^5/mL, FACS buffer) and incubated with saturating concentration (20 μg/mL) of mouse-anti-human-CD62E-FITC mAb for 45 min on ice. A total of 10,000 gated events were used for analysis on a BD FACScan flow cytometer. An IgG1-FITC isotype control was performed as a control to ensure binding specificity. Change in E-selectin expression for each of the four conditions in study (a) above was determined by fraction of total gated cells positive for E-selectin, relative to control. A total of eight experiments were conducted for each set of conditions (Table 1).

Statistical Analyses

All statistical analyses for multiple comparisons of PAS, PAR, and E-selectin expression on ECs, were performed with two-way or one-way ANOVA (SPSS Inc., Chicago, IL). Post-hoc tests to determine individual statistical differences within groups were conducted after Bonferroni's correction. A significance level of $p = 0.05$ was used for all analyses. Specifically, the following parameters across experimental groups were compared:

Platelet Activation State (PAS): The PAS values at times $t = 0$ min and $t = 54$ min obtained for all experimental conditions (Table 2) were averaged over total number of experiments ($n = 5$). Separate comparisons were made for PAS differences at the start ($t = 0$ min) and the end of the experiment ($t = 54$ min) for each set of conditions. We conducted a two-way ANOVA to investigate whether PAS was affected by (a) smoke extracts or nicotine, and (b) presence of ECs.

Platelet Activation Rate (PAR): To examine the effect of nicotine-free smoke extract on platelet activation with time, the PAS values obtained during the 54-min experiment for four conditions (Table 2) were fit to a two-degree polynomial curve. We additionally compared goodness of fit of this data between a 2-degree polynomial and a linear model.

E-Selectin Expression on ECs: E-selectin positive ECs were determined from cytometry for each condition in Table 1. The data obtained from cytometry corresponding to % positive gated cells for each condition fit better to a log-normal distribution (Shapiro-Wilk and Kolmogorov-Smirnov test for normality). The data was therefore log-transformed before multiple comparisons were made with one-way ANOVA separately for the two groups (Table 1A and 1B).

RESULTS

Platelet Activation State (PAS) Increases with Nicotine-Free Smoke Extract

We investigated platelet activation (PAS) in the presence of 10% nicotine-free smoke extract (NFE) and shear stress, both for platelets alone (Fig. 2a) and platelets with ECs (Fig. 2b). The data for each plot represents an average of five experiments. The corresponding results for the average PAS have been reported in Table 3. We found that platelet activation (PAS) was significantly increased upon addition of the nicotine-free extract ($p = 0.001$).

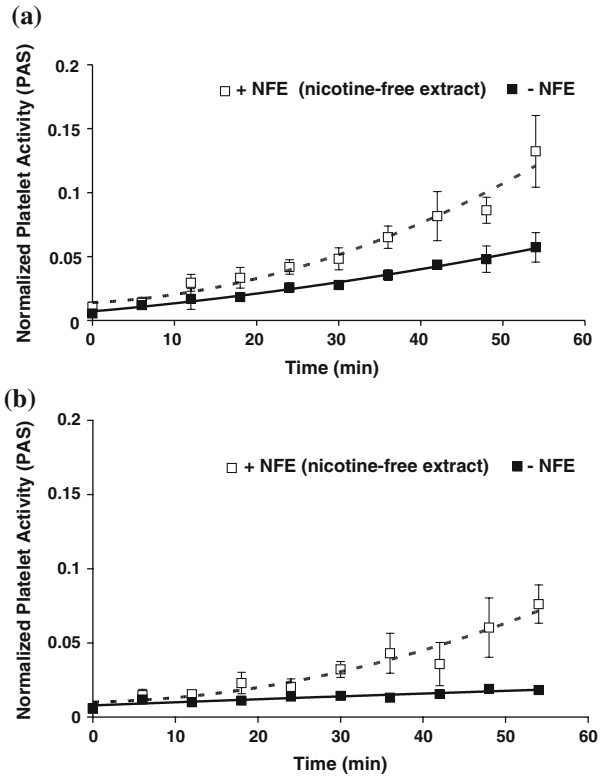


FIGURE 2. Platelet activation state (PAS) over 54 min in the HSD for (a) platelets (±smoke) and (b) platelets + ECs (± smoke). Note 10% nicotine-free smoke extract (NFE) was used. The data are fit to a 2-degree polynomial curve and the average platelet activation rates (PAR) are illustrated in Table 3.

TABLE 3. Summary of platelet activation (PAS and PAR, mean ± s.e.m.) upon exposure to nicotine-free smoke extract.

Condition	PAS at $T = 54$ min	PAR (1×10^{-5} min^{-1})
Platelets	0.06 ± 0.01	1.00 ± 0.60
Platelets + NFE	0.13 ± 0.03	3.02 ± 1.00
Platelets, ECs	0.02 ± 0.00	0.02 ± 0.15
Platelets, ECs + NFE	0.08 ± 0.01	3.00 ± 1.00

All experiments were done under the same shear stress in the HSD.

Platelet Activation Rate (PAR) Increases with Nicotine-Free Smoke Extract

We found that the 2-degree polynomial model was a better overall fit for our PAS data in 3 out of 4 cases shown in Fig. 2 and reported above: $p = 0.01$, 0.02 (platelets ± NFE) and $p = 0.87$, 0.04 (platelets + ECs ± NFE). To maintain consistency, we determined the PAR corresponding to the curvature or quadratic coefficient for the fit for each condition, prior to statistical test with two-way ANOVA. We found that PAR increased significantly due to the presence of nicotine-free smoke extract ($p = 0.006$). The average PAR values have been reported in Table 3.

Control Experiments for Initial Platelet Activation State (PAS at $t = 0$ h)

To ensure that purified platelets did not activate significantly over the 3 h time course of use each day, we performed control experiments and measured the platelet activity overtime with the modified prothrombinase assay. We found that during this time course platelets activated less than 1% when left on the shaker. The mean (±s.e.m.) PAS for this control experiment (repeated 3 times with different platelet batches) at times $T = 0$, 1, 2, 3 h was found to be 0.0051 ± 0.0016, 0.0051 ± 0.0007, 0.0099 ± 0.0021, and 0.0071 ± 0.0009, respectively. Additionally, we compared the PAS data at the beginning of all our experiments as outlined in Table 2 (i.e., PAS at $t = 0$ min) and found that the initial level of platelet activation did not differ between different experimental groups and conditions ($p > 0.05$) and was less than 1% as found in control experiments above. The mean (±s.e.m.) starting PAS ($t = 0$ min) was calculated as 0.0082 ± 0.0008.

E-Selectin Expression on ECs

Three representative cytometry histograms for expression of E-selectin on ECs, when exposed to (i) shear stress alone (dotted line), (ii) shear stress and platelets (solid line), (iii) shear stress and NFE (dashed-dotted line), and (iv) shear stress and NFE and platelets (thick solid line), are shown in Fig 3a. In all cases, the shift in fluorescence intensity histogram is distinct upon simultaneous exposure of ECs to platelets and smoke (condition iv, thick solid line). The average increase in E-selectin expression for each of these conditions is illustrated in Fig. 3c. The data is an average of eight individual experiments, and mean ± s.e.m values for % positive gated cells are shown. This was statistically confirmed by ANOVA over the log-normalized data for the four conditions mentioned above. The pair-wise differences were then determined after post-hoc Bonferroni's correction after significance was established with ANOVA ($p = 0.001$), and the results are illustrated in Table 4. E-selectin expression obtained upon concurrent exposure of ECs to the nicotine-free extract and platelets, was significantly different from all other conditions and proves the hypothesis of current study.

To confirm this additive effect, another agonist (TRAP-6) which is known to maximally activate P-selectin expression on platelets,[11] was used in place of NFE and the above study was repeated. In contrast with smoke-activated platelets that had a final PAS of 6%, TRAP-6 activated platelets had a PAS of over 90% for all experiments. It should be noted that despite the high PAS, in the absence of fibrinogen there was no platelet aggregation in these studies (confirmed by flow cytometry to P-selectin). An identically statistically significant effect was observed with TRAP-6 ($p < 0.05$, statistical data not shown) as reported above for NFE. As above, three representative cytometry histograms for the expression of E-selectin on ECs when NFE is replaced with TRAP-6 are shown in Fig. 3b. The shift in fluorescence intensity when ECs are concurrently exposed to platelets and TRAP-6 is distinctly identified from individual exposure conditions (Fig. 3b). The average results for % E-selectin positive gated ECs (mean ± s.e.m.) from eight experiments are illustrated in Fig. 3c. Similar statistical results confirming the additive effect were found as in the case of nicotine-free extracts. This further supports the above conclusion that expression of E-selectin on ECs upon concurrent exposure of platelets in combination with the agonist that activates them was significantly increased relative to E-selectin expression obtained upon individual exposure of ECs to either platelets or agonists.

Conventional Cigarette Extracts and PAS

Extracts were prepared from two commercially available cigarettes (that contain nicotine): (1) Marlboro Red (1.2 mg nicotine); and (2) Quest-1 (0.6 mg nicotine) as outlined in the methods. Addition of these

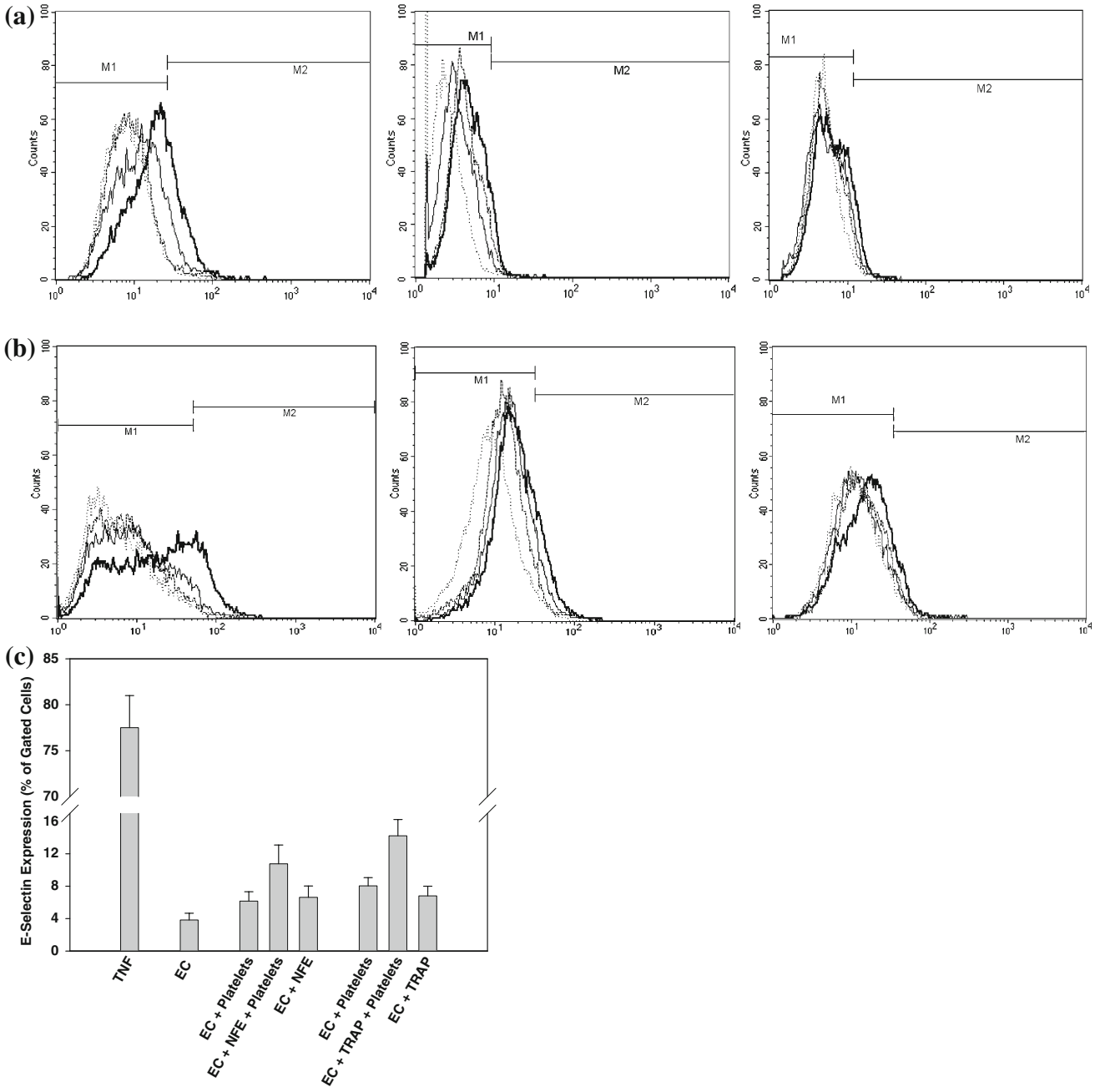


FIGURE 3. (a) Three typical cytometry histograms for E-selectin expression on ECs subject to conditions outlined in Table 1A: (i) shear stress alone (dotted line), (ii) shear stress and platelets (solid line), (iii) shear stress and NFE (dashed-dotted line), and (iv) shear stress and NFE and platelets (thick solid line). (b) Three typical cytometry histograms for E-selectin expression on ECs subject to conditions outlined in Table 1B: (i) shear stress alone (dotted line), (ii) shear stress and platelets (solid line), (iii) shear stress and TRAP-6 (dashed-dotted line), and (iv) shear stress and TRAP-6 and platelets (thick solid line). (c) Summary of % E-selectin positive gated ECs (mean ± s.e.m.) determined by flow cytometry. All experiments (n = 8) were done under shear stress in HSD as per conditions on horizontal axis, except TNF-α control. Smoke (10% NFE) and TRAP-6 (100 μM final) were used where stated.

extracts (10%) in place of the NFE, reduced platelet activation (PAS) relative to the latter ($p = 0.02$, Fig. 4). PAS was different in the presence of ECs (Fig. 4), but this difference was not influenced by the smoke extracts or nicotine ($p = 0.8$). The pair-wise differences between PAS for each condition (Table 2) were ascertained by post-hoc tests and the results are illustrated in Table 5. Despite the higher tar content of Marlboro cigarettes (16 mg) relative to the Quest cigarettes (10 mg), platelet activation was significantly

TABLE 4. Statistical summary for the expression of E-selectin on ECs.

Conditions	EC	EC + NFE	EC + platelets	EC + platelets + NFE
EC	–	1.00	1.00	0.01*
EC + NFE	1.00	–	1.00	0.03*
EC + platelets	1.00	1.00	–	0.01*
EC + platelets + NFE	0.01*	0.03*	0.01*	–

p-values from post-hoc pair-wise statistical tests for E-selectin expression in the presence of nicotine-free smoke extract, corresponding to conditions in Table 1A. Note that (*) indicates statistical difference.

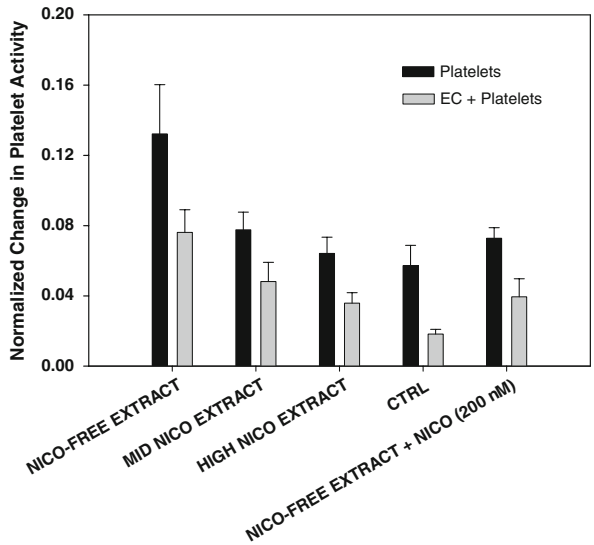


FIGURE 4. PAS for platelets ± ECs, in the HSD for (*left to right*): (1) nicotine-free (10% NFE), (2) medium-nicotine (10%), (3) high-nicotine (10%) extracts, (4) control (no smoke, no nicotine), and (5) condition (1) with pure nicotine (200 nM) added. Data (mean ± s.e.m.) in each bar is representative of five experiments.

reduced by the 2-fold higher nicotine content in the former ($p = 0.001$). Specifically, the PAS measured with Marlboro extract was less than that measured with the NFE by 48% (platelets only) and 47% (platelets + ECs). The PAS measured with the Quest-1 extract was also significantly lower compared to the NFE extract ($p = 0.03$). These findings clearly support the role of nicotine as a key modulator of platelet activation in smokers, at least within the short experimental durations as in the current study.

Further, when 200 nM nicotine was added in addition to 10% NFE, the resulting PAS decreased by 45% (platelets only) and 49% (platelets + ECs) relative to activation in the presence of NFE as reported above ($p = 0.004$). This confirms our earlier findings that nicotine is a key modulator of smoke-induced platelet activation.

DISCUSSION

Hemodynamic Shear Device

Platelet activation and aggregation at elevated shear stresses has been successfully investigated previously in the cone-plate viscometer.[15,30] The interaction between platelets and ECs has also been investigated in parallel-plate flow chambers[17] and also the cone-plate viscometer.[20] Equally well described is the annular Couette viscometer with a narrow annular gap and inner rotating cylinder, which has been used to investigate shear-induced blood damage and hemolysis of red blood cells.[21] The HSD design incorporates features of these two viscometers to maintain the same shear stress and facilitate sampling, as discussed under methods and in our recently published study.[19] In the present study, we used an intermittent shear stress of 9 dynes/cm^2 which is representative of flow in a normal coronary artery. Under these conditions, the flow is laminar ($\tilde{Re} < 0.5$) and secondary flow effects in the cone region therefore may not impose significant

TABLE 5. Statistical summary for the effect of nicotine on platelet activation (PAS at *T* = 54 min).

Conditions	Nicotine-free	Medium-nicotine	High-nicotine	Control	NFE + 200 nM nicotine
Nicotine-free	–	0.03*	0.00*	0.00*	0.00*
Medium-nicotine	0.03*	–	1.00	0.36	1.00
High-nicotine	0.00*	1.00	–	1.00	1.00
Control	0.00*	0.36	1.00	–	1.00
NFE + 200 nM nicotine	0.00*	1.00	1.00	1.00	–

The experimental conditions are described in Table 2. The table shows *p* values, determined from post-hoc pair-wise tests after significant difference for effect of experimental condition on PAS was established with two-way ANOVA ($p = 0.02$). Note that (*) indicates statistical difference.

localized forces and edge effects in the present system.[27] The present system can therefore be utilized to investigate EC-platelet or EC-leukocyte interactions under physiological or pathophysiological shear stresses.

E-Selectin Expression on ECs

We show that nicotine-free smoke extract or TRAP-6, when introduced together with platelets over ECs, result in a relatively greater increase in E-selectin expression on ECs. Although the mechanisms are unknown, it is likely that both smoke extract (or TRAP-6) and platelets contribute to the enhanced expression: (1) Downstream signaling resulting from engagement of receptor–ligand pairs on apposite cells may increase E-selectin expression on ECs. For instance, the presence of endothelial vWf in the cell suspension may act as bridging molecule between platelets and ECs, and this could be supplemented with integrin-like interactions between adhesion receptors on both cell types.[4] (2) ECs have been previously shown to increase E-selectin expression in response to smoke extracts[6] and also by TRAP-6 binding receptors on the surface.[26] We did not get significantly different E-selectin expression with platelets or nicotine-free extract introduced alone over ECs relative to ECs exposed to shear stress (control). However upon simultaneous introduction of platelets and nicotine-free extract on ECs, the E-selectin expression obtained is significantly different from the other three conditions (Table 4). We obtained similar results with TRAP-6 (in place of NFE), thus confirming the above mechanism of additive E-selectin expression.

Peak E-selectin expression on ECs in the presence of pro-inflammatory cytokines such as TNF-α and IL-1-β typically occurs at 4–6 h. The duration of our experiments was 54 min, after which ECs were immediately fixed. This may explain the lower E-selectin expression rates after 54 min in our experiments, but is comparable to levels found after TNF-α treatment beyond 2 h.[3] Such levels may be sufficient to induce leukocyte adhesion and progression of inflammation. The time limitation in our study arises from maintaining identical experimental conditions with the same passage of ECs and batch of platelets for one set of results (Table 1, four conditions) involving four experiments of 54 min each, done on the same day. Nevertheless, a relatively smaller but significant increase in E-selectin is observed and confirms our hypothesis. We may therefore conclude that such expression of E-selectin observed when activated platelets and agonists are introduced together over ECs, may represent the initial inflammatory response similar to the one induced by cytokines.

Modulation of PAS by Nicotine

The addition of pure nicotine to nicotine-free smoke extract has been previously shown to reduce platelet activation due to smoke extracts and shear stress.[22,23] We confirmed this protective effect of nicotine in our present study, albeit in the presence of ECs (Fig. 4). ECs have been known to express a subset (alpha-7) of cholinergic receptors on the surface, which can bind nicotine.[24] A recent *in vivo* study has demonstrated that nicotine may confer immunosuppressive effects on ECs by this mechanism.[24] Possible modulatory effects of nicotine on ECs (as mentioned above) may influence the differences observed with respect to reduction in PAS relative to nicotine-free extract (Fig. 4), however longer experiments are needed to investigate such effects. Platelet activation (PAS) was not statistically significant in the presence of medium- and high-nicotine extract relative to control ($p = 0.36$ and $p = 1.00$, respectively, Table 5). This shows that the presence of nicotine in the cigarette at concentrations above 200 nM may compensate for the harmful effects of non-nicotine cigarette components on platelet activation. Additionally, our conclusions regarding the protective effects of nicotine on platelet activation are well supported by our recent study with adult smokers whereby smoking zero-nicotine cigarettes caused a significantly higher (~3-fold) increase in platelet activation, than smoking medium-nicotine cigarettes.[11]

It should, however, be noted that the conclusion holds only for the short experimental duration of the study. Longer experiments with purified platelets are not feasible and the effects of nicotine and cigarette smoke extract over extended periods can only be investigated in animal models (*in vivo*). An alternative approach that involves pre-treatment of ECs with nicotine and cigarette smoke extract prior to exposure with platelets, under physiological conditions, will be investigated in the future.

PAS in Presence of ECs

Endothelial cells protect platelets from shear-induced activation by a variety of mechanisms.[2] Such mechanisms may be initiated by mechanotransduction under shear stresses, and may be influenced by the topography and the net-forward magnitude of the stresses.[7] Our shear stress condition is laminar and atheroprone effects associated with disturbed flow conferring an inflammatory phenotype to ECs may therefore not be expected.[7,12] Given the short duration and lower shear stress conditions in our study, antithrombotic effects due to release of NO and prostacyclins may be significant only during the acceleration and peak shear stress phase (~25% experimental time or 14 min). We believe that the difference in PAS arise

due to a combination of the above factor and the fact that ECs may form a passivating layer for the platelets, which may be less thrombogenic than the material surfaces of our device. As observed in Fig. 4, PAS was different in the presence of ECs, however this difference was not influenced by nicotine or smoke extracts. This is indicated by the lack of significance of the effect of interaction between group (platelets, ECs and platelets) and experimental conditions (i.e., nicotine and smoke) in two-way ANOVA ($p > 0.05$).

Our primary focus here is to demonstrate the pro-inflammatory effects of nicotine-free smoke on platelets in the presence of ECs, thereby bringing the system close to physiology. We clearly show that PAS increases significantly in the presence of the nicotine-free extract, relative to control (Fig. 2). Additionally, we observe that the rate of increase of PAS in the presence of such extracts (i.e., PAR) is non-linear. This fact corroborates our earlier findings of non-linear increases in PAS due to platelet-platelet co-operativity at physiological (2×10^8/mL) concentrations. We attributed such increase to the enhanced Factor Va release by activated platelets. In the present study, we observed that the PAR was statistically more non-linear (Figs. 2a and 2b) when smoke extract was added to the platelets (irrespective of the presence of ECs). Such non-linearity may be enhanced by external agonists such as cigarette smoke which may induce much higher Factor Va release by platelets.

In summary, we show that the HSD is a compact *in vitro* device to study the interaction between circulating and cultured cells under physiological shear stresses. More importantly, we also show that E-selectin expression on ECs is enhanced significantly only upon simultaneous exposure to platelets and nicotine-free cigarette smoke extract. We additionally confirm that nicotine decreases platelet activation due to cigarette smoke, in the presence of ECs. In this *in vitro* study this effect of nicotine was studied within a combined system of ECs and platelets, which brings this study closer to a physiologically relevant scenario. Such a system may therefore be used to develop precursor models to study inflammation *in vitro*. More studies that predispose the ECs to an inflammatory phenotype, before platelets or agonists are added are clearly needed.

In conclusion, our study supports the hypothesis that cigarette smoke is likely associated with increased cardiovascular disease risk, and this may be effectively modulated by nicotine.

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