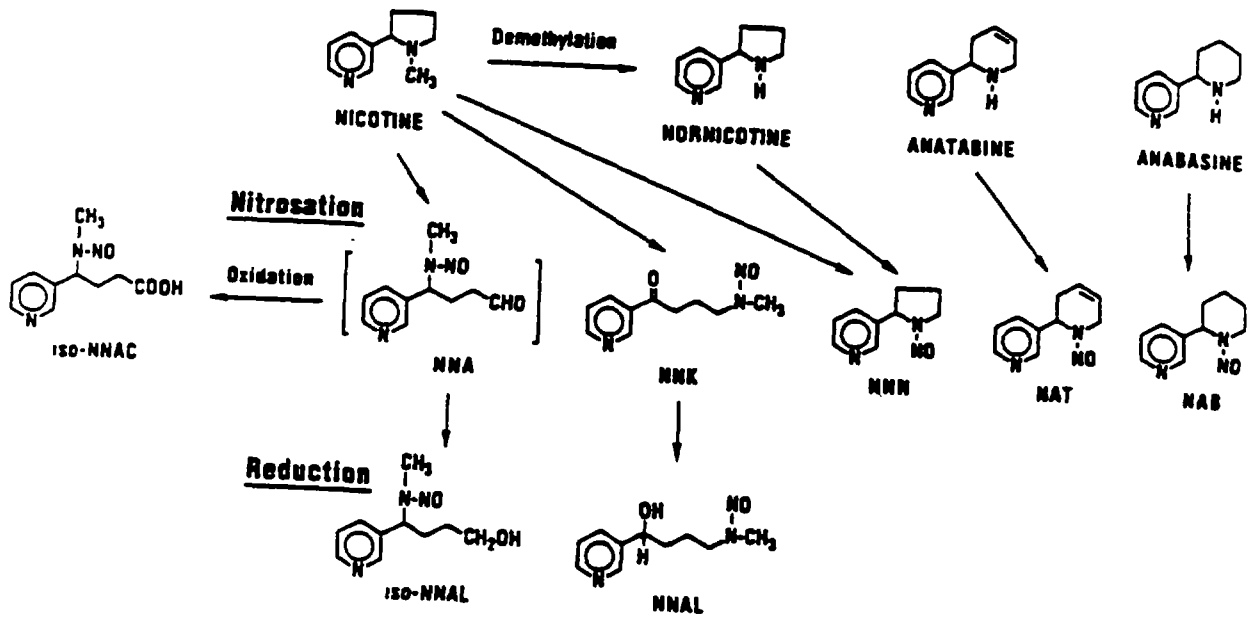
Figure 7 Formation of Tobacco-Specific N-Nitrosamines (20)



Glossary of Terms

carcinogen	Substance that results in the production of tumors
ciliatoxic agent	Substance that inhibits the movement of cilia in the mucus-secreting respiratory epithelium. Total inhibition of such movement ciliastasis) prevents lung clearance and leads to accumulation of foreign substances in the respiratory airways.
cocarcinogen	Not a carcinogen by itself but potentiates the activity of a carcinogen when co-administered.
complete carcinogen	A carcinogen with both tumor initiating and tumor promoting activity.
draw resistance	Pressure developed by the full length of a cigarette when air is drawn through at a rate of 17.5 ml/sec (20^0C, 760 torr). Expressed as inches (or mm) of water column.
flavor additives	Plant extracts or synthesized chemicals that are added to the tobacco to impart flavor to the smoke.
mainstream smoke	For analytical purposes and for collecting the smoke in a closed system, mainstream is the smoke issuing from the mouth end of a cigarette. For the smoker, mainstream is the smoke that is drawn from the mouth end of a cigarette during puffing.
nonvolatile smoke constituents	All particulate phase constituents.
organ-specific carcinogen	Exerts carcinogenic activity in host tissues that have appropriate

	activating enzymes; acts on specific organs regardless of the route of application.
overwrap	Tipping paper, opaque-white paper, or cork wrapped around the filter and the cigarette rod to join both.
perforation	Tiny holes in cigarette paper or filter wrapper increases air permeability. Can be made by mechanical or electrostatic methods or by laser beams.
redox potential	Potential for electrobiochemical reduction/oxidation.
sidestream smoke	For analytical purposes, sidestream is that part of the smoke of a cigarette that emits from the burning end during puff intervals and that diffuses through the paper. In free-smoking situations, some sidestream smoke exits from the mouthpiece during puff intervals.
total particulate matter (TPM)	Fraction of smoke collected on a Cambridge (glass fiber) filter in machine smoking.
TPM dry	Total particulate matter minus water and minus nicotine.
toxic agent	Adversely pharmacoactive substance, for example, nicotine.
tumorigenic agent	Substance known to elicit neoplasms in animal assays.
tumor initiator	A substance or an active metabolite that forms chemical lesions with DNA having potential to develop into benign and malignant neoplasms.
tumor promoter	An agent that facilitates tumor development in an initiated cell when applied subsequent to initiation.

vapor phase (gas phase)	The fraction of tobacco smoke that passes through a Cambridge filter. Contains some condensable smoke particulates that have not been retained on the glass fiber filter.
volatile smoke constituent	Chemical having more than 50% in the vapor phase.

Estimated Costs For the Analysis of Individual Cigarette Smoke Components[1,2]

Smoke Component	Estimated Cost
Total Particulate Matter, dry	$350
pH	$250
Redox Potential	$500
Carbon Monoxide	$250
Nitrogen Oxides	$400
Hydrogen Cyanide	$350
Volatile Hydrocarbons (esp. 1,3-Butadiene, Isoprene, Benzene, Toluene)	$600
Volatile Aldehydes	$700
Volatile N-Nitrosamines	$800
Nicotine (by GC)	$250
Phenols	$500
Catechols	$350
Polynuclar Aromatic Hydrocarbons (GC)	$1,500
Benzo(a)pyrene only	$500
Tobacco-Specific N-Nitrosamines	$800

[1] These estimates pertain to the direct cost of each determination (duplicate analyses) for one brand or prototype of cigarette. They exclude overhead as approved for the individual institute by the U.S. Dept. of Health and Human Services.

[2] For practical purposes, we recommend that analytical profiles of the smoke of cigarettes are done for at least two different brands or prototypes and one reference cigarette at any time.

Chapter E

SHORT-TERM TESTS FOR THE EVALUATION OF CIGARETTE SMOKE TOXICITY

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Chapter Contents

1. Introduction:

Several epidemiological and experimental studies have implicated cigarette smoking with the increased incidence of a variety of human diseases. Major health consequences of smoking have been discussed in Chapter A. Also the physicochemical complexity of tobacco smoke and its major toxic constituents have been described in Chapter D. The main aim of this Chapter is to describe a testing strategy for comparing the toxicity of low ignition propensity cigarette prototypes using short-term tests.

Cigarette smoke is a complex aerosol which is composed of gaseous and particulate phases and contains thousands of different types of compounds. Any alteration in cigarette design and/or composition is likely to alter the physical and chemical characteristics of smoke and thereby its potential to cause toxicity. Some examples of such alterations have been described in Chapter A. To provide a comparison of toxicity of different cigarette prototypes, it is important that the tests selected for their toxicological evaluation are quantitative and have relevance to smoking-related health consequences described in Chapter A.

During the past several years, a number of short-term tests have been developed for routine assessment of the toxicity of chemicals. The main focus of these tests has been the identification of potential carcinogens in the environment. As a result of such testing, large databases of chemical genotoxicity and carcinogenicity have been developed by the National Toxicity Program, US Environmental Protection Agency, and IPCS (Parodi and Waters 1991). Analyses of these data have demonstrated a relationship between genotoxicity and carcinogenicity of chemicals (Huff and Haseman 1991, Tennant 1991, Parodi et al. 1991).

Currently, there are several genotoxicity tests that are used for identification of chemical carcinogens. These tests can be generally classified into four categories;tests for detecting (i) gene mutations, (ii) chromosomal aberrations, (iii) primary DNA damage, and (iv) induction of mammalian cell transformation (Brusick 1987). Many of the tests from these four categories have been applied to genotoxic assessment of cigarette smoke and its condensates. These tests demonstrate that tobacco smoke possesses cytotoxic, mutagenic and carcinogenic activities (DeMarini 1983, Hoffmann et al. 1987).

A plan for the evaluation of toxicity of low ignition potential cigarettes is presented. Two types of tests are proposed. The prokaryotic Ames' Salmonella mutagenicity assay and a eukaryotic mammalian cell transformation assay are included to assess the genotoxic potential of the test cigarette smoke condensates. An inflammatory lung cell response bioassay is

proposed to assess the general pulmonary toxicity of the whole smoke from cigarette prototypes. These three tests should be included in a reasonable evaluation of test cigarette smoke toxicity.

2. General Considerations:

There are three types of smoke preparations that are used for in vitro testing of cigarette smoke: i) condensate collected by freezing smoke in a cold trap, ii) smoke particulates collected at room temperature on Cambridge filters, and iii) freshly generated whole smoke or its gas phase. The first two preparations have been used quite often as a solution/suspension in DMSO or acetone for testing in various short-term tests (DeMarini 1983). The use of fresh whole smoke for in vitro testing has been limited.

Since most biological assays have baseline noise,it is important that the toxicity of test cigarettes be compared to known reference compounds and cigarettes that serve as positive control. Compounds like benzo(a)pyrene or 3-methylcholanthrene and the University of Kentucky reference research cigarettes are included in each experiment to serve as references for comparison of test cigarettes. In our hands, the condensates from the University of Kentucky reference cigarettes have given fairly consistent response in the Ames' Salmonella assay and has allowed identification of condensates of low and high mutagenic activity (Gairola 1979).

There are several research cigarettes of different tar and nicotine delivery that are available from the University of Kentucky Tobacco and Health Research Institute (Davis et al. 1984). These cigarettes were developed to minimize the experimental variability introduced by the use of commercial cigarettes in analytical and biological studies of smoke (Benner 1970) and have been used worldwide for experimental studies of tobacco smoke. Two newer research cigarettes, designated 1R4F and 1R5F, were manufactured in the 1980s and contain 0.8 and 0.16 mg nicotine/cigarette, respectively. These two cigarettes probably represent low and ultralow nicotine cigarettes currently sold on the market.

Standard reference materials for complex environmental mixtures developed by the National Institute of Standards and Technology (May et al. 1992) may also be useful as reference materials, but have not been tested in conjunction with tobacco smoke studies.

3. Recommended Tests:

The Salmonella mutagenicity assay and a mammalian cell transformation assay (C3H/10T1/2 or BALB/3T3) are proposed for routine testing of genotoxicity of smoke condensates. To evaluate general pulmonary toxicity of whole smoke, a lung inflammatory cell response assay in mice has been proposed. Gene mutation and cell transformation assays are selected because of the high relevance of these two genotoxic endpoints in the overall carcinogenic process and tobacco smoke carcinogenesis in particular, which is known to involve the action of both tumor initiators and tumor promoters (Hoffmann and Wynder 1971, Melikian et al. 1989).

The Ames Salmonella assay is a gene mutation assay which quantitatively measures the ability of the test condensates to induce specific point mutations in a prokaryotic bacterial system. Since mutational events are generally believed to be essential for the initiation phase of carcinogenesis, this test may provide an estimation of the potential tumor initiators in tobacco smoke preparations.

Mammalian cell transformation assays use eukaryotic mammalian cell culture systems to measure the potential of smoke to induce malignant cell transformation. Cell transformation is a multistep process and is considered a close approximation of events occurring during in vivo oncogenesis. This assay may, therefore, measure the activity of both tumor initiators and promoters. Past studies have shown that cigarette smoke condensates give a positive response in both of these tests (Kier et al.1974, Benedict et al. 1975). A good dose response relationship between the amount of test preparation and genotoxic response is generally observed in at least the Salmonella assay.

Selection of the bronchoalveolar lavage (BAL) cell response assay for pulmonary toxicity is based on the observation that chronic inflammatory conditions exist in the lungs of smokers and the BAL cells obtained from smokers exhibit altered characterstics which play an important role in the development of chronic obstructive pulmonary diseases associated with cigarette smoking (Hunninghake et al. 1979, Niewoehner 1988). Studies in the animal models have also demonstrated several alterations in the lungs of mice that resemble those reported in human smokers (Matulionis 1984, Gairola 1986).
This test, however, has never been employed to differentiate the toxicity of different types of cigarettes and may, therefore, need developmental work before routine use in toxicity evaluation.

3.a. Tests for Evaluating Genotoxicity of Smoke Condensates:

The Ames Salmonella assay can be performed on dimethylsulfoxide solutions of freshly collected particulates from 1-5 test cigarettes. The particulates are collected on 0.3

μm Cambridge filters (Wartman et al. 1959), as noted in Chapter B. Larger quantities of condensates, needed for transformation assays, are collected in cold traps. The condensates are suspended in acetone for testing in mammalian cell assays.

Test 1: AMES' SALMONELLA TYPHIMURIUM MUTAGENICITY ASSAY:

Ames' Salmonella assay is the most widely used mutagenicity assay which has been used to predict the carcinogenicity of pure chemicals and complex mixtures (Ashby and Tennant 1991, Claxton et al. 1992). This is a simple assay which is easy to perform and provides results within a relatively short period of time. The test employs genetically constructed strains of a bacterium, Salmonella typhimurium, to detect the ability of chemicals to induce gene mutations. The tester strains possess defined mutations which prevent them from growing in a medium that does not contain an essential aminoacid, histidine. Upon treatment with a mutagenic chemical, some of these mutants revert back to their wild type form and regain capacity to grow in the absence of histidine (Ames et al. 1975). This simple feature of the test allows detection of chemicals that interact with bacterial DNA to cause reverse mutations. Up to five strains of S.typhimurium have been used for routine testing of chemicals.

The test is performed by mixing the low-histidine top agar, tester strain, test compound, and the S-9 mix in a sterile tube. The mixture is poured into petridishes containing bottom agar and the dishes are incubated at 37^0 C for 48-72 hrs. Revertant colonies are scored and the data are analysed. The assay procedure has been described in detail by Ames and coworkers (Ames, McCann and Yamasaki, 1975, Maron and Ames, 1983).

Past studies indicate that two strains of S.typhimurium, TA98 and TA100, provide an adequate evaluation of condensate mutagenicity (Mizusaki et al. 1977, Sato et al. 1977, Gairola 1979, Yoshida and Matsumoto 1980). The most sensitive strains for condensate evaluation are strains TA1538 and TA98 (DeMarini 1983), which detect those substances that induce frameshift types of mutations. Since genetic backgrounds of these two strains are basically the same and TA98 is somewhat more sensitive due to the presence of a plasmid, use of the latter strain is recommended. Strain TA1535 and TA100 which detect chemicals inducing base substitution types of mutations also give a positive response for smoke condensates and are especially useful for assaying the levels of direct mutagens eg.,nitrate-rich tobacco smoke condensates (Kier et al. 1974, Sugimura et al.1977). In view of increased concern for the role of oxy-radicals in tobacco carcinogenesis (Church and Pryor 1985), a newer tester strain TA 102 may also be considered in the evaluation of condensate mutagenicity. This strain has been successfully used to detect the mutagenicity of oxidizing agents (Levin et al. 1982) and some

metal species that act via oxidative mechanisms, e.g., selenite, (Kramer and Ames 1988). However, data on the mutagenic evaluation of smoke condensates using strain TA102 are presently not available. Also this strain has a high spontaneous reversion rate which may necessitate a feasibility study of this strain for evaluation of smoke condensate mutagenicity before its use for routine genotoxicity evalation.

Tests are performed in the absence and presence of Aroclor-induced rat liver S-9 fraction to evaluate the presence of direct mutagens and those that require metabolic activation. In our experience, freshly prepared condensates give a near linear dose response curve when tested at concentrations of 100-400 μg/plate. If the condensates are stored for more than a week or two, a flattening effect is observed at higher concentrations, possibly due to cytotoxicity.

A general description of the procedure is provided below:

a) Culture Maintenance and Growing Conditions: Bacterial cultures are maintained as frozen stocks and are grown in Oxoid nutrient broth No. 2 to a density of 1-2 x 10^9 cells/ml. This is achieved by inoculating media flask with a tester strain and incubating at 37°C overnight as a stationary culture. Early next morning the cultures are placed in a gyrorotary incubator running at approximately 200 rpm at 37°C for 4-6 hrs. Once the cultures are ready they are stored on ice before use.

b) Preparation of Rat Liver S-9: Rat liver enzymes are induced with Aroclor 1254 suspension in corn oil (200 mg/ml). A single intraperitoneal injection of 500 mg/kg body wt of rat is given 5 days prior to sacrifice. Rats are sacrificed by cervical dislocation and their livers are excised under sterile conditions for placing in ice cold sterile PBS. All the steps are performed at 0-4°C under sterile conditions. The liver is chopped into small pieces and homogenized in chilled 0.15 M KCl using 3 ml of solution for each gm wet liver weight. The homogenate is centrifuged at 9,000G for 10 minutes and the supernatants are collected for storage in sterile plastic tubes at -80°C. S-9 preparations are standardized with respect to their cytochrome P-450 content and mixed function oxidase activity.

c) Preparation of S-9 mix: S-9 mix is composed of 100 mM phosphate buffer, pH 7.4, 8 mM magnesium chloride, 33 mM potassium chloride, 4 mM NADP and 0.05-0.1 ml of S-9/ml of mix. The mix is made fresh for each experiment and can be stored on ice for the day without significant loss of activity.

d) Assay Procedure: Bottom agar plates and top agar are prepared in advance as described by Moran and Ames (1983). On the day of the experiment, to the 100 ml of melted top agar, 10 ml of a solution containing 0.5 mM histidine and 0.5 mM biotin

are added and thoroughly mixed. This low histidine top agar is then dispensed at 2 mls per sterile 13 x 100 mm tube and maintained at 45°C in heating blocks. To each tube, the test sample and 0.1 ml of the tester strain culture with and without 0.5 ml of S-9 mix are added and mixed before pouring onto plates containing 25 ml of bottom agar. The plates are incubated at 37°C for 48 to 72 hours and the revertant colonies are counted to obtain the number of induced revertants/plate.

e) Experimental Design: Each experiment should include negative (spontaneous revertants) and positive controls (one compound which is a direct mutagen and one which requires metabolic activation). A solvent control is also necessary. Four to 5 concentrations of each condensate sample with three replicates for each concentration are generally tested in each experiment. For reference cigarettes, 50 to 300 μg and 100 to 500 μg condensate/plate are tested in the absence and presence of S-9 mix, respectively. Numbers of revertants/plate for each concentration are obtained by taking an average of three replicates for that concentration of the sample. The number of revertants vs. concentration of condensate/plate are plotted to obtain a dose-response curve. If necessary, log transformations of the response can be plotted against condensate concentration to obtain a dose-response curve.

f) Data Evaluation and Interpretation: The data are generally presented as the number of induced revertants per plate, which are obtained by subtracting the number of spontaneous revertants from the total revertants/plate for each concentration of the test sample. When plots are developed for induced revertants vs. condensate concentration, a near linear dose-response curve is obtained; this is particularly common at lower concentrations. Specific activity of each condensate can be calculated from these plots. Specific activity is defined as the number of induced revertants/mg of condensate in the absence of metabolic activation, or the number of revertants/mg condensate, when metabolically activated with S-9 containing a given amount of cyt. P-450 or S-9 capable of metabolizing a given amount of benzo(a)pyrene/min/mg protein. Once the data have been generated for each of the test condensates, direct comparisons can be made to a reference or with each other to determine their mutagenic potential. Given the delivery of total particulate matter for each cigarette, the total number of revertants/cigarette can also be calculated to compare cigarette types.

In the mutagenic evaluation of tobacco smoke condensates, it has been our experience and that of other's (Matsumoto et al. 1977) that condensates generally do not show significant activity for direct mutagens, except for some experimental cigarettes, e.g., high nitrate tobacco cigarettes. Therefore, it is difficult

to obtain a dose-response curve when condensates are tested in the absence of S-9s.

g) Limitations of the Assay: Because of the high toxicity of fresh whole smoke to bacterial strains, the assay is limited to providing a genotoxic evaluation of only the particulate phase of cigarette smoke. Any changes in the constituents of gas phase resulting from low ignition modifications of cigarettes are, therefore, not evaluated in the plate incorporation version of this assay.

Certain mutagens and carcinogens, e.g., metallic salts, some organometallics, halogenated compounds, give negative results in the Ames' test (Ashby and Tennant 1988). If such chemicals are present in the low ignition potential cigarettes, their mutagenic activity will not be detected by the Ames' test.

Test-2: MAMMALIAN CELL TRANSFORMATION ASSAY:

A number of mammalian cell transformation assays have been used in the evaluation of genotoxicity of chemicals. C3H/10T1/2 mouse embryo, BALB/c 3T3 mouse fibroblast and Syrian hamster embryo (SHE) cell assay systems are the most commonly used assays for which reasonable baseline data exist (IARC/NCI/EPA 1985, IARC 1985, Dunkel et al.1991). Cell transformation has been defined as the induction of certain neoplasia-related phenotypic changes in the cultured cells.

The most commonly examined endpoints in cell transformation assays have been the morphological alteration of cell colonies. Normally, cells in culture grow to form a confluent monolayer and then stop dividing when surrounded by the cells (contact inhibition). However, if the cells have been treated with a carcinogen, some cells continue to grow and form foci of transformed cells. These foci exhibit dense, haphazard overgrowth on the monolayer. When injected into appropriate host animals, the transformed cells form tumors, but the normal cells generally do not give rise to tumors. Because of their ability to form a tumor in the host, the cells from such foci are considered malignantly transformed (Landolph 1985).

The selection of the C3H/10T1/2 cell assay for condensate evaluation is based on a very low rate of spontaneous transformation of these cells in culture and positive results with smoke condensates (Benedict et al. 1975). Furthermore, C3H/10T1/2 and BALB/c 3T3 cells are continuous cell lines which give relatively more reproducible results than SHE cells, which are primary culture cells and are by nature variable. Since the main purpose of this testing program is to provide a quantitative comparison of the activity of different smoke condensates, it is desirable that the selected assays are reproducible.

The theory that tumor initiator and tumor promoter activities of chemicals play important roles in the carcinogenic process reinforces the relevance of the cell transformation assay. Cigarette smoke condensates are known to contain both tumor initiators and promoters (Hoffman and Wynder 1971).

Mammalian cell transformation assays can be performed with either C3H/10T1/2 or BALB/c 3T3 cells. Detail test protocols for these assays have been described (IARC 1985). The basic features of the C3H/10T1/2 assay procedure are described below which also apply to BALB/c 3T3 cell assay.

Cell Transformation Assay: Transformation assays are performed essentially as described by Reznikoff et al., (1973) with some later modifications (Landolph 1985, Dunkel et al. 1991). For each concentration of the test condensate, 30-40 60-mm petri dishes are seeded with 2 x 10^3 cells. In all experiments a set of dishes for positive control (3-methylcholanthrene) and another set for solvent control are run in parallel. After 24 hr of incubation at 37°C in a 5% CO_2 atmosphere, appropriate concentrations of the test condensates in dimethylsulfoxide (DMSO) or acetone are added to the cultures. After another 24 hr incubation, the old medium is changed with fresh medium. Thereafter, the medium is replaced every fourth day until the cultures are confluent. Once the cultures have reached confluency, the medium is changed every seventh day with Eagle's basal medium (BME) containing 5% heat-inactivated fetal calf serum (FCS). The experiments are terminated after 6 weeks of incubation. The medium is removed and the cultures are washed with phosphate buffer solution (PBS), fixed with methanol, stained with Giemsa and scored for type 2 and type 3 foci of transformants (Reznikoff et al. 1973).

Cell Culture Methods: Mouse embryo fibroblast cell line (C3H/10T1/2), clone 8 is grown in BME supplemented with 10% heat inactivated FCS and 5 mg/ml gentamycin (Kennedy, 1985). It is important that several batches of sera are pretested for plating efficiency, cell attachment and transformation with 1 mg/ml 3-methylcholanthrene prior to purchase to insure the consistency of the transformation assay. Only those lots that give a plating efficiency of over 20% are purchased for use in the assays. The C3H/10T1/2 stock cultures are seeded at a density of 2 x 10^4 cells per 25 cm^2 flask and grown in a humidified incubator in a 5% CO_2/air atmosphere at 37°C. Every 7 days, the stock cultures are passaged using trypsin (0.1% in PBS for 3 min) to detach the cells from the flask. After detachment, the cells are resuspended in complete BME, counted, and reseeded into 25 cm^2 flasks.

It is important that the cultures used for the assay are in early passage. For storage, the cells from the log phase of growth are harvested by trypsinization and suspended in antibiotic-free medium containing 10% FCS and 10% DMSO. The cell suspensions are frozen and stored in liquid nitrogen.

Cytotoxicity Assay: Cytotoxicity assays are performed in advance and in parallel with the cell transformation assays. The information derived from the cytotoxicity experiments is necessary for running the transformation experiments and calculating the transformation frequencies. The toxicity of the test solution is assessed by determining the plating efficiency of untreated and treated cells in each experiment. Five 60-mm Petri dishes/group are seeded with 200 cells and treated with different concentrations of the test solution or the solvent in the same manner as described above for the cell transformation assay. After 10-12 days of culture, the cell monolayers are washed and stained. Colonies are counted and the plating efficiency, which is defined as the number of colonies formed as a percentage of the number of cells seeded per dish, is calculated.

This assay is capable of detecting the cell transformation activity of 3-methylcholanthrene in the absence of a metabolic activation system (Reznikoff et al. 1973) and has also been reported positive for condensates in the absence of S-9 (Benedict et al. 1975). Presence of some metabolic activation capacity in C3H/10T1/2 cells for polycyclic aromatic hydrocarbons has been demonstrated (Gehly and Heidelberger 1982).

Data Evaluation and Interpretation: Data from these experiments are generally expressed as transformation frequencies, defined as the percentage of type 2 and type 3 foci, based on the number of survivors that form colonies in the dishes. The data generated can be plotted as % transformed colonies on a log scale vs the concentration of test condensate. Cytotoxicity data can also be plotted on the same graph to express percent cell survival in treated cultures.

Transformation frequencies depend heavily on the number of survivors. The frequencies decrease if the surviving number of cells/dish is too high or too low. In view of this problem, tabulation is currently preferred of the number of type 2 and type 3 foci per total number of treated dishes and the total number of dishes containing type 2 and type 3 foci out of the total number of dishes treated. Both formats of data expression allow the development of a dose-response curve.

When polycyclic aromatic hydrocarbons are tested in the assay, a dose response is usually obtained. Comparison of the transformation potential of smoke condensates from different test cigarettes will require tightly controlled conditions. In each

experiment a reference compound or a reference condensate or both are run in parallel. The data from the test condensate can then be compared to the values for the reference substance in each experiment and a potency value assigned to each test condensate. These manipulations are necessary to minimize the impact of variability of response in the assay from experiment to experiment.

Limitations of the Assay: Because of the high toxicity of fresh cigarette smoke to mammalian cells, C3H/10T1/2 assay may also be limited to assessing the genotoxicity of cigarette smoke condensates. Any changes in gas phase constituents resulting from low ignition modifications of cigarettes will,therefore, not be evaluated in this assay. Very few studies of smoke condensates in cell transformation assays have been performed. It will be necessary to further develop assay conditions for routine evaluation of condensate activity.

Other limitations of the cell transformation assay exist (Landolph 1985, IARC 1985, Dunkel et al. 1991). First, C3H/10T1/2 cells have a maximum plating efficiency of about 30%. In the standard transformation assay when toxic concentrations of the smoke condensates are tested the plating efficiency will be further reduced and the number of survivors may be too few for a valid transformation assay. Second, too few or too many cells per dish can affect the number of transformed colonies thus making the test somewhat semiquantitative even under tightly controlled conditions.

Third, the spontaneous transformation rate of C3H/10T1/2 cells is extremely low, which makes it difficult to obtain a baseline transformation frequency in each experiment. As many as 500 control plates may be needed to obtain the true spontaneous transformation rate of C3H/10T1/2 cells. However, this characteristic of C3H/10T1/2 cells also makes the test attractive, because the induction of even a few transformed colonies on the plates can be attributed to the action of the test condensate with confidence. In this respect, it may be noted that the BALB/c 3T3 cell system, which has a low but still easily obtainable basal transformation rate, has been often utilized for routine testing of various chemicals but sufficient information about smoke condensate activity in this system is presently not available. In contrast, the C3H/10T1/2 system has been successfully used to detect cell transformation activity of smoke condensate and its fraction (Benedict et al. 1975).

3.b. Tests for Evaluating Genotoxicity of Fresh Whole Cigarette Smoke:

While microgram quantities (50 to 500 μg/plate) of smoke condensates can be tested in the bacterial and mammalian cell assays, direct exposure of tester cells to fresh whole cigarette

smoke can cause high cell mortality due to the presence of highly toxic constituents, such as carbon monoxide. Therefore, the above assays are generally difficult to use in their standard format for genotoxic evaluation of fresh cigarette smoke. A yeast cell system which tolerates cigarette smoke much better than do bacterial or mammalian cell systems may be used for fresh smoke evaluation, if necessary.

In cases where chemical and physical analyses indicate a significant alteration in smoke composition, especially that of the gas phase constituents, the induction of mutations and other types of genetic damage by fresh whole smoke may be assessed by using the D-7 strain of Saccharromyces cerevisiae. This tester organism is a diploid strain of yeast which is used to identify chemicals that induce mitotic crossing-over, mitotic gene conversion, and gene mutations (Zimmermann et al. 1975). An exposure system has been developed to directly expose tester yeast cells to fresh smoke and has been found useful in quantitating various types of genetic damage by fresh smoke from different types of tobacco cigarettes (Gairola 1982).

3.c. Tests for Evaluating Pulmonary Inflammatory Response in Animals

i) Rationale: Cigarette smoking is a major risk factor in the development of chronic obstructive pulmonary disease, as discussed in Chapter A. Considerable evidence exists to suggest that chronic pulmonary inflammation plays an important role in the development of various pulmonary diseases, including those associated with cigarette smoking (Hunninghake et al. 1979, Neiwoehner 1988, Costabel and Guzman 1992). A test that evaluates the ability of test smokes to induce pulmonary inflammation will therefore be a useful indicator of the in vivo biological activity of cigarette smoke. The assay described below utilizes an analysis of the BAL cells from smoke-exposed mice for toxicological evaluation of fresh cigarette smoke.

ii) Bioassay procedure

Animal model: Rodents (mice, rats, guinea pigs and hamsters) have been generally used as animal models for studying the inhalation toxicity of cigarette smoke. Past studies have shown that exposure of rodents to cigarette smoke induces an inflammatory cell response in their lungs (Rylander 1974, Hoidal and Niewoehner 1982, Matulionis 1984, Gairola 1986). The studies have further shown that the pulmonary response of mice to cigarette smoke inhalation, as monitored by bronchoalveolar lavage (BAL), is significantly more pronounced than that of rats (Gairola 1986). Furthermore, it has been found that, as in human smokers, the BAL cells recovered from smoke-exposed mice show an infiltration of inflammatory cells including polymorphonuclear neutrophils (PMN) into the lungs, while those recovered from

smoke-exposed rats hardly show any PMNs. Among other changes induced in BAL cells of smoke-exposed mice are increased oxidant production and lysosomal enzyme content of pulmonary alveolar macrophage (Gairola 1986) which resemble those reported in human smokers (Hunninghake et al. 1979, Finch et al. 1982, Fisher et al. 1982).

In view of the observations described above, mouse is proposed as an animal model for this assay. An additional advantage of using mouse is its smaller size which allows for increasing the number of animals in treatment and control groups at relatively lower costs.

Smoke exposure protocol: Male or female C57Bl mice are exposed to smoke in a nose-only exposure system (Griffith and Standafer 1985) to fresh smoke from one cigarette in the morning and one in the afternoon. Since nose-only exposures require the use of restrainers for animals during exposures, it is important that a group of animals be given the same treatment as smoke-exposed animals but in the absence of smoke (sham control) to simulate stress conditions similar to those of the smoke-exposed group. The first week of the experiment is a "break in" period during which the animals are gradually acclimatized to treatments by exposing them to 3, 6, and 9 puffs of smoke each session for two days at a time. Thereafter, the animals receive exposure to 10 puffs of smoke per session, twice a day, seven days a week. Groups of animals (6-8) are sacrificed at different exposure points and the free lung cells are obtained by bronchoalveolar lavage (BAL) for further study.

Markers of smoke exposure: A number of markers are monitored to ascertain the inhalation of smoke by the animals. Total particulate matter (TPM) intake, blood carboxyhemoglobin, and urinary cotinine excretion are generally measured, (Griffith and Standafer 1985, Gairola 1986, 1987, Stanley et al. 1991). Animal TPM intake values are determined by measuring the removal of smoke particulates by the animals from the exposure chamber during each exposure session (Griffith and Hancock 1985). Some of the exposure markers, e.g., urinary cotinine and blood carboxyhemoglobin levels, used in animal studies are the same as those described for human studies in Chapter C.

Bronchoalveolar lavage: The morning after the last treatment, animals are anesthetized by an ip injection of pentobarbital and are exsanguinated by severing the abdominal aorta. The lungs are lavaged and the BAL cells are obtained for analyses (Gairola 1986).

Assessment of BAL cells: Total cell counts and viable cell counts by trypan blue exclusion are made with a hemocytometer. Small aliquots of BAL cell suspension are used to prepare Diff-Quik stained slides for differential leukocyte

counts. These data allow an assessment of the degree of macrophage, lymphocyte and polymorphonuclear neutrophil (PMN) infiltration into the lungs.

Differential counts for each batch of lavage cells at different exposure points indicate the time course of PMN infiltration into the lungs and can be plotted against exposure duration to determine the rate and extent of their influx. Since neutrophilic alveolitis has been implicated in the development of cigarette smoke-induced pulmonary diseases, such plots prepared for animals exposed to various test cigarette smokes are likely to provide a reasonable indication of their toxicity and the data can be used to differentiate the biological activity of test smokes.

The cells remaining after total, viable, and differential counts are cultured for one hour at 37°C to isolate macrophages as monolayer. 5'-nucleotidase activity of the cell lysates is measured by a radiometric procedure, which is a good indicator of smoke-induced macrophage activation in mice (Gairola 1986).

Data Evaluation and Interpretation: Graphs of the BAL cell data obtained from the study can be used to determine the time course of inflammatory cell influx into the bronchoalveolar lumen, which will indicate the toxicity of smoke. A useful endpoint is the time of PMN infiltration which may reflect the potency of the test smoke toxicity. Macrophage enzyme activities can also be used to assess the potential biological activity of test cigarette smoke.

iii) Limitation of the Bioassay: Even though markers of smoke exposure are used, it is difficult to ascertain the dose of smoke inhaled by the animals. Also, interanimal variation may complicate interpretation of data, but this problem can be controlled by increasing the number of animals per group. The use of this assay has been limited for comparative assessment of smoke from different cigarette types and therefore may require further development of standardized assay conditions before use in any routine evaluation program. Also the expense of performing this assay may be a deterent for its routine use.

The presence of particulates in the ambient atmosphere, respiratory infections,etc., can also induce an inflammatory response in the lungs of animals, thus complicating an assessment of smoke effects. Therefore, it is very important to house the animals in Bioclean rooms equipped with HEPA filters and maintained at higher (30 to 40) air changes/hour to minimize the exposure of animals to particulates and/or any infectious agents.

4. Estimates of Cost and Duration of Tests:

Ames' Test: The length of time for testing five concentrations of one reference and four unknown condensate samples, in the

absence and presence of S-9s, is estimated at about 4-6 weeks. The current cost of testing five concentrations of one compound in the absence and presence of S-9, in two to five strains of the test bacteria, varies between $1,500-2,200/condensate sample.

Cell Transformation Assay: The length of time for testing 5 concentrations of one condensate in the absence of S-9 in cell transformation assay is about 6 months and costs range from $7,000-8,000/condensate sample.

Inflammatory Cell Response Bioassay: The length of time for testing smoke from one type of cigarette in this bioassay will take 8-10 months. The cost of testing one cigarette type in this bioassay is estimated at about $50,000. If more than one cigarette type is tested at the same time, the cost will be reduced.

5. Other Toxicological Endpoints Associated with Smoke Exposure-related Health Problems:

In addition to genotoxicity and pulmonary toxicity, cigarette smoke has been implicated in other health effects discussed in Chapter A. Smoking-induced immunosuppression in human smokers may be one of the mechanisms contributing to the development of smoking-associated health effects. Although the incidence of immunosuppression in smokers is well documented (Holt 1987, Johnson et al. 1990), the exact nature of immunotoxicants in smoke is not known. It may be useful to include some tests for evaluating the immunotoxic activity of low-ignition potential cigarette smoke. Unfortunately very few studies addressing immunotoxicity of smoke have been performed in animal models. Only recently some experimental evidence suggesting an impairment of thymic-dependent and independent functions of lymphocytes from long-term smoke-exposed mice and rats has been reported (Sopori et al. 1989, Chang et al. 1990, Goud et al. 1992).

Reproductive toxicity and atherogenesis are two other health problems associated with cigarette smoking. A number of epidemiological studies have suggested that chronic smoking is deleterious to female reproductive health and fetal development (Abel 1980, Mattison et al. 1989, Werler 1986). Relatively fewer data regarding the effects of smoke on reproductive system are available in animal models (Mattison 1982). Preliminary studies in mice have shown that long-term exposure to cigarette smoke lengthens the murine estrous cycle and causes accelerated loss of oocytes from the ovaries (Gulati et al. 1989, Gairola and Gulati 1991).

Still fewer studies have examined the formation of atherosclerotic plaques following exposure to cigarette smoke in animal models. One study reported the formation of aortic

lesions in Syrian hamsters following a 12-15 month exposure to cigarette smoke (Haley and Axelrad 1982). Recently, enhanced development of atherosclerosis has also been reported in rabbits which had been fed high cholesterol diets and were exposed to environmental tobacco smoke (Zhu et al.1993).

The long duration of exposures required to induce many of the above described immunological, reproductive, and atherogenic toxicities in animals would make bioassays based on these endpoints prohibitively expensive for routine use in the evaluation of low-ignition cigarette prototypes.

REFERENCES

Abel, E.L.; Smoking during pregnancy: A review of effects on growth and development of offspring. Human Biology 52:593-625, 1980.

Ames, B.N., McCann, J. and Yamasaki, E.; Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res. 31:347-364, 1975.

Benedict, W.F., Rucker, N., Faust, J. and Kouri, R.E.; Malignant transformation of mouse cells by cigarette smoke condensate. Cancer Res. 35:857-860, 1975.

Benner, J.F.; Tentative summary of leaf and smoke analysis of the University of Kentucky reference and alkaloid series cigarettes. Pro.Tobacco and Health Conf. 2:30-34,1970.

Brusick, D.; Principles of Genetic Toxicology, Plenum Press, New York, 1987.

Chang, J.C.C., Distler, S.G. and Kaplan, A.M.; Tobacco smoke suppresses T-cells but not antigen presenting cells in the lung associated lymph nodes. Toxicol.Appl.Pharmacol. 102:514-523, 1990.

Church, D.F. and Pryor, W.A.; Free-radical chemistry of cigarette smoke and its toxicological implications. Environ. Hlth. Perspect. 64:111-126, 1985.

Claxton, L.D., Douglas, G., Krewski, D., Lewtas, J., Matsushita, H., and Rosenkranz, H.; Overview, conclusion,and recommendations of the IPCS collaborative study on complex mixtures. Mutation Res. 276:61-80, 1992.

Costabel, U. and Guzman, J.; Effect of smoking on bronchoalveolar lavage constituents. Eur.Respir.J. 5:776-779, 1992.

Davis, D.L., Vaught, A., Tso, T.C.,and Bush, L.P.; Analysis of a new lower yield research cigarette. CORESTA meetings,pp 3, 1984.

Dunkel, V.C., Rogers, C., Swierenga, S.H.H., Brillinger, R., Gilman, J.P.W. and Nestmann, E.R.; Recommended protocols based on a survey of current practice in genotoxicity testing laboratories: III.Cell transformation in C3H/10T1/2 mouse embryo cell, BALB/c 3T3 mouse fibroblast and Syrian hamster embryo cell cultures. Mutation Res. 246:285-300, 1991.

Finch, G.L., Fisher, G.L., Hayes, T.L.,and Golde, D.W.; Surface morphology and functional studies of human macrophages from cigarette smokers and nonsmokers. J.Reticuloendothel.Soc. 32:1-32, 1982.

Fisher, G.L., McNeill, K.L.,Finch, G.L., Wilson, F.D. and Golde, D.W.; Functional evaluation of lung macrophages from cigarette smokers and nonsmokers. J.Reticuloendothel.Soc. 32:311-321, 1982.

Gairola, C.G.; Short-term bioassays for the evaluation of cigarette smoke condensates. Proc. Fifth Workshop Conf.Tobacco and Health Research Institute, Lexington, KY 5:284-302, 1979.

Gairola, C.G.; Genetic effects of fresh cigarette smoke in Saccharomyces cerevisiae Mutation Res. 102:123-136, 1982.

Gairola, C.G., ; Free lung response of mice and rats to mainstream cigarette smoke exposure. Toxicol. Appl. Pharmacol. 84:567-575, 1986.

Gairola, C.G.; Pulmonary aryl hydrocarbon hydroxylase activity of mice, rats and guinea pigs following long term exposure to mainstream and sidestream cigarette smoke. Toxicology 45:177-184, 1987.

Gairola, C.G. and Gulati, D.K.; Ovarian toxicity of cigarette smoke in mice. Toxicologist 11:109, 1991.

Gehly, E.B. and Heidelberger, C.; Metabolic activation of benzo(a)pyrene by transformable and nontransformable C3H mouse fibroblasts in culture. Cancer Res. 42:2697-2704, 1982.

Goud, S.N., Kaplan, A.M. and Subbaroa, B.; Effects of cigarette smoke on the antibody responses to thymic independent antigens from different lymphoid tissues of mice. Arch.Toxicol. 66:164-169, 1992.

Griffith, R.B. and Hancock, R.; Simultaneous mainstream-sidestream smoke exposure systems.I. Equipment and procedures. Toxicology 34: 123-138, 1985.

Griffith, R.B. and Standafer, S.; Simultaneous mainstream-sidestream smoke exposure systems. II. the rat exposure system. Toxicology 35:13-24, 1985.

Gulati, D.K., Russell, S.R., Cocanougher, T.A., Thomas, M.A. and Gairola, C.G.; Cigarette smok-induced alterations of estrual cyclicity in mice. Toxicologist 9:185, 1989.

Haley, N.J. and Axelrad, C.; Cigarette smoke-induced changes in aortic tissue from Syrian golden hamsters. Fed.Proc. 41:452, 1982.

Hoffmann, D. and Wynder, E.L.; A study of tobacco carcinogenesis, XI. Tumor initiators, tumor accelerators, and tumor promoting activity of condensate fractions. Cancer 27: 848-864, 1971.

Hoffmann, D., Wynder, E.L., Hecht, S.S., Brunnemann, K.D., LaVoie, E.J. and Haley, N.J.; Chemical carcinogens in tobacco, in: P. Bannasch (ed.), Cancer Risks. Strategies for Elimination. Springer, New York, pp 101-103, 1987.

Hoidal, J.R. and Niewoehner, D.E.; Lung phagocyte recruitment and and metabolic alterations induced by cigarette smoke in humans and hamsters. Amer.Rev.Respir.Dis. 126:548-552, 1982.

Holt, P.G.; Immune and inflammatory function in cigarette smokers. Thorax 42:241-249, 1987.

Huff, J. and Haseman, J.; Long-term chemical carcinogenesis experiments for identifying potential human cancer hazards: Collective database of the National Cancer Institute and National Toxicology Program (1976-1991). Environ.Hlth.Perspect. 96:23-31, 1991.

Hunninghake, G.W., Gadek, J.E., Kawanami, O., Ferrans, V.J. and Crystal, R.G.; Inflammatory and immune processes in the human lung in health and disease: Evaluation by bronchoalveolar lavage. Am. J. Pathol. 97:149-206, 1979.

IARC/NCI/EPA Working Group ; Cellular and molecular mechanisms of cell transformation and standardization of transformation assays of established cell lines for the prediction of carcinogenic chemicals: overview and recommended protocols. Cancer Res. 45:2395-2399, 1985.

IARC; Transformation assay of established cell lines: Mechanisms and application. T. Kakanuga and H. Yamasaki (Eds), IARC Scient.Publ.# 67, Lyon, 1985.

Johnson, J.D., Houchens, D.P., Kluwe, W.M., Craig, D.K.,and Fisher, G.L.; Effects of mainstream and environmental tobacco smoke on the immune system in animals and humans: a Review. Crit.Rev.Toxicol. 20:369-395, 1990.

Kennedy, A.R.; Conditions for modification of radiation transformation in vitro by a tumor promoter and protease inhibitors. Carcinogenesis 6:1441-1445, 1985.

Kramer, G.F. and Ames, B.N.; Mechanisms of mutagenicity and toxicity of sodium selenite (Na_2SeO_3) in Salmonella typhimurium. Mutation Res.201:169-180, 1988.

Landolph, J.R.; Chemical transformation in C3H10T1/2 Cl8 mouse embryo fibroblast:historical background, assessment of the transformation assay,and evolution and optimization of the transformation assay protocol, in: Transformation assay of

established cell lines: Mechanisms and application, T.Kakanuga, and H.Yamasaki, IARC Scient.Publ.No.67:185-203, 1985.

Levin, D.E., Hollstein, M., Christman, M.F., Schwiers, E.A., and Ames, B.N.; A newer Salmonella tester strain (TA 102) with A.T base pairs at the site of mutation detects oxidative mutagens. Proc.Natl.Acad.Sci.(USA), 79:7445-7449, 1982.

Maron, D.M. and Ames, B.N.; Revised methods for the Salmonella mutagenicity test. Mutation Res. 113:173-215, 1983.

Matsumoto, T., Yoshida, D., Mizusaki, S. and Okamoto, H.; Mutagenic activity of aminoacid pyrolyzates in Salmonella typhimurium TA 98. Mutation Res. 48:279-286, 1977.

Mattison, D.R.; The effects of smoking on fertility from gametogenesis to implantation. Environ. Res. 28:410-433, 1982.

Mattison, D.R., Plowchalk, D.R., Meadows, M.J., Miller, M.M., Malek, A. and London, S.; Seminars Reprod.Endocrinolog. 7:291-304, 1989.

Matulionis, D.H.; Effects of cigarette smoke generated by different machines on pulmonary macrophages in mice and rats. J.Anal.Toxicol. 8:187-191, 1984.

May, W.E., Benner, B.A., Wise, S.A., Schuetzle, D. and Lewtas, J.; Standard reference materials for chemical and biological studies of complex environmental samples. Mutation Res. 276:11-22, 1992.

Melikian, A.A., Hecht, S.S., and Hoffmann, D.; Mechanistic studies of tobacco carcinogenesis in mouse epidermis and lung tissues, in: Progress in Clinical and Biological Research 298:331-345, Skin Carcinogenesis: Mechanisms and Human Relevance, Liss, New York, 1989.

Mizusaki, S.H., Okamoto, H., Akiyama, A. and Fukuhara, Y.; Relation between chemical constituents of tobacco and mutagenic activity of cigarette smoke condensate. Mutation Res. 48:319-326, 1977.

Niewoehner, D.E.; Cigarette smoking, lung inflammation and the development of emphysema. Review. J. Lab. Clin. Med. 111:15-27, 1988.

Parodi, S., Malacarne, D. and Taningher, M.; Examples of uses of databases for quantitative and qualitative correlation studies between genotoxicity and carcinogenicity. Environ.Hlth.Perspect. 96:61-66, 1991.

Parodi, S. and Waters, M.D.; Introduction and Summary. Genotoxicity and carcinogenicity databases : An assessment of the present situation. Environ. Hlth.Perspect. 96:3-4, 1991.

Reznikoff, C.A., Bertram, J.S., Brankow, D.W., and Heidelberger, C.; Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res. 33:3239-3249, 1973.

Rylander, R.; Pulmonary cell responses to inhaled cigarette smoke. Arch.Environ.Health 29:329-333, 1974.

Sato, S., Seino, Y., Ohka, T., Yahagi, T., Nagao, M., Matsushima, T., and Sugimura, T.; Mutagenicity of smoke condensates from cigarettes, cigars, and pipe tobacco. Cancer Lett. 3:1-8, 1977.

Sopori, M.L., Cherian, S., Chilukuri, R. and Shopp, G.M.; Cigarette smoke causes inhibition of the immune response to intratracheally administered antigens. Toxicol.Appl.Pharmacol. 97:489-499, 1989.

Stanley, S., Huffman, M., Sadove, R., Gairola, C.G., Diana, J.N., Dowell, R.T., Tai, H-H.. Watt, D. and Tobin, T.; Nicotine and cotinine: Detection by ELISA in blood, saliva, and urine. The Toxicologist 11:134, 1991.

Sugimura, T., Nagao, N., Kawachi, T., Honda, M, Yahagi, T., Seino, Y., Sato, S., Matsukura, N., Matsushima, T., Shirai, A., Sawamura, M., and Matsumoto, H.; Mutagens-carcinogens in food, with special reference to highly mutagenic pyrolytic products in broiled foods, in: H.H. Hiatt, J.D. Watson and J.A. Winsten (eds), Origins of human cancer, Cold Spring Harbor Laboratory, New York, pp 1561-1577, 1977.

Tennant, R.W.; The genetic toxicity database of the National Toxicology Program: Evaluation of the relationships between genetic toxicity and carcinogenicity. Environ.Hlth.Perspect. 96:47-51, 1991.

Wartman, W.B., Cogbill, E.C., and Harlow, E.; Determination of particulate matter in concentrated aerosols. Application to analysis of cigarette smoke. Anal.Chem. 31:1705-1709, 1959.

Werler, M.M., Pober, B.R. and Holmes, L.B.; Smoking and pregnancy, in: J.L.Sever and R.L. Brent (eds), Teratogen Update: Environmentally Induced Birth Defects Risks, Alan R. Liss, New York, pp 131-139, 1986.

Yoshida, D. and Matsumoto, T.; Amino-a-carboline as mutagenic agents in cigarette smoke condensate. Cancer Lett. 10:141-149, 1980.

Zhu,B.Q., Sun,Y.P., Sievers,R.E., Isenberg,W.M., Glantz,S.A., Parmley,W.W.; Passive smoking increases experimental atherosclerosis in cholesterol-fed rabbits. J.Am.Coll.Cardiol. 21:225-232,1993.

Zimmermann, F.K., Kern, R. and Rasenberger, H.; A yeast strain for simultaneous detection of induced mitotic crossing-over, mitotic gene conversion, and reverse mutation. Mutation Res. 28:381-388, 1975.

GLOSSARY

Carcinogenesis: or **Oncogenesis:**	Processes through which tumors are produced.
Genotoxicity:	Any adverse effect on structure, function, or expression of genetic material in a living cell.
Initiators:	Agents that begin but do not necessarily promote the carcinogenic process.
Malignant Cell Transformation:	Change in the phenotypic characterstics of cells in culture that indicates their capability to induce tumors when injected into animals.
Mutagenesis:	Processes by which heritable alterations in genetic material or functions are produced.
Mutations:	Any heritable alterations in the expression of genetic material or functions.
Promoters:	Agents that encourage but do not necessarily initiate cancer development.

Chapter I

In Vivo Bioassays for Carcinogenicity

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Chapter Contents

Introduction

In the 1989 Report on the Health Consequences of Smoking, the Surgeon General of the U.S. Public Health Service, in evaluating the health effects of smoking, concluded that "smoking is responsible for more than one of every six deaths in the United States" (1). Cigarette smoking alone increases the risk for coronary heart disease from 23/1000 to 54/1000, together with hypercholesterolemia cigarette smoking raises the risk to 103/1000, and together with high blood pressure, to 92/1000. All three risk factors, smoking, hypercholesterolemia, and high blood pressure, synergistically increase the risk for coronary heart disease to 189/1000 (1).

Cigarette smoking is also a major risk factor for chronic obstructive pulmonary disease, and here primarily for emphysema and chronic bronchitis. Eighty to 90% of the morbidity from chronic obstructive lung disease in the United States has been attributed to cigarette smoking (2).

Smoking of cigarettes is causally associated with cancer of the lung, larynx, oral cavity, esophagus, pancreas, renal pelvis and urinary bladder and is also linked with an increased risk for cancer of the nasal cavity, liver and the uterine cervix and possibly, related to cancer of the stomach (1). In 1992, the National Cancer Institute published a population-based case-control study that provided evidence for the association of cigarette smoking with several types of leukemia and thereby confirmed earlier prospective and case control studies (3). The National Cancer Institute estimated that in 1991 of the 514,000 cancer deaths at the seven sites causally associated with cigarette smoking, 30.6% are due to smoking (4).

Chemical analyses for the major known carcinogens offer a meaningful indication of the carcinogenic potential of cigarette smoke, especially in conjunction with chemical analytical data for the smoke of cigarettes already bioassayed for carcinogenic activity. *In vitro* assays for genotoxicity such as the Ames test with various bacterial strains, the DNA repair assay with primary rat liver cells, and the sister chromatid exchange assay have remained inconclusive in regard to the quantitative aspects of the genotoxic potencies of cigarette smokes (5). At present, conclusive data on the carcinogenicity of the smoke of new cigarettes can only be ascertained with long-term bioassays with laboratory animals (5-7).

Three animal species are primarily utilized for bioassays of whole cigarette smoke in inhalation experiments. These are mice, rats, and Syrian golden hamsters. All of the inhalation studies have the inherent shortcoming that the animals are obligated to breathe through the nose and that their inhalation of tobacco

smoke is shallow. They do not inhale smoke through the mouth as human smokers do. Nevertheless, cigarette smoke inhalation studies with mice, rats and Syrian golden hamsters have led to the induction of significant numbers of benign and malignant tumors in the respiratory tract of these animals.

The data from cigarette smoke inhalation studies with mice have not been fully accepted since only lung adenoma and lung adenocarcinoma have been elicited and not squamous cell tumors in the lung. Furthermore, most strains of mice have a fairly high rate of spontaneous lung adenoma. The rates of both adenocarcinoma and squamous cell carcinoma of the lung have increased in cigarette smokers and the current ratio of lung adenocarcinoma to squamous cell carcinoma in male smokers is 1:2-3 (8).

As will be discussed under "Inhalation Bioassays", the critique on the data from smoke inhalation studies with mice is no longer fully justified. A large-scale inhalation study with rats using highly advanced methodology presents encouraging data (9). However, until additional long-term inhalation bioassays have been completed with this exposure system, the database is too limited to recommend this rat bioassay for routine studies.

The largest database from cigarette smoke inhalation studies stems from assays with Syrian golden hamsters. As will be discussed, these long-term inhalation studies have only in a few cases led to lung tumors; however, they have induced highly significant incidences of benign and malignant tumors in the upper respiratory tract of hamsters. The tumors occurred primarily in the larynx.

Since the early 1960's, remarkable progress has been achieved in respiratory carcinogenesis. We have become well aware of the existence of carcinogens with organ-specificity for the respiratory tract of laboratory animals, and bioassays of aerosols and volatilized chemicals have also provided considerable evidence for their potential to induce tumors in the respiratory tract of mice, rats and hamsters (10).

I. Inhalation Bioassays

Three decades ago, the Leuchtenbergers (11) reported the first extensive inhalation experiments in which mice were exposed daily to air-diluted cigarette smoke in specially designed chambers. This smoke exposure led to early histological, cytological, and cytochemical changes in the major bronchi of the mice. The smoke exposure also caused various degrees of bronchitis associated with atypical proliferation of the bronchial epithelium. The investigators observed extracellular deposition of a brown pigment in the lungs of all the mice that

underwent long-term exposure to cigarette smoke aerosols. After about 12-15 months, the smoke-exposed mice began to develop lung adenoma and lung adenocarcinoma in significantly higher numbers than did the control mice. In inhalation studies with the gas phase of cigarette smoke, lung adenomas have also been observed, though to a significantly lesser extent than with the whole smoke (12). The findings of the Leuchtenbergers (11, 12) were confirmed by Otto (13) who exposed inbred albino mice to cigarette smoke daily. After at least 12 months of smoke exposure, 23 of 60 mice developed lung adenomas, while only 3 of 60 control mice were found with such tumors. One mouse in the exposed group developed a squamous cell carcinoma of the lung after 16 months (13).

Several criticisms have been voiced in regard to the induction of lung adenoma and lung adenocarcinoma in mice by exposure to cigarette smoke. Concerns include the fact that such exposures caused tumors in the peripheral lung, and not in the bronchi, and that some of the tested strains of mice had a relatively high rate of spontaneous lung adenomas. It has been observed that not only the carcinoma in the bronchi, but also the incidence of lung adenocarcinoma, has significantly increased in cigarette smokers and that such tumors are now even seen in nonsmokers who have been exposed to environmental tobacco smoke, to carcinogenic chemicals, or to radiation (1).

In the past, it was not understood how the topical application of tobacco "tar" to the skin of mice could lead to the development of lung adenoma and adenocarcinoma. Today, we are aware that tobacco smoke contains also organ-specific carcinogens such as the tobacco-specific N-nitrosamines, which can induce adenoma and adenocarcinoma in the lung upon application to the skin (14) and other sites in mice.

A major breakthrough in inhalation assays came with the development of new smoke-inhalation devices that facilitate the exposure to diluted tobacco smoke aerosols (15-17). When 80 rats were exposed seven times daily for intermittent periods (8.4 x 30 seconds) to 10% cigarette smoke aerosol for up to 2.5 years, most animals developed hyperplastic and metaplastic changes in the nasal turbinals, larynges and tracheas. Seven of the 80 smoke-exposed F344 female rats developed tumors in the respiratory tract, including 1 adenocarcinoma and 1 squamous cell carcinoma in the lung, compared to 1 alveologenic carcinoma only in the 93 control rats (9).

In another study, rats were exposed to diluted cigarette smoke twice a day for 10 minutes, 5 days a week for up to 40 weeks (18). Subsequently, DNA from nasal, lung and liver tissues was extracted and analyzed by the ^{32}P-postlabeling procedure. In the nasal mucosa at least four new DNA-adducts were seen; the

amount of these adducts increased with the duration of smoke exposure. In the lung, one new DNA-adduct was detected; it also accumulated as smoke exposure progressed. It appears that the DNA adducts were aromatic and/or hydrophobic in nature (18). In a similar assay, rats were exposed for 22 days to diluted cigarette smoke. In the nose-only intermittent exposure and nose-only continuous exposure 14 ± 0.9 and 9.9 ± 0.7 DNA adducts per 10^9 bases were determined in the lung (19). These studies demonstrate that minute amounts of genotoxic smoke components reach the lungs of rats in inhalation assays.

Dontenwill and associates developed the "Hamburg II" smoke inhalation device in which small animals can be exposed to air-diluted smoke (Figure 1). Eighteen groups, each consisting of 80 female and 80 male random-bred Syrian golden hamsters, comprised this cigarette smoke inhalation lifetime assay. Animals in group 1 were exposed once daily for about 10 minutes, seven times each week to air-diluted smoke (7:1); those in group 2 had twice daily exposures to diluted smoke, hamsters in group 3 had 3 exposures to diluted smoke; and those in group 4 were exposed twice daily to the gas phase of diluted smoke, while group 5 consisted of sham-treated controls. In group 1, 38 animals developed papilloma and one animal had a carcinoma of the larynx (total 24%), hamsters in group 2 developed 69 papilloma and 17 carcinoma of the larynx (total 54%), corresponding tumor yields in group 3 were 77 papilloma and 11 carcinoma of the larynx (total 55%). Laryngeal tumors were not observed in group 4 (gas phase only) nor in group 5 (controls). Three hamsters in group 2 developed papilloma of the pharynx; tumors of the lung were not seen in any of the hamsters in this study (20).

In another assay, male Syrian golden hamsters from 2 inbred lines were exposed five times a week for up to 100 weeks to air-diluted smoke (21). In one inbred strain, 7 of 84 hamsters developed papilloma in the larynx, 9 had microinvasive cancer; in the second inbred strain, 11 of 87 animals had papilloma and 2 microinvasive cancers occurred in the larynx; none of the control hamsters developed laryngeal tumors (21).

In a dose-response lifetime study with a hamster strain susceptible to the induction of laryngeal tumors, twice daily exposures to 22% cigarette smoke resulted in 70% with papilloma and 47% with carcinoma of the larynx (22). The corresponding incidences in the hamsters exposed twice daily to 11% cigarette smoke were 27% and 7%. Those in the control group were 6% and 0%, respectively. In the high-dose group, 3 of 62 hamsters also developed tracheal papilloma (22).

These studies demonstrated the dose-repsonse carcinogenic effect of cigarettes. The Syrian golden hamsters are less susceptible than other laboratory animals to the toxicity of

nicotine and of carbon monoxide and are therefore preferred for inhalation studies with tobacco smoke.

II. Bioassays with Cigarette Smoke Particulate Matter

Inhalation assays with Syrian golden hamsters have demonstrated that only whole smoke induces benign and malignant tumors of the respiratory tract in a dose-dependent fashion. However, inhalation of smoke which is free of particulate matter ("tar") does not lead to tumors. This indicates that the dose of carcinogens in the gas phase by itself is not sufficient to induce tumors and that the majority of the carcinogens reside in the particulate matter of tobacco smoke. This consideration has led to in-depth fractionation studies and bioassays with tobacco smoke condensate in mice, rats and rabbits (6, 23, 24). The neutral subfractions B and BI that contain a concentrate of the polynuclear aromatic hydrocarbons (PAH), harbor the major tumor initiators (Figure 2). The PAH subfraction is also the only portion of the tar that, upon repeated intratracheal instillation, elicits tumors in the respiratory tract of rats (25).

Assays of the PAH concentrate explain only a small fraction of the total carcinogenicity of the tar. Results from bioassays of the PAH-subfraction in combination with the weakly acidic, non-carcinogenic fraction explain 70-90% of the carcinogenicity of the whole tar (23, 24). The weakly acidic fraction contains the major tumor promoters, volatile phenols, and the major cocarcinogens, catechols. In addition to tumor initiators, tumor promoters and cocarcinogens, tobacco smoke also contains carcinogens with organ-specificity. These act independently of the mode of exposure or site of application, by inducing benign and malignant tumors in specific organs. Table 1 presents a list of the known tumorigenic agents in tobacco smoke, their concentrations in the smoke of one cigarette, and the evaluation of evidence of their carcinogenicity by the International Agency for Research on Cancer (26 27). Table 2 is a listing of the likely causative agents for tobacco smoke-related cancers on the basis of organ-specificity of carcinogens and their various biological activities and concentrations in cigarette smoke.

The agents in tobacco smoke most likely to cause induction of cancer of the respiratory tract are PAH, the tobacco-specific N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the volatile aldehydes, acetylaldehyde and formaldehyde and, to a minor extent, polonium-210 (from agricultural and environmental sources).

III. Bioassay on Mouse Skin

Inhalation studies with Syrian golden hamsters have clearly demonstrated that the major carcinogenic activity of whole cigarette smoke resides in its particulate matter (tar), as discussed earlier. This has led to extensive bioassays of cigarette tar in both the connective tissue of rats and the skin of mice (6). Since the induction of sarcoma in the connective tissue of rats can be influenced by the physical form of the tar, by the presence of insoluble particles (Oppenheimer-Nothdurft effect; 28, 29) the mouse skin bioassay is now the preferred method for estimating the tumor potency of smoke condensates especially when comparing tars of experimental cigarettes that vary from the control cigarette only in a few aspects.

The tars obtained from a smoking machine (Chapter B), such as a Borgwaldt-30 cigarette smoker (30) or other devices (6), are stored in the dark at refrigerator temperature until needed for biological testing, but should not be older than 3 weeks when applied. Before use, the suspensions in acetone are thoroughly mixed in a mechanical shaker for at least 3 hours, a sample is poured into a 60-ml glass-stoppered reagent bottle. Since tars are not always fully dissolved in the acetone (1:1), vigorous shaking of the bottle is essential before each use.

Anywhere from 30 to 100, usually 50 but preferably 100, Ha/ICR/Mil (Swiss albino) female mice are used for each tar to be tested. The random bred Ha/ICR/Mil (Swiss albino) mice are sturdy animals, and they are quite resistant to nicotine toxicity. Compared to two inbred strains of mice often used in skin carcinogenesis (CAF_1, C57BL), they are more susceptible to the carcinogenic activity of tobacco tars (31). Female mice are used for the bioassay since they do not fight as do the males, which results in skin scratches. Thus, females can be housed 5 to a cage, while males require one cage for each mouse. The maintenance of female mice is therefore significantly more cost effective (6). At the onset of bioassays the mice are 5 to 7 weeks of age and weigh 22 to 25 g. They receive feed and water ad libitum. Their cages are cleaned twice weekly.

Before each tar application, the dorsal hair of the mice is shaved with a Model A2 (size 40) Oster animal clipper. The tar is then applied in 0.1 ml of an acetone suspension containing 50 mg tar with a full No. 5 camel hair brush, or by pipette. The treatment is repeated three times weekly, allowing at least one day between applications for absorption of the tar before the next application. It is sometimes necessary, especially at the onset of the experiment, to skip a painting if the mice exhibit poor absorption or low tolerance of the tar.

Mice that survive the first month usually tolerate the toxic effects of the tar solutions (LD_5). All mice lost during the first month in an experiment are replaced by mice of the same age. Therefore, the initial number of animals to be scheduled for each assay must exceed the requirements for the control and experimental groups by about 10%. If the toxicity persists, even though the number of applications is cut down, the tar must be applied at a lower concentration with the necessary revision of the protocol. In recent years, however, such modifications have usually not been necessary, owing to the generally lower levels of nicotine in tobaccos. The bioassay is not terminated until 90% of the mice in the tar group with the longest survival rate have died or were moribund and had to be killed; this takes usually 18-20 months of tar application.

Average weights of the mice are recorded at the onset of the experiment and again at 2 weeks, 4 weeks, and thereafter at monthly intervals as an indicator of the general health of the mice. A reduction in weight could be associated with a reduced tumor yield, particularly if the weight loss takes place in the tumor promotion phase. It is essential that weight records be kept in experiments with tobacco carcinogenesis.

Constant observation of the animals is also essential. Any change in appearance, habit, or reaction is noted; any lesion on the back is described as to [1] type, i.e. ulcer, infection, or tumor; [2] date of appearance or change in appearance; and [3] exact location. When such a notation is first made, the animal, is marked on the head with a yellow dye (picric acid). Diagrammatic representations of the animal's back are used to facilitate the recording.

The application of the test material by painting or pipetting may lead to benign skin tumors which are recorded when they have attained a diameter of 1 mm. They enlarge by nodular growth (papilloma) or by lateral invasion (carcinoma); some may not enlarge, but regress. Those tumors that remain 1 mm or grow larger for 21 consecutive calendar days are counted and become the raw tumor yield data. Continued growth of such lesions, however, is required before they can be recorded as macroscopically observed carcinomas (raw tumor yield data). A revised count is reported after histopathological confirmation of the macroscopically observed lesions. Lateral invasion of the tumor into adjacent skin is considered as transformation into a carcinoma.

Mice with carcinomas are killed by cervical dislocation and tumors are excised for subsequent histopathological analysis. All suspicious lesions are likewise examined. The animals are autopsied for distant metastases and the occurrence of other tumors, especially pulmonary adenomas and lymphomas.

The most careful recording of experimental observations demands equally careful statistical evaluation of the final data. Therefore, some pertinent statistical considerations will be discussed.

More than 90 mice, rats, or hamsters per group should be used in carcinogenicity bioassays with tobacco smoke condensates. We calculated that to find a difference with 80% statistical power between the carcinogenicity of condensates from an experimental cigarette [with reduced ignition propensity] vs. a control would require at least 90 animals in each group. An additional 30 are also needed for the negative control group.

The lethal toxicity of some smoke condensates require that bioassays be carried out with sufficient animals to achieve statistical soundness. Mice lost during the first month of an experiment are replaced.

Assuming, as an example, that none of the animals in a control group has a tumor and 6 or more animals in the experimental group have tumors, one may utilize the table prepared by Vos based on chi-square analysis with "Yates correction". This shows that the difference between the groups is significant at $P < 0.05$ when the number of animals in each group varies from 10 to 50 or more.

This situation merely identifies that one is dealing with a tumorigenic agent and does not allow a quantitative assay of tumor-producing agents of varying potency. If, for example, there are as many as 4 tumor-bearing animals in a given group treated with a weak carcinogen, the number of animals in each group becomes important. If there were 10 animals in each group, all of them must bear tumors to indicate a difference at the $P < 0.05$ level of significance. In a group of 50 animals treated with a stronger tumorigenic substance, only 12 need to show tumors. Information about differences in tumor yield necessary for significance at $P < 0.05$ between two groups for groups of 10 to 50 animals may be readily extracted from tables by the Food Protection Committee of the U.S. Academy of Science (30).

IV. Inhalation Bioassay with Syrian Golden Hamsters

Inhalation bioassays with whole smoke are also strongly indicated to confirm the relative carcinogenic potencies of respective condensates in mouse skin painting bioassays. As discussed earlier, the Syrian golden hamster (SGH) is presently the animal of choice for long-term inhalation assays with whole cigarette smoke (6, 20-22). It should be noted that inhalation bioassays with whole cigarette smoke will rarely lead to lung tumors in SGH or rats (9), but it will lead to papilloma and carcinoma in the larynges of the animals. Since the larynges of

inbred strains of male SGH are apparently most susceptible to the carcinogenic effects of cigarette smoke (21, 22) one is inclined to prefer this animal model.

Inbred strains may be difficult to obtain are not as resistant to the acute toxicity of the smoke as are random-bred SGH. Thus, the latter are generally used for inhalation studies (7, 31).

Three inhalation devices have been developed for exposure of SGH and rats to cigarette smoke. These are the "Hamburg II" device (20, 34), the "Oak Ridge" smoke inhalation exposure device (9, 17) and the "Walton-reverse smoker" (21). All 3 machines are well developed and the "Oak Ridge" device has especially favorable features in respect to forced smoke inhalation by laboratory animals. However, the "Hamburg II" device with SGH is recommended for comparing the tumorigenicity of whole smoke from various cigarettes. The device has been widely used and most data on the tumorigenicity of whole cigarette smoking, including a dose-response study, were generated with it (15, 20, 34). In general this bioassay requires 24-26 months.

The inhalation studies with SGH generally consists of twice daily exposures to air-diluted smoke (7:1) of one cigarette each, seven times weekly, for the entire lifespan of the animals. Because of the high CO concentration in undiluted smoke (2.8 - 4.6 vol%) the maximum tolerated dose is typically a 10 minute exposure twice daily of cigarette smoke diluted by air 1:7. Ten SGH at at time can be exposed concurrently to diluted cigarette smoke from one Hamburg II device. Since 80 male hamsters are needed for each test cigarette, the bioassay is very labor intensive and is recommended only as a last step in the cascade of assays. Details for the inhalation assay with SGH are presented in Dontenwill (15, 20). These exposure protocols are not intended to mimic human smoking behavior (see Topography chapter), but are intended to produce tumorigenic effects that can be statistically evaluated.

Inhalation assays using F344 rats and employing the "Oak Ridge" inhalation device for exposure to cigarette smoke (9, 17) appear promising. Although it can not yet be recommended for toxicity testing, it is hoped that the methodology will be confirmed by additional studies.

Critique

Ideally one establishes the carcinogenicity of an inhalable substance by bioassays leading to the induction of benign and malignant tumors in the respiratory tract of laboratory animals. In the case of cigarette smoke this goal has been only partially reached. In the Syrian golden hamster, papilloma and carcinoma

have been induced in the larynx with cigarette smoke in a dose-related fashion. However, with a few exceptions, squamous cell tumors of the lung, which are associated with cigarette smoking in humans, are not produced by this model (15, 20-22). Even with an advanced smoke inhalation device, only a few lung carcinomas were produced in rats (9, 17).

Epidemiologists identify several hundred prospective and case-control studies demonstrating that cigarette smokers face an increased risk for lung cancer. Therefore, confirmation by inhalation bioassays is not necessary.

Simulation of human smoking behavior in terms of deep inhalation of cigarette smoke into the lungs has not been successful in laboratory rodents. However, in comparisons of the relative tumorigenicity of the whole smoke of cigarettes with reduced ignition propensity to that of a control, the inhalation bioassay with hamsters should clearly reflect possible changes in the carcinogenic potential in the number of tumors observed in the larynges.

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References

1. U.S. Department of Health and Human Services. Reducing the Health Consequences of Smoking. 25 Years of Progress. A Report of the Surgeon General. DHHS Publ. No. (CDC) 89-8411, 1989, 703 pp.

2. U.S. Department of Health and Human Services. The Health Consequences of Smoking. Chronic Obstructive Lung Disease. DHHS (PHS) 84-50205, 1984.

3. Brown, L.M., Gibson, R., Blair, A., Burmeister, L.F., Schuman, L.M., Cantor, K.P., and Fraumeni, Jr. J.F. Smoking and risk of leukemia. Am. J. Epidemiol. 1992; 136: 763-768.

4. Shopland, D.R., Eyre, H.J., and Pechacek, J.F. Smoking - attributable cancer mortality in 1991: Is lung cancer now the leading cause of death among smokers in the United States? J. Natl. Cancer Inst. 1992; 83: 1142-1148.

5. International Agency for Research on Cancer. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. 1986; 38: 421 p.

6. Wynder, E.L., and Hoffmann, D. Tobacco and Tobacco Smoke Studies in Experimental Carcinogenesis. Academic Press, New York, 1967; 730 pp.

7. Mohr, U., and Reznik, G. Tobacco carcinogenesis. In: Pathogenesis and Therapy of Lung Cancer. C.C. Harris, ed., Marcel Dekker, Inc., New York, 1978; pp. 263-367.

8. Wynder, E.L., and Covey, L.S. Epidemiologic patterns in lung cancer by histologic type. Europ. J. Cancer Clin. Oncol. 1987; 23: 1491-1496.

9. Dalbey, W.E., Nettesheim, P., Griesemer, R., Caton, J.E., and Guerin, M.R. Chronic inhalation of cigarette smoke by 344 rats. J. Natl. Cancer Inst. 1980; 64: 383-390.

10. Nettesheim, P., and Griesemer, R.A. Experimental models for studies of respiratory tract carcinogenesis. In: Pathogenesis and Therapy of Lung Cancer. C.C. Harris, ed., Marcel Dekker, Inc., New York, 1978; pp. 75-188.

11. Leuchtenberger, C., Leuchtenberger, R., and Doolin, P.F. A correlated histological, cytological and cytochemical study of tracheobronchial trees and lungs of mice exposed to cigarette smoke. I, II, and III. Cancer 1958, 11: 490-506; 1960, 13: 721-732; 1960, 13: 956-958.

12. Leuchtenberger, C. and Leuchtenberger, R. Einfluss chronischer Inhalationen von frischem Zigarettenrauch und dessen Gasphase auf die Entwicklung von Lungentumoren bei Snell's Maeusen. Z. Praeventivmed. 1970; 17: 457-462.

13. Otto, H. Experimentelle Untersuchungen an Maeusen mit passiver Zigarettenrauchbeatmung. Frankf. Z. Pathol. 1963; 73: 10-23.

14. LaVoie, E.J., Prokopczyk, B., Rigotty, J., Czech, A., Rivenson, A., Adams, J.D. Tumorigenic activity of the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (iso-NNAL) and N'-nitrosonornicotine (NNN) on topical application to Sencar mice. Cancer Lett. 1987; 37: 277-283.

15. Dontenwill, W. Experimental investigations on the effect of cigarette smoke inhalation on small laboratory animals. In "Inhalation Carcinogenesis", M.G. Hanna, Jr., R. Nettesheim, and P. Gilbert, Jr., eds. AEC Symp. Ser. 1970; 18: 389-412.

16. Hoffmann, D. and Wynder, E.L. Chamber development and aerosol dispersion. In "Inhalation carcinogenesis", M.O. Hanna, Jr., P. Nettesheim, and P. Gilbert, Jr., eds., AEC Symp. Ser. 1970; 18: 173-189.

17. Maddox, W.L., Dalbey, W.E., Guerin, M.R., Stockley, J.R., Creasia, D.A., and Kendrick, J. A tobacco smoke inhalation exposure device for rodents. Arch. Envrion. Health 1978; 33: 64-71.

18. Gupta, R.C., Sopori, M.L., and Gairola, C.G. Formation of cigarette smoked-induced DNA adducts in the rat lung and nasal mucosa. Cancer Res. 1989; 49: 1916-1920.

19. Bond, J.A., Chen, B.T., Griffith, W.C., and Mauderly, J.L. Inhaled cigarette smoke induces the formation of DNA adducts in lungs of rats. Toxicol. Appl. Pharmacol. 1989; 99: 161-172.

20. Dontenwill, W., Chevalier, H.-J., Harke, H.-P., Lafrenz, U., Reckzeh, G., and Schneider, B. Investigations on the effect of chronic cigarette smoke inhalation in Syrian golden hamsters. J. Natl. Cancer Inst. 1973; 51: 1781-1832.

21. Bernfeld, P., Homburger, F., and Russfield, A.B. Strain differences in the response of inbred Syrian hamsters to cigarette smoke inhalation. J. Natl. Cancer Inst. 1974; 53: 1141-1157.

22. Bernfeld, P., Homburger, F., Soto, E., and Pai, K.J. Cigarette smoke inhalation studies in inbred Syrian golden hamsters. J. Natl. Cancer Inst. 1979; 63: 675-689.

23. Hoffmann, D. and Wynder, E.L. A study of tobacco carcinogenesis XI. Tumor initiators, tumor accelerators, and tumor promoting activity of condensate fractions. Cancer 1971; 27: 848-864.

24. Dontenwill, W., Chevalier, H.-J., Harke, H.-P., Klimisch, H.-J., Brune, H., Fleischmann, B., and Keller, W. Experimentelle Untersuchungen ueber die tumorerzeugende Wirkung von Zigarettenrauch-Kondensaten an der Maeusehaut. VI Untersuchungen zur Fraktionierung von Zigarettenrauch-Kondensat. Z. Krebsforsch. 1976; 85: 155-167.

25. Davis, B.R., Whitehead, J.K., Gill, M.E., Lee, P.N., Butterworth, A.D., and Roe, F.J.C. Response of rat lung to tobacco smoke condensate or fractions derived of it administered repeatedly by intratracheal instillation. Brit. J. Cancer 1975; 31: 453-461.

26. International Agency for Research on Cancer. Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs 1 to 42. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans 1987; Suppl. 7: 440, p.

27. International Agency for Research Cancer. Radon. IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans, 1988; 43: 173-259.

28. Oppenheimer, B.S., Oppenheimer, E.T., Stout, A.P. and Danishefsky, I. Malignant tumors resulting from embedding plastics in rodents. Science, 1955; 118: 305-306.

29. Nothdurft, H. Uber die Sarkomausloesung durch Fremdkrperimplantationen bei Ratten. Abhangigkeit von der Form der Implantate. Naturwissenschaften, 1955; 42: 106.

30. Elmenhorst, H. Eine vollautomatische Rauchmaschine für die Herstellung grosser Mengen von Cigarettenrauchkondensaten. Beitr. Tabakforsch., 1966; 3: 545-553.

31. Wynder, E.L., Graham, E.A., and Croninger, A.B. Experimental production of carcinoma with cigarette tar. II. Tests with different mouse strains. Cancer Res. 1955; 15: 445-448.

32. Food Protection Committee, Food Nutrition Board. Natl. Acad. Sci-Natl. Res. Council Publ. 1959; 749 pp.

33. Karbe, E. and Park, J.F. Experimental Lung Cancer. Carcinogenesis and Bioassays. Springer Publ. New York, 1974, 611 pp.

34. Kobayashi, N., Hoffmann, D., and Wynder, E.L. A study of tobacco carcinogenesis. XII. Epithelial changes induced in the upper respiratory tracts of Syrian golden hamsters by cigarette smoke. J. Natl. Cancer Inst., 1974; 53: 1085-1089.

Table 1 Tumorigenic agents in tobacco and tobacco smoke

Compounds	In processed tobacco (per g)	In Mainstream smoke (per cigarette)	IARC evaluation of evidence of carcinogenicity[a]	
			In laboratory animals	In humans
PAH				
Benz[a]anthracene		20 - 70 ng	sufficient	
Benzo[b]fluoranthene		4 - 22 ng	sufficient	
Benzo[j]fluoranthene		6 - 21 ng	sufficient	
Benzo[k]fluoranthene		6 - 12 ng	sufficient	
Benzo[a]pyrene	0.1 - 90 ng	20 - 40 ng	sufficient	probable
Chrysene		40 - 60 ng	sufficient	
Dibenz[a,h]anthracene		4 ng	sufficient	
Dibenzo[a,i]pyrene		1.7 - 3.2 ng	sufficient	
Dibenzo[a,l]pyrene		present	sufficient	
Indeno[1,2,3-cd]pyrene		4 - 20 ng	sufficient	
5-Methylchrysene		0.6 ng	sufficient	
Aza-arenes				
Quinoline	1 - 2 µg			
Dibenzo[a,h]acridine		0.1 ng	sufficient	
Dibenzo[a,j]acridine		3 - 10 ng	sufficient	
7H-Dibenzo[c,g]-carbasole		0.7 ng	sufficient	
N-Nitrosamines				
N-Nitrosodimethylamine	ND - 215 ng	0.1 - 180 ng	sufficient	
N-Nitrosoethylmethylamine		3 - 13 ng	sufficient	
N-Nitrosodiethylamine		ND - 25 ng	sufficient	
N-Nitrosonornicotine	0.3 - 89 µg	0.12 - 3.7 µg	sufficient	
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	0.2 - 7 µg	0.08 - 0.77 µg	sufficient	
N'-Nitrosoanabasine	0.01 - 1.9 µg	0.14 - 4.6 µg	limited	
N-Nitrosomorpholine	ND - 690 ng		sufficient	
Aromatic amines				
2-Toluidine		30 - 200 ng	sufficient	inadequate
2-Napthylamine		1 - 22 ng	sufficient	sufficient
4-Aminobiphenyl		2 - 5 ng	sufficient	sufficient
Aldehydes				
Formaldehyde	1.6 - 7.4 µg	70 - 100 µg[b]	sufficient	
Acetaldehyde	1.4 - 7.4 µg	18 - 1400 µg[b]	sufficient	
Crotonaldehyde	0.2 - 2.4 µg	10 - 20 µg		
Miscellanous organic compounds				
Benzene		12 - 48 µg	sufficient	sufficient
Acrylonitrile		3.2 - 15 µg	sufficient	limited
1,1-Dimethylhydrazine	60 - 147 µg		sufficient	
2-Nitropropane		0.73 - 1.21 µg	sufficient	
Ethylcarbamate	310 - 375 ng	20 - 38 ng	sufficient	
Vinyl chloride		1 - 16 ng	sufficient	sufficient
Inorganic compounds				
Hydrazine	14 - 51 ng	24 - 43 ng	sufficient	inadequate
Arsenic	500 - 900 ng	40 - 120 ng	inadequate	sufficient
Nickel	2000 - 6000 ng	0 - 600 ng	sufficient	limited
Chromium	1000 - 2000 ng	4 - 70 ng	sufficient	sufficient
Cadmium	1300 - 1600 ng	41 - 62 ng	sufficient	limited
Lead	8 - 10 µg	35 - 85 ng	sufficient	inadequate
Polonium-210	0.2 - 1.2 pCi	0.03 - 1.0 pCi	sufficient	sufficient

[a] No designation indicates that an evaluation by IARC has not been carried out. [b] The 4th report of the Independent Scientific Committee on Smoking and Health (1988) published values for the 14 leading British cigarettes in 1986 (51.4% of the market) of 20-105 µg/cigarette (mean 59 µg) for formaldehyde and 550-1150 µg/cigarette (mean 910 µg) for acetyladehyde. PAH, polynuclear aromatic hydrocarbons; ND, not detected.

Table 2

Likely Causative Agents for Tobacco-Related Cancers

Organ	Initiator or Carcinogen	Enhancing Agents
lung, larynx	NNK acetaldehyde formaldehyde polonium^{210} (minor)	acrolein, crotonaldehyde(?)
	PAH	catechol (cocarcinogen) weakly acidic tumor promoters
esophagus	NNN NAB	ethanol, catechol
pancreas	NNK NNAL	nutrition
bladder	4-aminobiphenyl 2-naphthylamine other aromatic amines	infectious agents(?)
oral cavity	PAH NNN NNK	ethanol herpes simplex

NAB - N'-nitrosoanabasine
NNAL - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN - N'-nitrosonornicotine
PAH - polyaromatic hydrocarbons

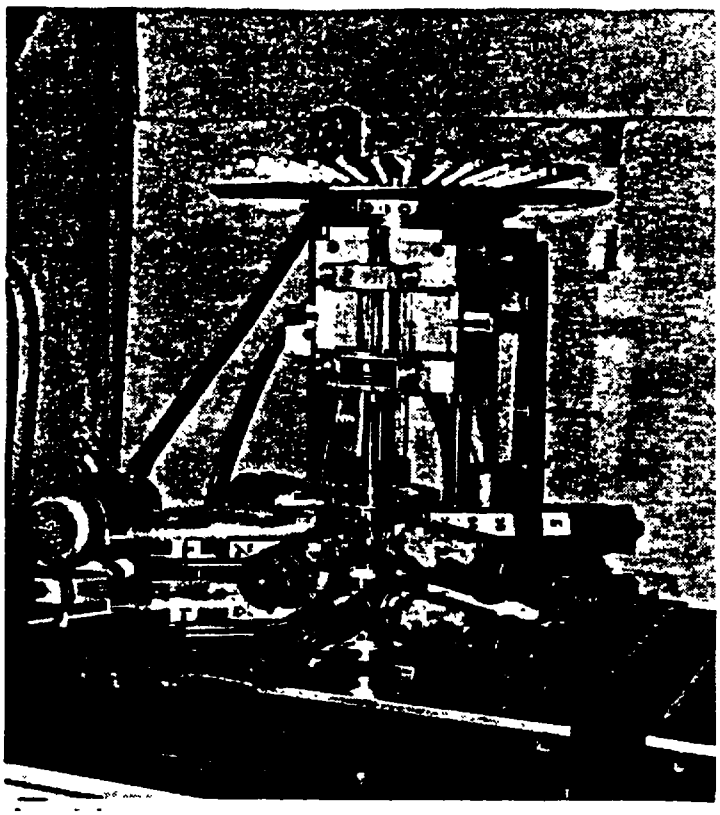

Fig.1 Hamburg II Smoke-Inhalation Device for 10 Hamsters (16).

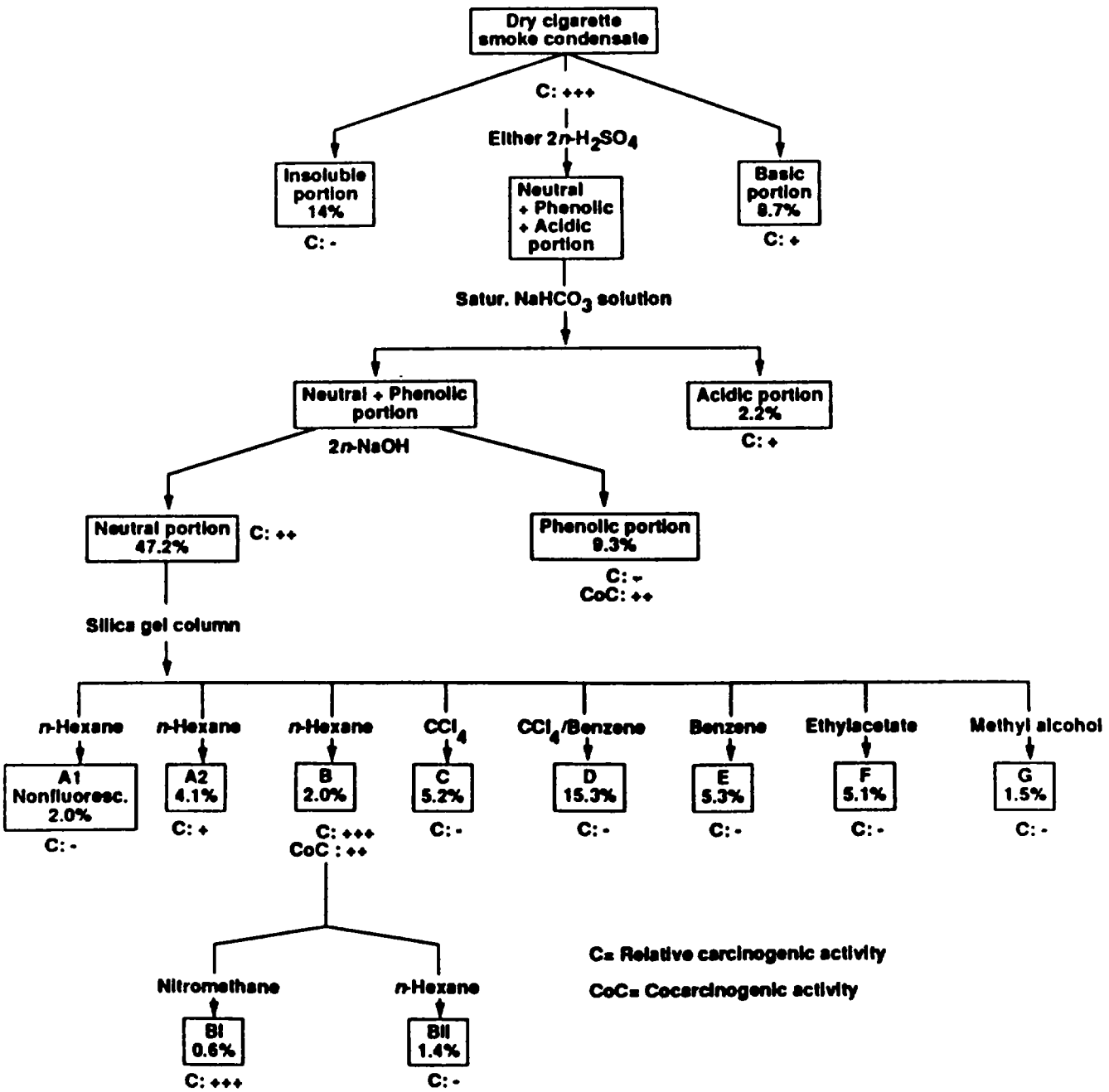

Fig.2 Fractionation of Cigarette Smoke Condensate (23).

Glossary of Terms

adenocarcinoma	malignant tumor of a glandular structure, such as in the peripheral lung
adenoma	benign tumor of a glandular structure, such as in the peripheral lung
carcinoma	malignant tumor of epithelial orgin
DNA	deoxyribonucleic acid. DNA is localized in the cell nucleus and is the molecular basis of heredity in many organisms
genotoxicity	damage to the DNA structure
in vitro	experimentation with microorganisms, isolated cells, tissues, or isolated organs in biological media
in vivo	experimentation with live animals, such as mice, rats and hamsters
papilloma	benign tumors (warts) due to a proliferation of epithelial tissue

Cost Estimates for Carcinogenicity Bioassays

Bioassay	Animal	Groups	Number of Animals[2]	Cost[1]
Inhalation	SG hamster (random bred)	exptl. cigarette I	60	$185,000-$250,000[3]
		exptl. cigarette II	60	
		sham control	60	
Skin	mouse (Ha/ICR/Mil)	exptl. cigarette I	90	$39,000
		exptl. cigarette II	90	
		sham control	60	

[1] Estimates pertain only to direct cost. The costs exclude the overhead as approved for individual institutes by the U.S. Department of Health and Human services. Direct total costs include animal purchase, health screening of the animals, maintenance, treatment (smoking of hamsters or tar application to mouse skin), weighing (first 8 weeks weekly, subsequently monthly), recording, autopsy and histology.

Estimates do not include purchase of cigarettes (inhalation study requires about 280,000 cigarettes/group; mouse skin bioassay 1.5 kg/group requires about 75,000 cigarettes, assuming one cigarette yields 20 mg tar), or the smoking of cigarettes for the preparation of the tar for the mouse skin bioassays.

[2] The number of animals per group is calculated for a difference between two groups with 80% statistical power either for the tumorigenic activity in the larynx of hamsters of whole cigarette smoke or for the tumorigenic activity on mouse skin of a tar.

[3] This includes the overtime for twice daily exposure on Saturday and Sunday.

Part II

VOLUME 5
PART II

LIMITED TIER I AND II DEMONSTRATION OF THE TOXICITY TESTING PLAN FOR LOW IGNITION-POTENTIAL CIGARETTES

U.S. Consumer Product Safety Commission
in consultation with the
U.S. Department of Health and Human Services

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July 27, 1993

I. Introduction

The Toxicity Testing Plan for Low Ignition-Potential Cigarettes was written to fulfill part of the responsibilities given to the Consumer Product Safety Commission (CPSC) by the Fire-Safe Cigarette Act of 1990. The Plan identified various toxicological tests and associated direct cost estimates. Limited testing from Tiers I and II was recommended as a first step toward the implementation of the Plan.

CPSC staff, in consultation with the Department of Health and Human Services (HHS), directed a demonstration of this limited testing to verify its feasibility and costs. This study was designed demonstrate the ability of the testing to distinguish among and between cigarette brands/types. It was not designed to directly compare brands/types against each other since the prototypes were not chosen as intended replacements for the commercial cigarettes selected. All testing recommended in the first step was conducted, except for the pH test.

II. Experimental

Five cigarette brands/types were tested in this demonstration. Two commercial brands, "K" and "L", were selected from among those with the highest current sales in the U.S. The two prototypes selected, #530 and #531, showed low ignition-potential in preliminary data from the National Institute of Standards and Technology (NIST). A University of Kentucky standard reference medium tar / medium nicotine cigarette, #1R3F, was included in the testing. All cigarettes contained filters.

Except for the reference type, the cigarettes tested were randomly selected from among 300 (or 1.5 cartons) of each brand/type sent to each laboratory by NIST and CPSC staff. The average values were derived from machine smoking 100 cigarettes of each brand/type according to the Federal Trade Commission (FTC) protocol (Chapter B in the Toxicity Testing Plan).

Two contract laboratories performed the testing. The Tobacco Health and Research Institute at the University of Kentucky measured the standard FTC parameters of tar, nicotine, total particulate matter, and water. The number of puffs per cigarette was recorded. The weight of tobacco burned was estimated by weighing the amount of tobacco that would normally be burned during machine smoking in 50 cigarettes of each type. This provided sufficient data to compare results on per cigarette, per tobacco weight burned, and per tar weight bases. Per puff data are not shown, but can be calculated from the data in Tables 1-6.

The American Health Foundation conducted analyses of benzo(a)pyrene and four tobacco-specific nitrosamines, N'-nitrosonornicotine (NNN),

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosoanabasine (NAB), and N'-nitrosoanatabine (NAT), as described in Chapter D of the Plan. The Foundation also ran the *Salmonella* mutagenicity (Ames) assay, as described in Chapter E. Data from the Tobacco Health and Research Institute were used to express the results on per tobacco weight burned and per tar weight bases.

III. Results

Tables 1 and 2 summarize the results of testing the standard FTC parameters for cigarettes #530, #531, K, L, and the #1R3F reference. The words "soft packed cigarette", " total particulate matter" and "carbon monoxide" are abbreviated as "SPF", "TPM", and "CO" respectively, in these tables. The averages are shown on a per cigarette basis in Table 1 and on a per tobacco weight burned in Table 2. The range of data for nicotine was 29-58 mg/g, "tar" 24-47 mg/g, and carbon monoxide 20-39 mg/g (Table 2). Tables 3 through 7 show the data according to run and port number, and also the estimated weight of tobacco burned for each brand/type tested.

Table 8 contains data for the levels of four tobacco-specific nitrosamines in the cigarettes. The range of data for NNN was 181-458 ng/g, NAT 210-421 ng/g, NAB 42-72 ng/g, and NNK 148-319. The range of the total tobacco-specific nitrosamine data was 582-1140 ng/g.

Results for benzopyrene levels could not generated in time for publication of this document due to laboratory technical difficulties. An addendum will contain this data.

Tables 9 through 12 summarize the mutagenicity as indicated by the number of revertant colonies in the Ames assay. A positive mutagenic effect is indicated when the response is greater than twice the spontaneous revertant rate and is denoted by an asterisk. Table 9 indicates the three tester strains are operating properly in the Ames test system.

All cigarette "tars" were mutagenic when the microsomal S-9 fraction was added (Tables 10-12). About 1.5 times more revertants occurred with the more mutagenic tars, in the presence of S-9, compared to the other tars.

IV. Discussion and Conclusions

The limited testing conducted in this demonstration can distinguish differences between and among cigarette brands/types. This indicates that the tests recommended in the Toxicity Testing Plan, at least in Tiers I and II, can generate data that will be

useful in evaluating the potential changes in toxicity of low ignition-potential cigarettes.

Minor changes in the specifications of the Ames assay are recommended. The TA1535 strain of Salmonella gave negative results for all condensates tested in the demonstration study. However, the positive control for that strain, sodium azide, indicated that the TA1535 strain was functioning properly. Only a small response was obtained with TA1538. This suggests that these two strains may not provide useful data about differences in toxicity for the condensates. After consultation with the testing laboratories, it is recommended that the Toxicity Testing Plan use TA98 and TA1537 strains, unless it is suspected that non-frameshift type mutations might occur.

A dose-related response is typically seen in the Ames assay when testing tobacco smoke condensates. However, the lack of a dose-related response in Tables 10-12 suggests that the concentration range tested might be too high or that other toxicities, such as cell death, might be occurring. Data from the dose-related response range is more useful for comparing toxicity. Therefore, the initial test concentration should be adjusted to define the beginning and slope of this range.

The testing costs for this demonstration were within the $6,900 in direct costs per brand/type estimated in the Plan for the first step of implementation. CPSC staff contracted the testing for under $3,000 per brand/type, but the commercial rate could be somewhat higher. On the other hand, the economies of large volume testing that could be conducted by manufacturers can be expected to reduce the cost per brand/type. The estimates stated in the Plan appear to be reasonable, at least for Tiers I and II.

TABLE 1

Summary of the Results of FTC Parameters
Average per Cigarette

CIGARETTE CODE	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
#530	28.00	1.93	3.67	22.40	18.7	8.26
sd	*1.62*	*0.06*	*0.54*	*1.50*	*0.9*	*0.32*
#531	16.97	1.27	1.84	13.86	12.1	6.60
sd	*1.06*	*0.06*	*0.45*	*0.65*	*1.4*	*0.29*
K	17.33	1.11	1.81	14.42	12.6	7.74
sd	*0.98*	*0.04*	*0.37*	*0.70*	*0.7*	*0.21*
L	19.80	1.50	1.68	16.61	12.7	8.97
sd	*0.63*	*0.05*	*0.27*	*0.45*	*0.6*	*0.33*
#1R3F	21.20	1.31	3.31	16.57	19.0	8.09
sd	*0.95*	*0.07*	*0.42*	*0.63*	*0.9*	*0.28*

TABLE 2

Summary of the Results of FTC Parameters
Average per Weight of Tobacco Burned

CIGARETTE CODE	TPM mg/g	NICOTINE mg/g	WATER mg/g	TAR mg/g	CO mg/g	PUFFS /g
#530	58.22	4.01	7.63	46.57	38.9	17.18
sd	*3.37*	*0.12*	*1.23*	*3.12*	*1.9*	*0.67*
#531	35.35	2.64	3.84	28.86	25.2	13.75
sd	*2.21*	*0.13*	*0.94*	*1.35*	*2.9*	*0.60*
K	29.18	1.86	3.04	24.28	21.2	13.04
sd	*1.65*	*0.07*	*0.62*	*1.18*	*1.2*	*0.35*
L	31.19	2.37	2.65	26.16	20.0	14.13
sd	*0.99*	*0.08*	*0.43*	*0.71*	*0.9*	*0.52*
#1R3F	30.50	1.88	4.76	23.98	27.34	11.64
sd	*1.37*	*0.10*	*0.60*	*0.91*	*1.29*	*0.40*

TABLE 3
Measurement of FTC Parameters
Cigarette #530

EXPERIMENTAL 92-30 FK 100'S
TIPPING LENGTH 32 MM
BUTT LENGTH 35 MM

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	2	27.22	1.94	3.39	21.89	18.2	7.90
C06	19APR93	12	28.56	1.94	3.73	22.89	20.0	8.98
C06	19APR93	17	27.34	1.88	3.33	22.13	19.6	8.08
C07	19APR93	3	27.84	1.97	3.83	22.04	19.2	8.24
C07	19APR93	13	26.08	1.82	3.25	21.00	17.7	8.40
C07	19APR93	18	26.48	2.03	3.05	21.40	17.6	9.02
C08	20APR93	4	27.42	1.90	3.66	21.86	18.7	7.98
C08	20APR93	9	28.42	1.99	3.69	22.73	18.8	8.04
C08	20APR93	14	27.10	1.86	3.12	22.12	18.9	8.62
C08	20APR93	19	28.78	2.02	3.60	23.16	19.6	8.24
C09	20APR93	5	28.40	1.93	4.35	22.12	19.6	8.40
C09	20APR93	10	27.32	1.95	3.90	21.48	18.1	8.24
C09	20APR93	15	26.42	1.87	3.47	21.08	16.4	8.56
C09	20APR93	20	28.56	1.98	3.94	22.63	19.4	8.32
C10	21APR93	1	27.58	1.88	4.04	21.66	18.9	7.92
C10	21APR93	6	28.16	1.97	3.59	22.60	19.3	8.12
C10	21APR93	11	30.46	1.96	4.87	23.64	19.7	8.14
C10	21APR93	16	32.80	1.91	2.95	27.94	18.4	8.00
C11	21APR93	4	29.50	2.01	4.70	22.79	18.5	8.06
C11	21APR93	7	25.60	1.81	2.92	20.86	17.6	8.00
	AVERAGE		28.00	1.93	3.67	22.40	18.7	8.26
	STD DEV		1.62	0.06	0.54	1.50	0.9	0.32
AVG/GM TOB BURNED 0.481			58.22	4.01	7.63	46.57	38.9	17.18

TABLE 4
Measurements of FTC Parameters
Cigarette #531

EXPERIMENTAL 92-31 FK 100'S
TIPPING LENGTH 32 MM
BUTT LENGTH 35 MM

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	3	16.24	1.21	1.72	13.31	11.0	6.22
C06	19APR93	8	17.44	1.25	2.01	14.18	12.3	6.52
C06	19APR93	13	16.04	1.25	1.45	13.34	10.5	6.26
C06	19APR93	18	15.46	1.26	1.16	13.03	16.6	6.96
C07	19APR93	4	17.56	1.33	2.01	14.21	12.2	7.02
C07	19APR93	9	16.12	1.22	1.43	13.47	10.7	6.88
C07	19APR93	14	15.64	1.20	1.41	13.03	11.0	6.62
C07	19APR93	19	15.66	1.23	1.60	12.83	10.0	6.38
C08	20APR93	5	15.92	1.18	1.50	13.24	11.3	6.46
C08	20APR93	10	18.08	1.32	2.20	14.56	12.4	6.54
C08	20APR93	15	17.94	1.32	2.14	14.48	12.0	6.92
C08	20APR93	20	16.50	1.21	1.42	13.88	12.2	6.46
C09	20APR93	1	17.22	1.24	2.07	13.91	12.3	6.30
C09	20APR93	6	17.20	1.28	1.69	14.23	12.1	6.96
C09	20APR93	11	16.46	1.19	1.80	13.47	11.3	6.46
C09	20APR93	16	17.08	1.26	2.06	13.76	11.3	6.88
C10	21APR93	2	18.72	1.30	3.12	14.30	12.0	6.34
C10	21APR93	7	18.30	1.32	2.19	14.79	13.4	7.00
C10	21APR93	12	19.38	1.41	2.52	15.45	13.2	7.02
C10	21APR93	17	16.70	1.26	1.52	13.92	12.3	6.50
C11	21APR93	3	16.40	1.27	1.72	13.40	14.4	6.20
C11	21APR93	12	17.20	1.34	1.83	14.02	11.9	6.26
	AVERAGE		16.97	1.27	1.84	13.86	12.1	6.60
	STD DEV		1.06	0.06	0.45	0.65	1.4	0.29
AVG/GM TOB BURNED 0.480			35.35	2.64	3.84	28.86	25.2	13.75

TABLE 5
Measurement of FTC Parameters
Cigarette K

80MM HPF
TIPPING LENGTH 24 MM
BUTT LENGTH 27 MM

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	4	18.00	1.24	1.75	15.01	13.0	8.32
C06	19APR93	9	17.36	1.15	1.55	14.66	12.2	7.94
C06	19APR93	14	17.48	1.11	1.62	14.75	13.4	7.82
C06	19APR93	19	17.96	1.15	1.85	14.96	12.5	7.92
C07	19APR93	5	17.60	1.14	1.99	14.47	12.6	7.74
C07	19APR93	10	17.40	1.14	1.77	14.49	11.9	7.72
C07	19APR93	15	15.66	1.06	1.65	12.95	10.8	8.28
C07	19APR93	20	15.48	1.06	1.42	13.00	12.2	7.68
C08	20APR93	1	17.02	1.07	1.60	14.35	12.7	7.60
C08	20APR93	6	16.66	1.06	1.52	14.08	12.4	7.64
C08	20APR93	11	17.20	1.09	1.64	14.46	12.2	7.50
C08	20APR93	16	17.58	1.06	1.87	14.65	12.6	7.72
C09	20APR93	2	17.90	1.08	2.47	14.35	13.3	7.52
C09	20APR93	7	16.30	1.04	1.54	13.72	12.6	7.32
C09	20APR93	12	18.28	1.12	2.25	14.91	13.9	7.88
C09	20APR93	17	17.36	1.09	1.89	14.38	13.3	7.72
C10	21APR93	3	17.96	1.13	2.12	14.71	12.8	7.64
C10	21APR93	8	18.38	1.17	1.97	15.24	13.3	7.74
C10	21APR93	13	19.32	1.12	2.75	15.45	12.9	7.50
C10	21APR93	18	17.68	1.12	1.52	15.04	12.1	7.92
C11	21APR93	2	15.42	1.04	1.19	13.19	11.9	7.50
	AVERAGE		17.33	1.11	1.81	14.42	12.6	7.74
	STD DEV		0.98	0.04	0.37	0.70	0.7	0.21
AVG/GM TOB BURNED 0.594			29.18	1.86	3.04	24.28	21.2	13.04

TABLE 6
Measurement of FTC Parameters
Cigarette L

85MM SPF
TIPPING LENGTH 25 MM
BUTT LENGTH 28 MM

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	5	19.10	1.42	1.57	16.11	12.3	9.28
C06	19APR93	10	20.32	1.59	1.83	16.90	12.4	9.32
C06	19APR93	15	19.02	1.55	1.39	16.08	12.1	9.46
C06	19APR93	20	19.12	1.53	1.42	16.16	13.6	9.00
C07	19APR93	1	19.68	1.55	1.76	16.37	12.2	8.80
C07	19APR93	6	20.34	1.46	1.97	16.91	13.6	8.74
C07	19APR93	11	20.96	1.56	2.11	17.29	12.9	9.42
C07	19APR93	16	20.52	1.55	2.14	16.82	12.9	9.08
C08	20APR93	2	18.76	1.42	1.67	15.67	11.6	8.68
C08	20APR93	7	19.52	1.45	1.68	16.39	12.6	9.18
C08	20APR93	12	20.26	1.55	1.46	17.25	13.0	9.16
C08	20APR93	17	20.32	1.50	1.62	17.21	13.4	8.86
C09	20APR93	3	19.74	1.45	1.80	16.49	12.7	8.62
C09	20APR93	8	20.10	1.48	1.75	16.86	13.4	9.12
C09	20APR93	13	20.60	1.54	2.08	16.97	13.1	8.96
C09	20APR93	18	20.58	1.57	1.73	17.28	12.4	9.36
C10	21APR93	4	19.36	1.43	1.60	16.33	12.9	8.18
C10	21APR93	9	19.28	1.47	1.63	16.18	11.5	8.92
C10	21APR93	14	19.20	1.50	1.20	16.49	12.5	8.76
C10	21APR93	19	19.40	1.45	1.18	16.77	13.2	8.46
C11	21APR93	8	19.68	1.52	1.78	16.38	12.9	9.06
	AVERAGE		19.80	1.50	1.68	16.61	12.7	8.97
	STD DEV		0.63	0.05	0.27	0.45	0.6	0.33
AVG/GM TOB BURNED 0.635			31.19	2.37	2.65	26.16	20.0	14.13

TABLE 7
Measurement of FTC Parameters
Cigarette #1R3F

CODE X 1R3F Reference Cigarette 85MM SPF
TIPPING LENGTH 25
BUTT LENGTH 28

RUN #	DATE	PORT	TPM mg/cig	NICOTINE mg/cig	WATER mg/cig	TAR mg/cig	CO mg/cig	PUFFS /cig
C06	19APR93	1	21.26	1.27	3.51	16.48	19.5	7.86
C06	19APR93	6	22.02	1.31	3.30	17.41	19.1	8.16
C06	19APR93	11	22.86	1.41	3.77	17.68	19.9	8.58
C06	19APR93	16	22.70	1.47	3.50	17.73	19.2	8.04
C07	19APR93	2	20.46	1.40	3.15	15.92	16.7	8.08
C07	19APR93	7	20.72	1.34	3.08	16.29	18.7	7.82
C07	19APR93	12	21.34	1.35	3.51	16.48	18.3	8.32
C07	19APR93	17	21.68	1.39	3.71	16.58	18.7	8.56
C08	20APR93	3	20.14	1.25	2.75	16.13	18.2	8.10
C08	20APR93	8	20.00	1.25	3.12	15.63	17.2	7.62
C08	20APR93	13	21.38	1.34	3.25	16.79	19.8	8.34
C08	20APR93	18	19.78	1.17	2.78	15.83	17.7	8.20
C09	20APR93	4	21.78	1.33	3.81	16.64	19.2	7.88
C09	20APR93	9	20.70	1.25	3.35	16.10	18.8	7.86
C09	20APR93	14	21.20	1.22	3.35	16.63	20.2	8.20
C09	20APR93	19	22.02	1.29	3.47	17.26	20.5	7.98
C10	21APR93	5	22.74	1.32	4.43	16.99	19.8	8.18
C10	21APR93	10	22.14	1.32	3.46	17.36	19.6	7.74
C10	21APR93	15	21.06	1.20	3.45	16.42	19.5	8.56
C10	21APR93	20	20.84	1.33	2.73	16.78	20.0	8.10
C11	21APR93	1	21.04	1.30	3.27	16.47	19.3	7.92
C11	21APR93	6	20.00	1.30	2.53	16.18	18.6	8.28
C11	21APR93	11	19.68	1.41	2.89	15.38	18.7	7.64
	AVERAGE		21.20	1.31	3.31	16.57	19.0	8.09
	ST DEV		0.95	0.07	0.42	0.63	0.9	0.28
AVG/GM TOB BURNED 0.695			30.50	1.88	4.76	23.98	27.34	11.64

TABLE 8
Tobacco-specific N-Nitrosamines in Test Cigarettes

brand	NNN	NAT	NAB	NNK	Total
			(ng/cig)		
KY 1R3F, 85 mm	193	210	29.3	222	654
L, 85 mm	287	216	26.5	194	724
K, 80 mm	272	250	25.9	173	721
#530, 100 mm	134	165	34.7	102	436
#531, 100 mm	86.7	101	20.5	70.9	279
			(ng/g tobacco)		
KY 1R3F	278	302	42.2	319	941
L	452	340	41.7	306	1140
K	458	421	43.6	184	1107
#530	279	343	72.1	212	841
#531	181	210	42.7	148	582
			(ng/mg tar)		
KY 1R3F	8.05	8.76	1.22	9.26	27.3
L	17.3	13.0	1.60	11.7	43.6
K	18.9	17.3	1.80	12.0	50.0
#530	5.98	7.36	1.55	4.55	19.4
#531	6.26	7.29	1.48	5.12	20.2

Ames Salmonella/Microsome Mutagenicity Test
Mean Summary Data

Sponsor:	Brunnemann	Date Initiated:	6/4/93
Study number:	LRD57	Date Scored:	6/7/93
Test Articles:	KY 1R3F, K, L, 92-30 (#530), 92-31 (#531)		
Description:	5 tobacco smoke condensates; 4 doses each		
Considerations:	TA98, TA1535, TA1538; +/- Rat S9		

TABLE 9
Controls

			Spontaneous Revertant Colonies/Plate (avg)		
Solvent Controls		S-9	TA98	TA1535	TA1538
DMSO		(-)	22	21	15
DMSO		(+)	34	14	23
Positive Controls (µg/pl)					
Sodium azide	5	(-)	-	1000	-
2-Nitrofluorene	5	(-)	382	-	115
2-Anthramine	5	(+)	1964	114	1874

TABLE 10
Test Compound: KY 1R3F

	Total Revertant Colonies/Plate (avg)			
Dose Level (µg/pl)	S-9	TA98	TA1535	TA1538
50	(-)	25	19	18
100	(-)	41	15	15
200	(-)	52*	21	20
300	(-)	57*	18	22
100	(+)	169*	11	73*
200	(+)	195*	11	69*
300	(+)	157*	12	73*
400	(+)	172*	13	56*

* Positive response (threshold=2.000 x corresponding solvent).

TABLE 11

Test Compound: K

Dose Level (µg/pl)	S-9	Total Revertant Colonies/Plate (avg) TA98	TA1535	TA1538
50	(-)	29	16	9
100	(-)	37	14	14
200	(-)	67*	20	26
300	(-)	81*	15	26
100	(+)	194*	14	76*
200	(+)	162*	12	72*
300	(+)	172*	14	77*
400	(+)	167*	15	71*

Test Compound: L

Dose Level (µg/pl)	S-9	Total Revertant Colonies/Plate (avg) TA98	TA1535	TA1538
50	(-)	36	19	17
100	(-)	40	18	18
200	(-)	47*	20	22
300	(-)	42	16	23
100	(+)	150*	16	71*
200	(+)	156*	14	61*
300	(+)	142*	18	58*
400	(+)	143*	18	59*

* Positive response (threshold=2.000 x corresponding solvent).

TABLE 12

Test Compound: 92-30 (#530)

Dose Level (µg/pl)	S-9	Total Revertant Colonies/Plate (avg) TA98	TA1535	TA1538
50	(-)	34	22	13
100	(-)	32	24	16
200	(-)	37	24	15
300	(-)	46*	17	16
100	(+)	83*	13	53*
200	(+)	102*	20	46
300	(+)	95*	14	54*
400	(+)	94*	11	53*

Test Compound: 92-31 (#531)

Dose Level (µg/pl)	S-9	Total Revertant Colonies/Plate (avg) TA98	TA1535	TA1538
50	(-)	27	15	16
100	(-)	32	19	14
200	(-)	36	20	18
300	(-)	44	19	22
100	(+)	112*	13	43
200	(+)	129*	27	53*
300	(+)	104*	15	51*
400	(+)	85*	18	48*

* Positive response (threshold=2.000 x corresponding solvent).