Safety of Processed Tobaccos:

The Dry Ice Expanded Tobacco (DIET) Process

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Executive Summary

(Is it "biologic" activity or "biological" activity? Which one is proper? It is used both ways in the document. If you can determine which one is most appropriate, I suggest you search the entire document. I am certainly not a grammarian.)

Expansion processes have a long history of use in food and tobacco manufacturing. When using an expansion process in the cigarette industry, the main goal is to increase cured tobacco filling capacity to levels approximating those of the uncured leaf. Tobacco expansion can be achieved using different expansion agents. When carbon dioxide (CO_2) is used as the expansion agent, the process is called DIET (Dry Ice Expanded Tobacco) **(Is this a universally used term or a term used by RJRT?).** Since CO_2 serves as a processing aid during tobacco expansion, a negligible amount of CO_2 may be present in DIET. This document will summarize the DIET process and studies conducted by R. J. Reynolds Tobacco Company (RJRT) to assess the safety of DIET. Data summarized in this paper demonstrate that mainstream cigarette smoke (and smoke condensate) from cigarettes containing DIET has comparable biologic activity to that of reference cigarettes.

In studies performed by the U. S. National Cancer Institute (NCI), use of DIET in cigarettes did not change the biologic activity of mainstream cigarette smoke. Moreover, NCI suggested that mainstream cigarette smoke condensate from cigarettes with expanded tobacco displayed lower *in vivo*, tumorigenic activity in mice compared to condensate from cigarettes without expanded tobacco. Besides *in vivo*, tumorigenicity, NCI studies included mainstream cigarette smoke chemistry and *in vitro* studies. In chemical analyses comparing smoke from cigarettes with expanded (CO_2, Freon 11, freeze-dried) and without expanded tobacco, most of the major smoke chemistry analytes remained unchanged while a few were lower in cigarette smoke from cigarettes with expanded compared to cigarettes without expanded tobacco. *In vitro* studies included **evaluation of** cytotoxicity in KB tumor cells, inhibition of phagocytosis by alveolar macrophages, and ~~cilliary~~ **ciliary** transport inhibition. The only noteworthy results were that smoke condensate from CO_2 expanded tobacco induced more cytotoxicity in tumor cells than condensate from cigarettes without expanded tobacco; however, *in vitro* results have limited relevance. **Because?**

In RJRT studies, mainstream cigarette smoke and smoke condensate from cigarettes with DIET did not exhibit increased biologic activity compared to smoke and smoke condensate from reference cigarettes (with or without Freon-expanded tobacco) **two different reference cigarettes? Which Freon? The NICI Studies used Freon 11. I think you should be specific at this point, as to which Freon was used. We have struggled with the use of the terminology of "reference" cigarettes in the past. Some people automatically consider that a "reference" cigarette is one such as the 1R4F. To avoid any confusion we occasionally use "control" cigarettes.** The RJRT testing strategy used to assess DIET safety included chemical and biological studies. Chemical studies included expansion agent purity verification and target compound level identification in mainstream smoke. Biologic **(Why is "biologic" studies used in this sentence whereas, two sentences earlier, "Biological" was used to modify studies. Which one is proper?)**, *in vitro*, and *in vivo* studies were used to characterize the effects of smoke or smoke condensate from cigarettes containing DIET compared to reference cigarettes. *In vitro* genotoxicity studies included Ames, sister chromatid exchange, and chromosomal aberration studies. *In vivo*, **(delete comma)** animal studies included **(a rat nose-only subchronic inhalation study and a mouse dermal tumor promotion study.)** sub-chronic, nose-only, rat inhalation and dermal, mouse tumor promotion studies. Overall, mainstream smoke from expanded (DIET, Freon **(which Freon? I mention this because I have not seen, to this point in the document, which Freon was used as the expander in the RJRT studies. Is Freon a registered tradename or trademark?)** and unexpanded tobacco cigarettes had similar biologic **(biological?)**activity. Smoke chemistry yields, *in vitro*, and *in vivo* data indicated that the mainstream smoke from unexpanded, Freon, and DIET expanded tobacco cigarettes is roughly similar.

RJRT concluded that there is no increase in biologic activity between mainstream smoke or smoke condensate from cigarettes with expanded tobacco compared to cigarettes without expanded tobacco. **RJRT concluded that there is no increase in biological activity of the mainstream smoke and smoke condensate from cigarettes with expanded tobacco when compared to the mainstream smoke and smoke condensate from cigarettes without expanded tobacco.)** This conclusion is more conservative

than that of other papers stating that the biologic activity of cigarette smoke from cigarettes with expanded tobacco is lower than that of cigarettes without expanded tobacco (Dontenwill *et al.*, 1977; Hoffman and Hecht, 1990; NCI, 1980).

Goal

This document will summarize the DIET process and studies conducted by R. J. Reynolds Tobacco Company (RJRT) to assess the safety of DIET.

Part A. Process: Tobacco Expansion and DIET

Tobacco Expansion Process

Processed tobaccos can be divided into 3 main classes: expanded,[a] reconstituted sheet, and processed stems (Borgerding *et al.*, 1999, p. 321). **(What does the superscript "a" refer to?)** In turn, each class of processed tobaccos can be manufactured in a number of ways. DIET is one of the processes by which expanded tobacco is manufactured. Expanded tobacco is included in cigarettes at levels ranging from 5 to 50% (Borgerding *et al.*, 1999, p. 326), with inclusion levels between 10-20% being preferred. Full flavor, full flavor low "tar", and ultra light brands can contain approximately 15, 25, and 50 percent expanded tobacco, respectively (Boxman, 1992).

The goal of expansion is to increase the filling capacity[b] of cured[c] tobacco **(What do the superscripts b and c indicate?)**, while optimizing the weight and density of the final tobacco blend used in cigarettes. Commercial expansion processes usually achieve 50-100% expansion. Cured tobacco leaves, depleted of water, shrink in the process of drying. Expansion allows for reinstatement of the original cell structure of the green leaves.

(Suggested wording for the previous paragraph: Cured tobacco leaves, depleted of water, shrink in the process of drying. Expansion allows for reinstatement of the original cell structure of the green leaves. Commercial expansion processes usually achieve 50-100% expansion. Expansion of the cured tobacco increases the filling capacity[d] of cured[c] tobacco, while optimizing the weight and density of the final tobacco blend used in cigarettes.)

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[a] *Expansion* is the method that allows cured, shredded tobacco to restore its volume to a green state equivalent (DeBardeleben, 1980, p. 26).

[b] *Filling capacity* is the capacity of tobacco to fill a cigarette rod firmly, at a certain moisture level. Filling capacity increases as a result of expansion (DeBardeleben, 1980, p. 27). The filling capacity is a volume measurement expressed in milliliters that is usually determined by use of a cylinder with piston. A set amount of tobacco is introduced into the cylinder and, when a pressure equivalent to the pressure that would be applied by wrapping paper to cigarettes is applied to the piston for a few seconds, the volume can be measured (Frederickson, 1968, p.5).

[c] *Curing* is a method of drying tobacco harvests. Curing can be accomplished by various techniques such as air-, flue-, fire-, or sun-curing (DeBardeleben, 1980, p. 19).

[d] *Filling capacity* is the capacity of tobacco to fill a cigarette rod firmly, at a certain moisture level. Filling capacity increases as a result of expansion (DeBardeleben, 1980, p. 27). The filling capacity is a volume measurement

The basic principle of tobacco expansion is impregnation of cured tobacco with an expansion agent (EA), followed by the rapid, "explosive" removal of the EA, resulting in expanded leaf cell structure (Browne, 1990, p. 50; Borgerding *et al.*, 1999, p. 325). This occurs because, while the EA is forced into the tobacco cell structure, the pressure builds up in the cells causing the cell volume to expand. The heat application increases the rate of expansion and "fixes" the expanded cell wall in such a way that it cannot collapse to the cured state. **(A comment to think about. In a "critical point drying" process, used typically for processing biological samples for scanning electron microscopy, liquid CO_2 replaces a solvent (typically ethanol), and penetrates the cell. Heat is applied gradually, the CO_2 is converted to a gas, and pressure is increased. The pressure is slowly reduced to ambient pressure by venting of the gas. The cell volume is not expanded. Picky Question. The pressure builds up in the cells? Is the expansion of volume created by the "forcing" of the expansion agent into the cell or by a rapid change of state? The second and third sentences of this paragraph seem to suggest a slightly different mechanism than the first sentence of the paragraph.)**

Over 150 patents have been issued on expansion processes (Davis and Nielsen, 1999, p. 380). Factors known to influence tobacco expansion volume include type of EA, type of tobacco used, part of tobacco leaf used, and a variety of operating conditions. Examples of EA's include, but are not limited to, CO_2, Freon, steam, nitrogen, and organic and inorganic compounds (Davies and Nelson, 1999, p. 380).

The DIET Process

The DIET process is currently among the most frequently used expansion processes in the cigarette industry (Borgerding *et al.*, 1999). RJRT's interest in DIET is rooted in the implementation of the Montreal protocol, which was signed in 1987 and became effective in 1989. The Montreal protocol stipulated that production and use of ozone depletion (depleting?) agents was to be phased out (Montreal Protocol, 1987). The implication was that the Freon used for tobacco expansion had to be replaced. Consequently, RJRT started investigating DIET prior to 1989.

DIET was initially developed and patented by Philip Morris, Inc., which has an extensive patent portfolio on DIET processes (e.g., de la Burde and Aument, 1974; Tyree, 1978). However, other companies also hold DIET process improvement patents, including RJRT (Guy and Poindexter, 1992; Poindexter *et al.*, 1996), Airco (Rothchild, 1979; 1981a; 1981b), Japan Tobacco, and Brown & Williamson Tobacco. A complete list of patents related to DIET was captured by Borschke in 2001 (Borschke, 2001, p. 57).

Using DIET presents advantages over other expansion processes. These **advantages** include: production of a more economical final product, increased profitability, increased product quality, increased process control ability, increased yield, restoration of some tobacco characteristics lost during curing (e.g., collapsed structure), decreased "tar", and decreased impurities. In addition, the process does not use additives.

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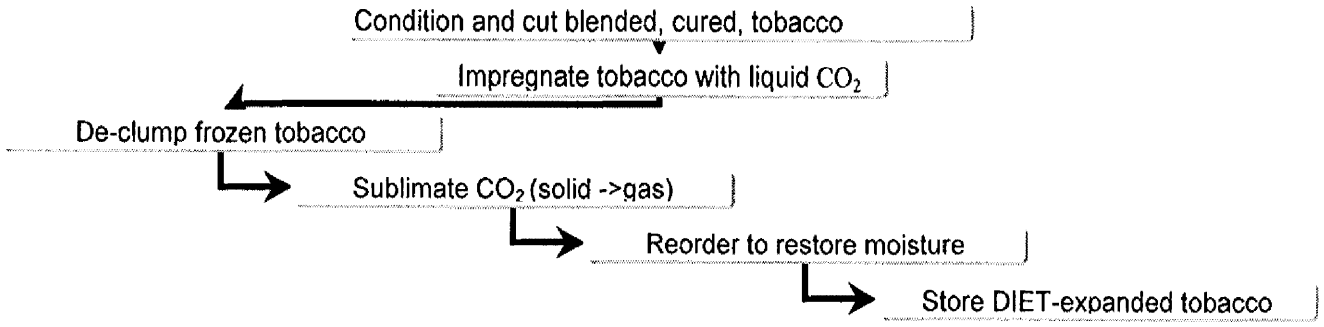
expressed in milliliters that is usually determined by use of a cylinder with piston. A set amount of tobacco is introduced into the cylinder and, when a pressure equivalent to the pressure that would be applied by wrapping paper to cigarettes is applied to the piston for a few seconds, the volume can be measured (Frederickson, 1968, p.5).

[c] *Curing* is a method of drying tobacco harvests. Curing can be accomplished by various techniques such as air-, flue-, fire-, or sun-curing (DeBardeleben, 1980, p. 19).

Basic Principle of the DIET Process (to be consistent with the previous header)

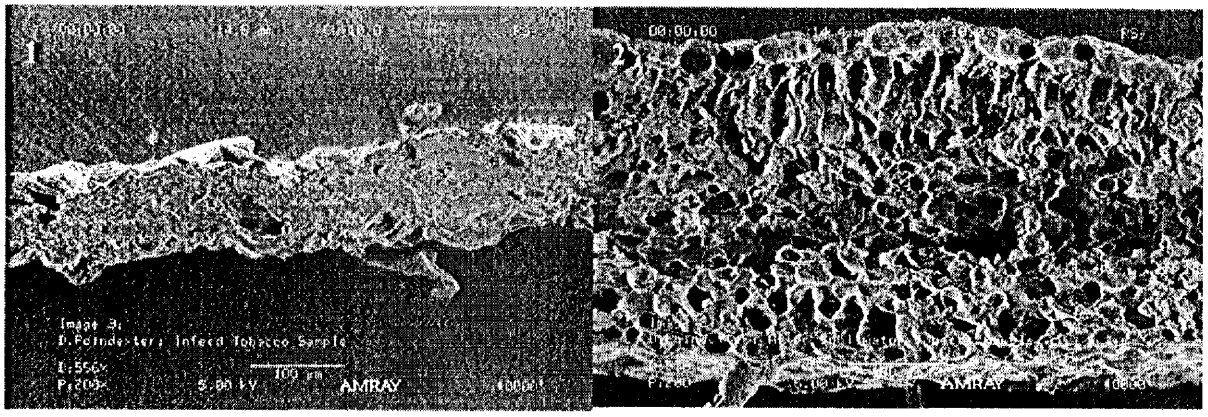
CO_2 is the EA in the DIET process. Tobacco expansion is associated with changes in the state of CO_2. During processing, tobacco is impregnated with CO_2 that changes from a liquid to a solid and then to a gas. The CO_2 phase transformation expands the tobacco. Sublimation is the process by which a solid becomes gas without going through the liquid phase. As a result of sublimation, frozen CO_2 in tobacco cells increases in volume more than 100,000 times. The goal of this process is to produce a target filling capacity that increases the volume of tobacco by about 80%. A simplified diagram is enclosed below (Figure 1).

Figure 1. Simplified diagram of DIET: main process steps



The Product of the DIET Process and RJRT Use

The end product of the DIET process is expanded tobacco. Figure 2 below shows how the tobacco cross-sections look before and after passing through the DIET process.



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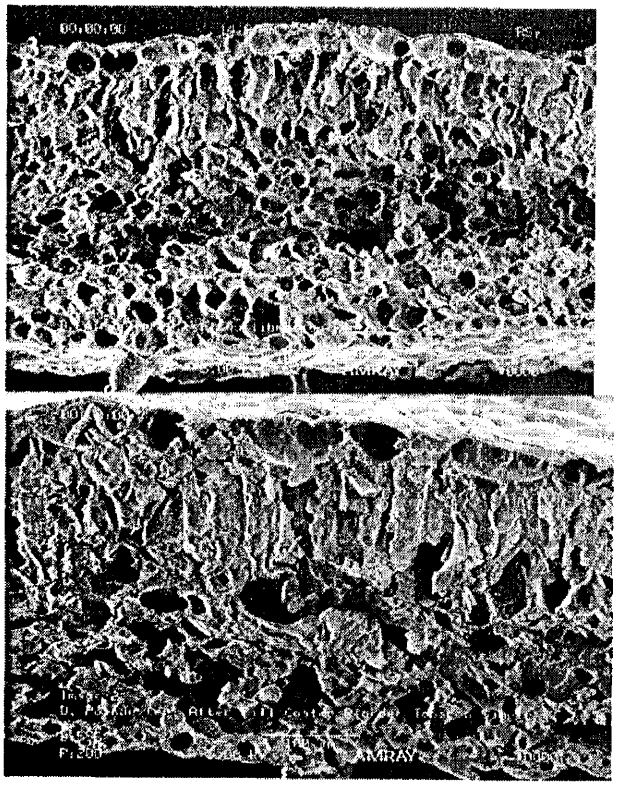
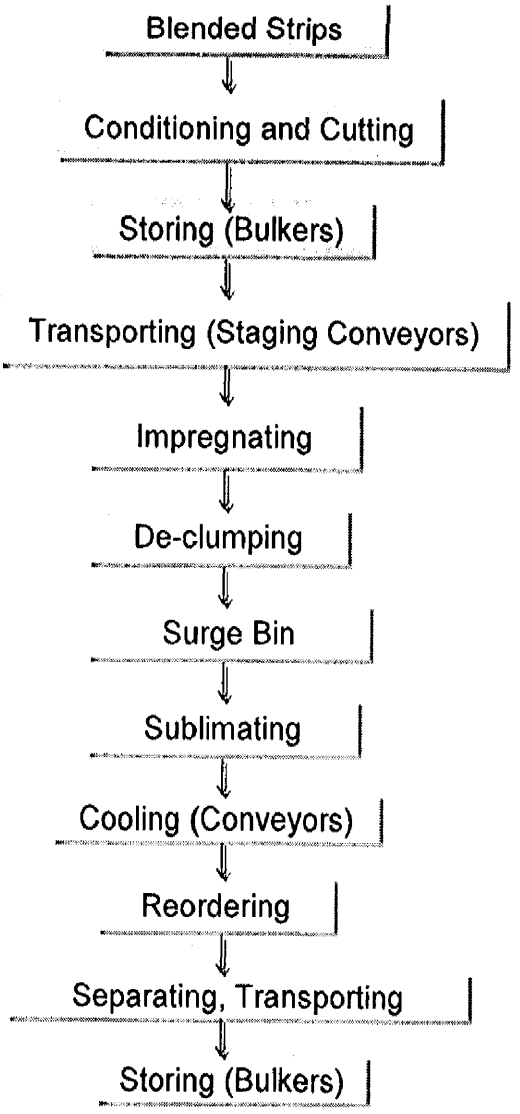


Figure 2. Tobacco input (1), sublimated (2), reordered (3), and output (4) from the DIET process.[f] Tobacco cross-sections from products in different phases of the process were photographed using Scanning Electron Microscopy.



Currently, RJRT has 7 active DIET products (G19-19, -22, -27, -29, -33A, -34, -36). The DIET products are included in 66 tobacco blends that include levels preferably between 8-18%, with some blends containing marginal ranges (5-8% and 25-50%). The main difference among the DIET product variations is the tobacco type combinations with different crop years and tobacco grades.

DIET Process Steps

[f] Photographs from D. Poindexter

A more detailed diagram of DIET is shown in Figure 3. Impregnation and sublimation are critical steps. Therefore, these steps will be described in more detail.

Impregnation and Declumping

Impregnation occurs in the impregnator vessel, which is essentially a large vessel that can be opened and closed at the top and at the bottom as needed. The impregnation step lasts **(requires)** about 13-15 minutes.

Figure 4 depicts the phase changes for CO_2 that occur during impregnation, and illustrates the concept of triple point, which is important to understanding CO_2 expansion. The triple point of any substance is that temperature and pressure at which the material can coexist in three phases (solid, liquid, and gas) in equilibrium. For most substances, atmospheric pressure is above the triple point pressure; as such, at ambient conditions, one can observe solid-liquid and liquid-vapor phase transitions (*e.g.*, water). For CO_2, the atmospheric pressure is below the triple point pressure and at ambient conditions one can only observe solid-vapor phase transitions. Thus, dry ice sublimes directly to vapor. To handle liquid CO_2, it has to be in a pressure vessel.

The triple point of CO_2 occurs at -69.9°F and 60.4 psig (Wittemann, 2002). During impregnation, from start to finish, there is a gradual pressure increase to a peak of about 420-460 psi (target ~435), followed by a decrease back to atmospheric pressure with a corresponding decrease in temperature from about 80°F to -109°F (Figure 4).

Figure 3. KRT DIET process steps

Figure 4. CO_2 phase diagram

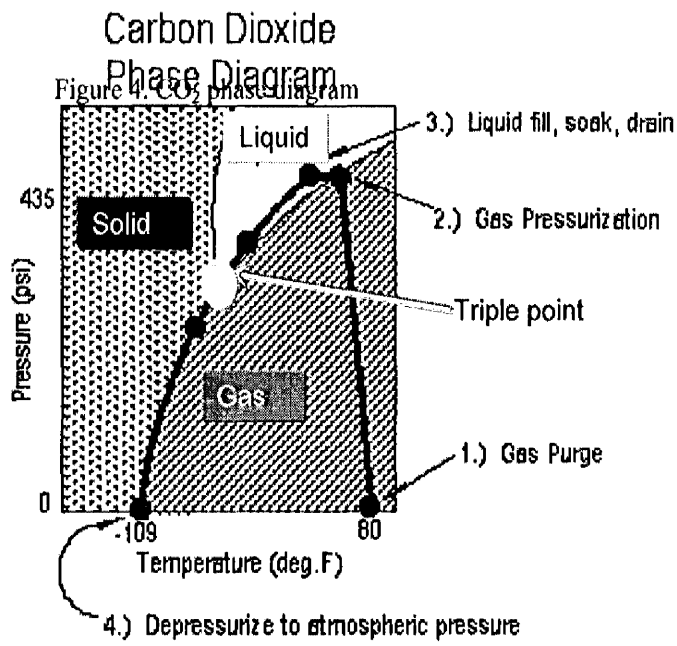


Figure 5 shows how impregnation and de-clumping occur. Figure 5, steps 1 and 2 show the filling and purging steps. Step 1 involves adding a set amount of cut tobacco from various plant parts, such as leaf or lamina to the top of the impregnator. Step 2 involves closing the impregnator and sealing it, then introducing CO_2 gas from the bottom. Figure 5, steps 3, 4, and 5 show the pressurization, liquid filling, and soaking phases. In these phases, the pressure in the impregnator increases while the temperature decreases (see Figure 4). CO_2 liquid fills the vessel and impregnates the tobacco by allowing it to soak briefly.

Figure 5, steps 6 and 7 show how draining and depressurization occur. The excess liquid is drained and recycled and the vessel is returned gradually to atmospheric pressure as the temperature decreases to -109°F and as the liquid converts to solid CO_2 (dry ice). The impregnated tobacco is released through opening the bottom door of the impregnator vessel and is then de-clumped or granulated.

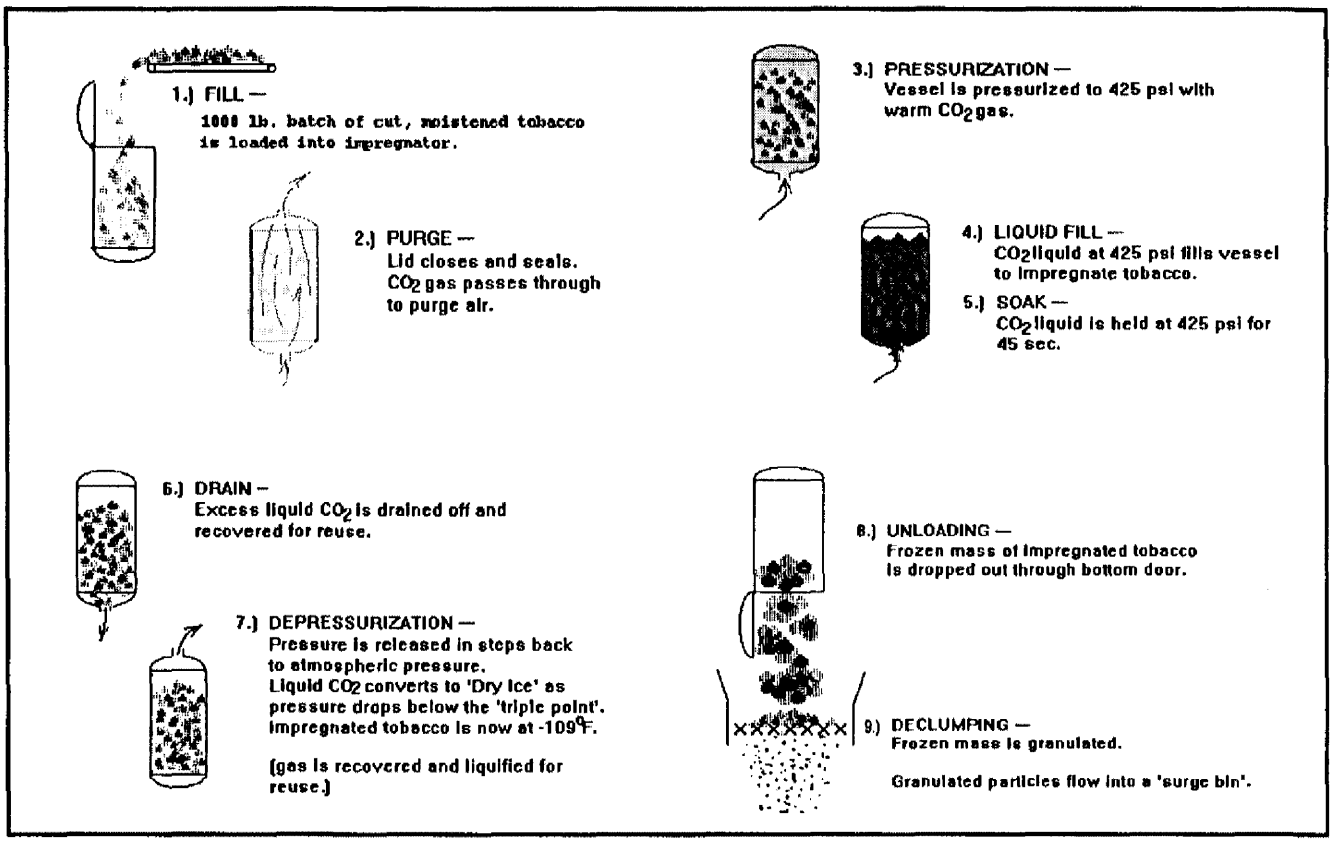
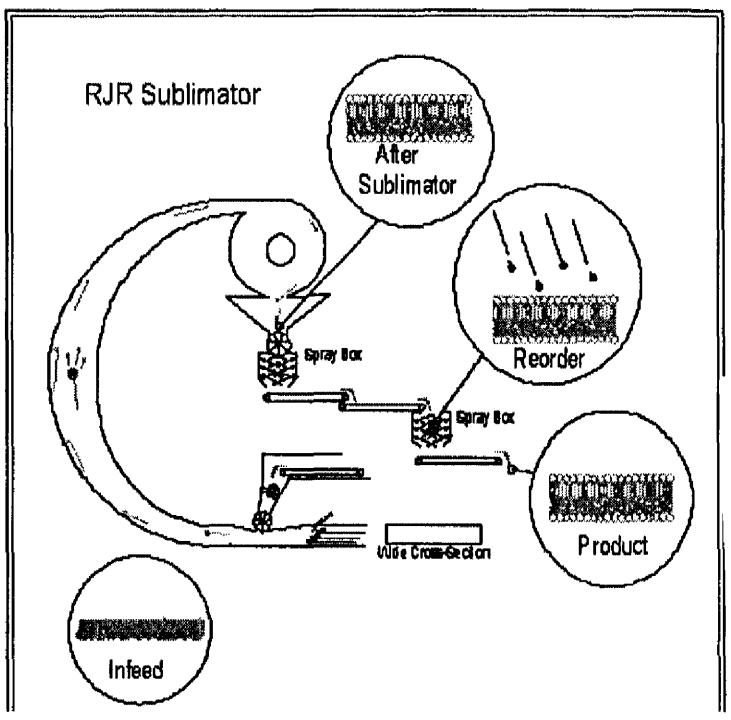


Figure 5. DIET impregnation step. This step involves 1) tobacco filling, 2) purging, 3) pressurization, 4) liquid filling, 5) soaking, 6) draining, 7) depressurization, 8) unloading, and 9) declumping (see text for additional explanations).

Sublimation

This step is depicted in Figure 6. The impregnated granulated tobacco from the de-clumper enters a surge bin container. The granules enter via a metering scale into the lower portion of the sublimator where hot gas, primarily air, passes at high velocity (Poindexter *et. al.*, 1996). The temperature range can span 450-700°F. Tobacco expands and exits onto cooling conveyors.

The tobacco volatiles from heating are collected, incinerated, and then vented into the atmosphere. Most excess CO_2 is



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collected and re-used, increasing efficiency and decreasing cost.

After the sublimator step, tobacco is cooled and then passes through reordering chambers where water is added to reestablish moisture to levels of ~12%. The product is then passed through the separator.

Figure 6. DIET sublimation step. This step involves tobacco infeeding, expansion, and reordering.

Separation

The separation step is presented in Figure 7. Expanded tobacco that has passed through sublimation and reordering is transported into the tower separator and is further pneumatically transported, passes through the tangential separator into product bulkers. From product bulkers, expanded tobacco can go to casing and cutting.

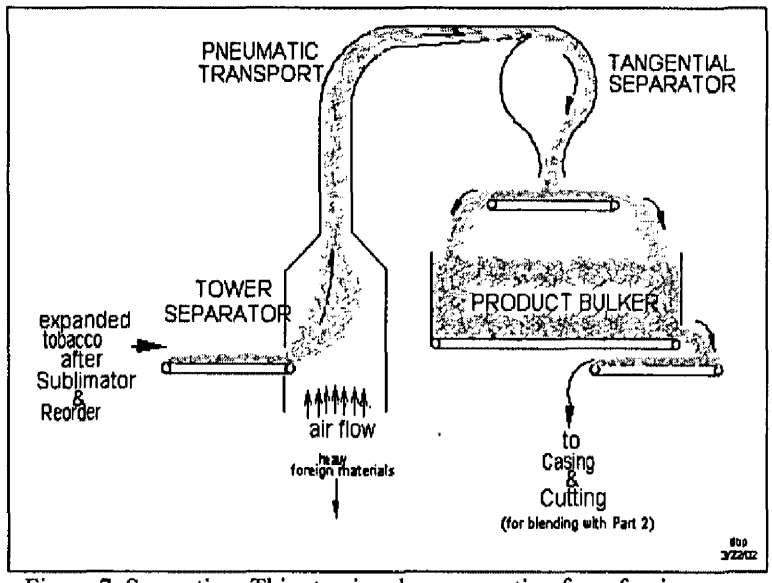


Figure 7. Separation. This step involves separation from foreign materials and pneumatic transport of tobacco through the tangential separator to bulkers.

Differences between the Pilot and the Commercial DIET Production Facilities

RJRT has 2 facilities for DIET production, a pilot plant and a commercial facility. While the DIET process and the product are roughly similar between the pilot and the commercial production facilities, there are a few differences. Most of the differences are due to the fact that the full production facility is larger. Otherwise, the DIET products are similar, especially from a chemistry and biologic activity point of view (Foy, 2001). Details of this comparison are addressed in Part B of this document. Some of the main differences are outlined in Tables 1a and 1b below.

Table 1a. General differences (pilot plant vs. commercial DIET production facilities)

Parameter	Pilot	Full scale (1999-2000 data)
Purpose	• Produce small quantities of DIET for prototype cigarettes that cannot be produced in the full scale facility • Produce DIET to include new materials for various R&D tests	• Produce DIET for RJRT cigarettes • Produce DIET for direct selling
Throughput/"Feed rate" (lbs/hr)	12	~6,200
Typical quantities (lbs)	15	10,000
Typical process total throughput time (min)	20 (with 1 week reordering)	30
Typical filling capacity (cc/100g)	800	800 (range: 690-820)
~End product moisture (%)	11.7	11.7

Table 1b. Equipment differences (pilot plant vs. commercial DIET production facilities)

Equipment and process step	Parameter	Pilot	Full scale
Infeed storage bulkers	Number	0	5
	Typical capacity (lb)	-	2 x 90,000 3 x 70,000
	Total time in this step	-	1 hr – 2 days
Impregnator	Number	1	2
	Typical capacity (lb)	4	1000
	Total time in this step (min)	15	15
Staging conveyors	Number	0	2
	Total capacity (lb)	-	1000
	Total time in this step (min)	-	7.5
Declumpers	Number	1	2
	Typical capacity (lb)	5	1000
	Total time in this step (sec)	5	30
Surge bin	Number	0	1
	Typical capacity (lb)	-	3000
	Total time in this step (min)	-	30
Sublimator	Number	1	1
	Typical capacity (lb/hr)	85	6200
	Total time in this step (sec)	1-3	1-3
Cooling conveyors	Number	0	3
	Typical capacity (lb)	-	6200
	Total time in this step (sec)	-	45
Reordering spray boxes	Number	0	2
	Typical capacity	-	6200
	Typical time in this step (sec)	-	1
Equilibration chamber	Number	1	0
	Typical capacity (lb)	1000	-
	Typical time in this step (week)	1	-
Product bulker	Number	0	3
	Typical capacity each (lb)	-	26,000
	Typical time in this step (hr)	-	3

Part B. Safety: Expanded Tobacco and DIET

General Considerations Supporting the Safety of Expanded Tobacco

There is a long history of use for expanded tobacco and botanicals. The idea of expanding botanical materials to increase filling capacity has been around since the 1800's (Borschke, 2001, p. 53). Some of

the early patents were in 1902, when Anderson received a patent for a method of expanding rice and wheat (Anderson, 1902). In 1929, Hawkins filed and then in 1931 received a patent for applying the same principle to tobacco using CO_2, air, and steam as expansion agents (Hawkins, 1929). In 1962, DeSouza and Rice filed and in 1964 received a patent for expanding tobacco with volatile organic solvents (DeSouza and Rice, 1962). Since this patent, many others have been granted with many expansion agents and, currently, many tobacco companies use expansion processes.

Historically, outside RJRT, tobacco expansion has played a role in reduced risk cigarettes. A main reason for using expanded tobacco is reduced cost, which brings about additional benefits of reduced "tar," and nicotine in the final cigarette. Because the goal of tobacco expansion is to increase filling capacity, it is expected that as less tobacco is used in cigarettes, less is burnt and less smoke is generated, with the potential of generating less biologic activity. In addition, the long use history of expanded tobacco increases confidence in the safety of the process itself. **?? Years of use increases confidence?**

CO_2 Natural Occurrence, Uses, and Regulatory Status

Normally, CO_2 is produced from human and animal respiration and from burning of **carbon-containing** ~~fossil~~ fuels. Therefore, CO_2 levels can be higher indoors where people live compared to outdoors. In this context, the American Society of Heating Refrigerating and Air-Conditioning Engineers, Inc. publishes "ASHRAE Standard 62-1989, Ventilation for Acceptable Indoor Air Quality." The ASHRAE standard requires that sufficient fresh air be provided to keep the indoor level below 1,000 ppm [http://www.ae.iastate.edu/aen125.htm]. As a reference, the outdoor levels are approximately 300 ppm. The contribution of **(CO2 within the final expanded tobacco is ...)** CO_2 to the final expanded tobacco is negligible at the end of the DIET process and well below the normal outdoor levels.

The quality and purity of the CO_2 used in DIET is high (99.8-99.99% pure). The CO_2 used in DIET currently comes from Airgas, AIRCO (a division of the BOC Group, Inc.) and Praxair suppliers. The CO_2 has been approved to comply with 21 CFR 184.1240 and the US Pharmacopoeia. Under 21 CFR 184.1240, the Food and Drug Administration classified CO_2 as Generally Recognized as Safe (GRAS) and approved CO_2 as a direct food additive. Consequently, the CO_2 used in DIET is food grade (e.g., like the CO_2 used in carbonate drinks or used to chill or freeze food products). In addition, CO_2 is used in foods as a propellant and an aerating agent (Food Chemicals Codex, 1996, p. 87). In 1994, the United States Environmental Protection Agency listed CO_2 as a possible tobacco expansion substitute for Freon 11 (CFC-11) (Federal Register, Vol. 59, No. 53, p. 13117).

The safety of CO_2 used in DIET is further supported by the fact that after processing, the levels of CO_2 in tobacco are negligible, as expected based on processing temperatures. Furthermore, DIET uses no additives. CO_2 is a processing aid and is recycled, limiting emissions into the atmosphere, ensuring the process is environmentally acceptable.

Safety Data

The safety profile of mainstream cigarette smoke from cigarettes with CO_2 expanded tobacco is similar to that of cigarettes containing prototypical tobacco (expanded using other methods or unexpanded). The studies summarized below demonstrate that CO_2 expansion does not alter the toxicological profile of tobacco. These studies come from two sources: NCI and RJRT.

NCI Studies

The NCI showed in the 1970's that the product of tobacco expansion using CO_2/ammonia did not display an increased toxicological activity profile compared to prototypical tobacco smoke. More than that, NCI indicated that the tumorigenicity of the CO_2/ammonia expanded cigarettes was *lower* than that of standard tobacco blends studied (NCI, 1980, p. 26).

The NCI experiments began in 1970 and were published under a series of reports called "Towards a Safer Cigarette" (NCI, 1976-1980). The expansion experiments started in 1972 (NCI, 1979, p. 1). These experiments evaluated the relative biologic activities of mainstream smoke from cigarettes containing a variety of modifications, including CO_2 /ammonia expanded tobacco. The CO_2/ammonia process is similar to DIET in terms of expansion agents except that DIET uses only CO_2. The ultimate objective of NCI was to identify characteristics of less hazardous cigarettes. The major focus of the NCI studies was to determine if mouse skin tumorigenicity of mainstream smoke condensates from experimental cigarettes increased, decreased, or did not change when compared to reference cigarettes (NCI, 1980, p. 2). The test battery employed by NCI included mainstream smoke chemistry, *in vitro* cytotoxicity, and *in vivo*, 18-month dermal carcinogenesis assays. Control cigarettes included the unexpanded, standard experimental blends (SEB I and II) and the 1R1 University of Kentucky reference cigarettes. The control cigarettes were representative of the 1970's cigarette market.

The SEB tested by NCI as control/non-expanded vs. expanded, were not filtered and had the same compositional breakdown (same tobacco types). The variables were manufacturers and crop years. The breakdown is shown below (Figure 8).

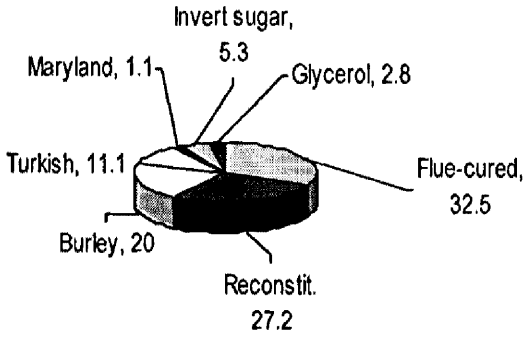


Figure 8. Composition of NCI SEB cigarettes (% components)

Smoke Chemistry

NCI indicated that the **(deliveries (per cigarette basis) of several)** major mainstream smoke components from CO_2/ammonia expanded tobacco ~~per-cigarette-deliveries-of-several-constituents~~ were lower than the unexpanded SEB blend (NCI Report No. 2, 1979). A table summary is presented below (Table 2).

Table 2. NCI mainstream smoke chemistry data

Analyte	Unit	CO_2 / Ammonia Expanded NCI	Unexpanded NCI (SEB II average of different crop years)
FTC Nicotine, "Tar," CO, and CO_2			
Total Particulate Matter	mg/cig	22.33	32.8
Nicotine		0.74	1.79
Water		3.33	3.79

"Tar"		18.2	27.1
CO	ml/cig	11.83	15.8
CO_2		21.28	30.5
Acetaldehyde	µg/cig	720	986
Acrolein		87.7	101
Formaldehyde		21.7	31
Hydrogen Cyanide		286.6	354
Phenols		71	133
NO_x		293.1	386

NCI noted that when results were analyzed as deliveries/puff, results were similar. The exceptions in this case were that the greatest reductions were seen for nicotine (-40%), phenolics (-24%), and increases were noted for acrolein (+20%) and hydrogen cyanide (+17%). Relative to TPM, nicotine and phenolics were lower than in SEB II, others were constant, while acrolein (+26%) and hydrogen cyanide (+19%) increased. The condensate concentration of most compounds from expanded tobacco was lower than SEB II except for indole and skatole, with the greatest reductions for phenols (25-35%), and benzo[a]pyrene (30-35%) (NCI, 1979, p. 40).

In Vivo Carcinogenicity Study

The dermal carcinogenicity study was conducted in groups of 100 ICR female Swiss mice (NCI, 1980, p. 2). Mice were exposed to 0.1 ml suspension of 25 or 50 mg dry smoke condensate per application, 6 days/week for 18 months. The control groups included mice with no treatment, mice with dorsal hair clipped but no skin painting (sham), mice painted with acetone (vehicle control), and mice painted with benzo[a]pyrene in acetone at 3 dose levels (positive controls). NCI assumed that a reduction in tumorigenicity from the mouse skin painting studies offered valid clues for future lines of investigation focused on inhalation. NCI acknowledged that there is uncertainty regarding the relationship between tumors from mouse skin painted with condensate and human cancer.

Condensate samples were prepared by smoking cigarettes on Process and Instruments Corporation smoking machines, using negative pressure, 1 puff/minute, 35 ml volume and 2 second duration, and using a maximum of 10 puffs/ cigarette. The condensate was collected in four traps at -70°C on either Pyrex beads or Teflon filaments. Then the condensate was extracted with acetone, concentrated under 40°C and reduced pressure until <6% water remained, then adjusted to final concentrations needed.

Observations included date of visual tumor development, tumor description, tumor number, animal weight, mortality, and necropsies. Tumors were confirmed using histopathology. Actuarial methods were used to estimates P_F, defined as the probability that an animal within a given group would not develop a tumor if the animal survived the 18 months experiment. In addition, estimates were made of the latent periods, that is, the number of days since the initiation of the experiment to 25, 50, or 75% survival ($T_{25, 50, 75}$).

The activity of condensate from the unexpanded SEB blend was compared to the activity of condensate from prototypes comprised of 100 percent CO_2/ammonia expanded tobacco. CO_2/ammonia expanded tobacco showed significantly lower carcinogenic activity than the unexpanded SEB blend (Table 3).

Table 3. NCI tumor data

Group	Low dose				High dose			
	P_F	T_{75}	Carc. Paps. Benign	Dead animals (no tumor)	P_F	T_{75}	Carc. Paps. Benign	Dead animals (no tumor)

Unexpanded	.442	390	93	97	9	97	.414	402	127	66	10	113
CO_2 / Ammonia expanded	.583	449	15	21	0	21	.456	430	25	22	0	24

(I think that the abbreviations of "Carc." And 'Paps" should be defined for Table 3. The header of Carc. Paps. and Benign need to be separated by vertical lines. Is "benign" the number of total benign tumors including papillomas? Is it the number of animals? Also, it might be wise to define Pf and T75 as a table footer just in case the table is separated from the text.) Collectively the mainstream smoke chemistry and the results of the dermal carcinogenicity assay suggested that tobacco expansion using CO_2/ammonia is unlikely to increase the biological activity of mainstream cigarette smoke.

In Vitro Studies

NCI conducted a battery of three *in vitro* studies, which assessed inhibitory potential of cigarette smoke condensate from cigarettes with or without expanded tobacco. These included **evaluation of** cytotoxicity in KB tumor cells, inhibition of phagocytosis by alveolar macrophages, and ciliary transport inhibition. The cytotoxicity assessment assessed the inhibitory **(inhibitory effects? growth inhibition?)** of water-soluble components of smoke condensate (or cytotoxic effect) on tumor cells. The phagocytosis test assessed the inhibition of removal of *Staphylococcus albus* bacteria from suspension by rabbit lung alveolar macrophages. Smoke samples were injected into the atmosphere above the macrophages and bacteria and bacterial survival were monitored after 2 hours. The ciliary transport tests measured the potency of mainstream smoke to inhibit ciliary transport. Table 4 presents a summary of the potencies of cigarette smoke condensates from cigarettes with CO_2 expanded vs. cigarettes without expanded tobacco. Ranges for all modifications are included for reference.

Table 4. NCI *in vitro* data

Endpoint	Cillia (Puffs/ED50)	Macrophage Inhibition (ml smoke/ID50)	KB Tumor Cell Cytotoxicity (ED50/Puff)
Treatment (Control??)	Range: 1.3-(>8)	3.6-7.8	13-36
Non-expanded SEB II	3.7	4.4	23.4
CO_2 expanded SEB II	1.3	5.1	30.5

(Cilia are misspelled in the table header. The reason I ask the following questions is because the units of measure are unclear. The first sentence in the paragraph prior to Table 4 distinctly indicates "condensate" and not puffs of smoke. What is the unit of measure for the parameter Puffs/ED50? Puffs divided by ED50 or the number of puffs required to reach the ED50? The mass of condensate equal to the yield of a certain number of puffs? Ml smoke/ID50. What does that mean? Ml of puff volume? Ml of a condensate suspension? If so, what is concentration of suspension?) Although some changes were observed and data are enclosed for completeness, the relevance of these data is minimal. **(Why considered minimal?)** The only noteworthy results were that smoke condensate from CO_2 expanded tobacco induced more cytotoxicity **(I don't understand thus unit of measurement? ED50/puff? A higher ED50 is usually less toxic. Is this unit of measure % toxicity per puff?)** in tumor cells than condensate from cigarettes without expanded tobacco; however, *in vitro* results have limited relevance **(Since this statement is juxtaposed against this concluding sentence, please consider removing the phrase "the relevance of these data is minimal" in the previous sentence.)** . An indication of the questionable relevance of these data is the fact that NCI did not stress these findings in their summary report (NCI, 1980).

RJRT Testing Strategy

The safety testing program at RJRT involves a battery of tests **including** , (delete comma?) chemical analysis, *in vitro* studies, and *in vivo*, **(delete comma?)** long-term animal tests. In the first tier, chemical studies are conducted to assess any of several parameters, such as purity, pyrolysis products, constituent levels in mainstream smoke, or residue or impurity levels. *In vitro* assays are used as a second tier. A change could be a new compound or a modification to a product or a process. Typical assays include the Ames assay, Sister Chromatid Exchange (SCE) assay, and cytotoxicity assay (Neutral Red). The third testing tier is *in vivo* **laboratory animal studies** and usually involves **(a rat nose-only subchronic inhalation study and a mouse 30-week dermal tumor promotion study.)** a 90-day sub-chronic, nose-only, rat inhalation study and a 30-week, mouse dermal, tumor promotion study.

RJRT DIET Studies

Tests were conducted to compare mainstream cigarette smoke or smoke condensate from cigarettes containing DIET expanded tobacco and cigarettes containing tobacco expanded using other established expansion methods, such as Freon 11 expansion. In addition, some tests were conducted to compare cigarettes with DIET to cigarettes without expanded tobacco. **(It is important here to distinguish which tests involved which cigarettes.)** The studies conducted for assessing the DIET safety included mainstream smoke chemistry analysis studies and toxicology studies. Toxicology studies included *in vivo* studies (a subchronic inhalation study in rats and a **30-week** dermal tumor promotion study in mice), and *in vitro* studies (Ames, SCE, and Chromosomal Aberration studies). These studies are presented in more detail in the next sections.

Smoke Chemistry

Tests for "tar", nicotine, carbon monoxide (CO) and carbon dioxide (CO_2) of smoke from cigarettes containing 100% DIET were conducted according to the Federal Trade Commission (FTC) methods. Mainstream cigarette smoke constituents analyzed are presented in Table 5. The table contains comparative ~~reference~~ values for mainstream cigarette smoke yields from commercial cigarettes (US market survey 1994-1995). The reference **(Here again, the term "reference" is used. In this paragraph the word "reference" could be eliminated and you wouldn't lose anything.)** values represent the mean and ranges of constituents in the mainstream smoke from 10 different, non-generic, non-menthol, and full flavor commercial cigarettes sold by 5 tobacco companies in the US **(Market survey?)**. The exception is the nitrosamine, representing 5 cigarette types manufactured by 3 companies. All mainstream smoke constituents from the cigarettes containing 100% DIET were within or below the range of the market survey values. These findings are consistent with the NCI data. **(Does the column for CO2 expanded represent the mean?)**

Table 5. RJRT mainstream smoke chemistry data (DIET vs. market surveys)

Analyte	Unit	CO_2 Expanded	Market Survey Mean	Market Survey Range
FTC Nicotine, "Tar," CO, CO_2	mg/cig			
Total Particulate Matter		16.7	21.1	18.3 - 31.4
Nicotine		0.74	1.24	0.96 - 1.66
Water		2.7	2.7	2.1 - 4.1
"Tar"		13.3	17.2	14.7 - 25.6
CO		12.0	13.8	9.8 - 16.6
CO_2		38.3	NA[3]	NA
Ammonia	µg/cig	17.3	37.4	27.6 - 47.8
Carbonyls				
Acetaldehyde		888	770	516 - 911
Acetone		355	355	244 - 433
Acrolein		137	110	72.3 - 193
Formaldehyde		39.4	24.0	13.7 - 39.7
Hydrogen Cyanide		278	245	159 - 285
Hydroxybenzenes (Phenols)				
Catechol		48.8	76.3	53.4 - 110
Hydroquinone		54.2	71.0	43.6 - 102
Phenol		11.0	30.2	14.9 - 64.1
p- + *m*-Cresol		11.0	20.7	12.1 - 40.8
Nitric Oxide		232	257	138 - 387
Nitropropanes				
1-Nitropropane		ND*	NA	NA
2-Nitropropane		ND	NA	NA
Benzo[a]pyrene	ng/cig	6.2	12.4	8.9 - 19.7
Nitrosamines				
N-nitrosoanabasine (NAB)		18	NA	NA
N-nitrosoanatabine (NAT)		182	195	150 - 235
N-nitrosonornicotine (NNN)		143	167	132 - 191
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)		80	143	117 - 181

*ND = not detectable (detection limit < 0.27 µg/cigarette)

[3]NA = not available

A comparison between the Freon and DIET mainstream smoke chemistry is presented in Table 6. In general, the results indicate that the levels of the mainstream cigarette smoke constituents were similar for the expanded tobacco cigarette prototypes. **(Some constituents up by 50% or greater = similar?)** Variability of analyses? Mean +/- s.d. not different? Within the normal range of variability? . Minor differences were observed in some constituents that are present in ng quantities. The variability of the yields of these constituents, present in ng quantities, is such that ??? **I think this table would be much better if mean and range are presented. Mean and s.d. possibly.)**

Table 6. Freon vs. DIET: direct comparison of mainstream smoke chemistry data

Analyte	Freon	DIET
FTC Nicotine, "Tar," CO, CO_2 (mg/cig)		
Total Particulate Matter	16.9	16.7
Nicotine	0.98	0.74
Water	3.1	2.7
"Tar"	12.8	13.3
CO	11.4	12.0
CO_2	35.2	38.3
Hydroxybenzenes (Phenols) (µg/cig)		
Catechol	43.5	48.8
Hydroquinone	46.6	54.2
Phenol	9.4	11.0
p- + *m*-Cresol	9.9	11.0
Carbonyls (µg/cig)		
Acetaldehyde	791.7	888.0
Acetone	310.1	354.7
Acrolein	117.5	136.8
Formaldehyde	31.6	39.4
Nitric Oxide (µg/cig)	188.3	231.7
Nitropropanes (µg/cig)		
1-Nitropropane	ND	ND
2-Nitropropane	ND	ND
Ammonia (µg/cig)	17.48	17.26
Benzo(a)pyrene (ng/cig)	6.83	6.18
Nitrosamines (ng/cig)		

NAB	11	18
NAT	114	182
NNN	88	143
NNK	75	80

*ND = Not detected at a limit of detection less than 0.27 µg/cigarette.

In Vivo Studies

Inhalation Study

A sub-chronic, inhalation study with mainstream cigarette smoke from cigarettes containing DIET expanded tobacco found no biologically significant ~~changes~~ **differences when** compared to Freon 11 (Freon) expanded tobacco (Higuchi, 1995).

Study Design

The study was a nose-only, 13 week inhalation study in rats ~~set-up~~ **conducted** to compare toxicological responses to mainstream smoke from cigarettes with **100%** DIET **relative to mainstream smoke of cigarettes composed of 100% Freon expanded tobacco? .** Groups of 30 male and 30 female, Sprague-Dawley rats were exposed to 0.08, 0.25, ~~and~~ **or** 0.8 mg WTPM/L of mainstream smoke from each cigarette type. The rats were exposed to mainstream smoke or air (sham) for 1 hour/day, 5 days/week for 13 weeks. At the end of exposure, 20 rats of each sex per group were used for histopathology, while remaining rats were observed for 13 additional weeks (recovery phase) without mainstream smoke exposure and then sacrificed **(euthanized)** to determine changes.

The controls in this experiment were cigarettes with Freon **(Freon 11?)** expanded tobacco, selected as control based, in part, on the NCI determination that expansion does not change the biologic activity of tobacco mainstream smoke (NCI, 1980). The samples tested by RJRT as control (Freon 11-expanded) vs. DIET-expanded had the same compositional breakdown (same tobacco types). Cigarettes contained 100% expanded tobacco (Figure 9).

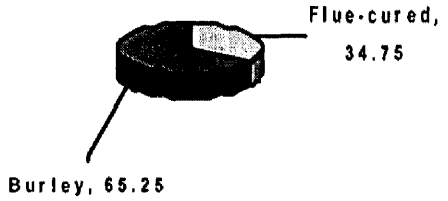


Figure 9. Composition of RJRT cigarettes (%)

Cigarettes were smoked by smoking machines using the standard FTC regimen (35 ml puff volumes of 2 second duration taken once per minute). Instrument-related and animal-related exposure parameters were rigorously controlled and indicated that rats received the incremental administered doses. For example, inhalation exposure systems were evaluated before starting the study to determine reproducibility and ability to deliver controlled, uniform concentrations of mainstream smoke to the animals' breathing zones. **From the exposure atmosphere, smoke particle size, and concentrations of smoke (wet total particulate matter/l), nicotine, and carbon monoxide were determined.** ~~and inhaled masses were determined~~. Tidal volume, respiratory rate, and minute volume were measured in 24 animals at weeks 2,

8, and 13 and then data were combined for males and females and compared to sham. **Calculated inhaled mass of WTPM was estimated for groups of smoke exposed rats based on the respiratory minute ventilation and exposure concentration data.**

Body weights, clinical observations, mortality, necropsy, organ weights, and pathology data were collected. Organ weights were determined for lungs, liver, kidneys, brain, testes with epididymis, adrenals, spleen, and heart. Pathology included respiratory tract organs (nasal passages, larynx, trachea, conducting airways, and lung), heart, thymic and peribronchial lymph nodes, ~~nasal tissue, and~~ brain, liver, kidneys, spleen, adrenals, **and** gonads (for the high exposure and sham groups). **For the mid and low exposure groups, histopathological evaluation included ...?.** In addition, all organs showing gross changes at necropsy were collected. **(A number of other organs are collected. Why not include the list as the dermal study does? It is probably more important to include the list for inhalation studies than for dermal studies. Let's talk about this list and the tissues examined.)** Body weights in DIET and Freon groups were similar after 90 days of exposure. **(This body weights sentence should probably be moved to a "results" section.)**

The Sham group was compared to each mainstream smoke exposed group , **by gender?(let's look for that detail), to determine if smoke related effects were observed. All smoke related effects were then compared between the different cigarette types at comparable exposure concentrations** ~~and then different cigarettes were compared at similar dose levels~~ to determine if there were statistical differences between different cigarette types **composed of 100% DIET or Freon expanded tobaccos.** In-life body weights and organ weights were initially analyzed using the Xybion/**Path-Tox** system by a one-way analysis of variance (ANOVA) and Bartlett's test for homogeneity. Homogeneous data were subsequently analyzed using the Dunnett's test. Non-homogeneous data were analyzed using the Cochran and Cox's modified t-test. Histopathology data were graded using 5 severity grades: 1 minimal, 2 mild, 3 moderate, 4 marked, and 5 severe. Histopathology data ~~from 20 rats per group~~ were analyzed with the Kolmogorov-Smirnov test which takes into account both incidence and severity.

The following statistical analyses were conducted using the Instat® software program. An unpaired t-test or ANOVA test and **or ?** Bartlett's test for homogeneity followed by Dunnett's test ($p<0.05$), if standard deviations of populations **("Populations" could be used synonymously for groups of animals or "populations" of data. I would substitute "group data" for "populations".)** were equal, or a Welch's alternate t-test or a nonparametric test (Mann-Whitney two sample test) were used to evaluate WTPM, CO and nicotine, calculated inhaled mass, physiological parameters, carboxyhemoglobin, and serum nicotine data. Statistical tests were conducted using a two-sided, 5% level of significance.

Results

Blood carboxyhemoglobin was consistent with the carbon monoxide level in the exposure atmosphere and serum nicotine was consistent with the nicotine in the exposure atmosphere. **(I think it is necessary to insert a table of WTPM, CO,**

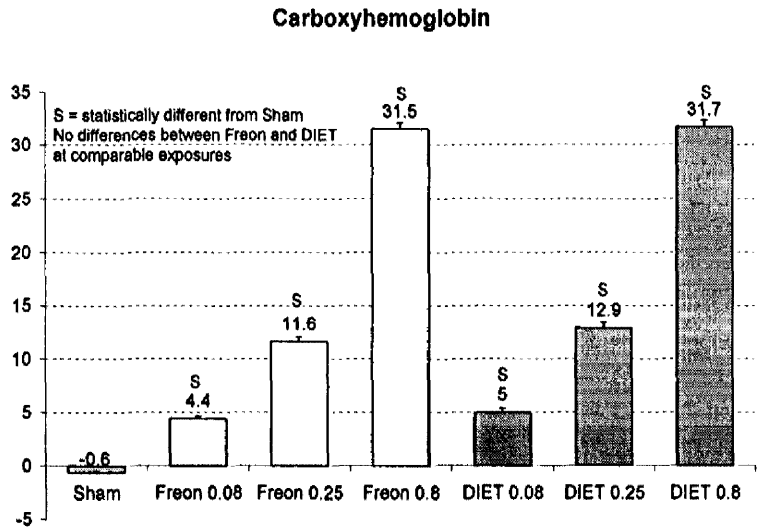


Figure 10. Carboxyhemoglobin level as indicator of smoke exposure (Sham, Freon, and DIET). Statistically significant differences from Sham at $p<0.05$ are noted as "S." There were no differences at comparable exposure levels between Freon and DIET.

and Nicotine summary exposure data so that the reader has confidence in the above statement.) Blood carboxyhemoglobin levels are shown in Figure 10 and nicotine levels in Table 7.

Table 7. Serum nicotine levels (Sham, Freon, and DIET)

Group name	Dose level (WTPM/L)	Animal serum nicotine levels (ng/ml)	± Standard error
Sham	-	0.4	0.2
Freon[S]	0.08	46.4	1.6
	0.25	78.9	4.8
	0.8	213.6	12.5
DIET[S]	0.08	37.8[F]	2
	0.25	69.2	4.5
	0.8	182.0[F]	8.4

[S] = Statistically significantly different from Sham
[F] = Statistically significant from different from Freon

Clinical observations are presented in Tables 8a and 8b for 30 animals per group. All animals were alive at the start of each observation interval. While minor adverse effects occurred at low incidence, ~~and~~ because there is no **apparent** dose response relationship **among exposure groups of either cigarette type**, the results were not considered biologically significant.

Table 8a. Males: Summary of clinical signs ~~after~~ **during the inhalation exposure period** ~~90 days of inhalation~~ (Sham, Freon, DIET)

Dosage (mg WTPM/L)	**0**			**0.08**				**0.25**				**0.8**			
Observation	**Sham**			**Freon**		**DIET**		**Freon**		**DIET**		**Freon**		**DIET**	
	a	b	c	a	b	a	c	a	b	a	c	a	b	a	c
Normal (no abnormalities)	30	100	14.8	28	93.3	29	13.1	30	100	30	13.5	27	90	28	13.9
Body surface															
Alopecia	6	20	7	6	20	10	7.4	7	23.3	6	11.2	8	26.7	7	9
Abrasion	0	0		0	0			1	3.3			1	3.3		
Scabbed area	0		0			0	0			0	0			1	9
Tail abrasion	0	0	0	0	0	0	0	1	3.3	1	1	1	3.3	1	3
Eyes															
Area around eye swollen	0	0		0	0			0	0			1	3.3		
Protruding eye	0		0			0	0			0	0			1	1
Ear (s) Hematoma	0	0	-	1	3.3	-	-	1	3.3	-	-	1	3.3	-	-

a = Number of animals affected
b = Percent of animals with positive observation noted at least once during interval
c = Mean number of animal weeks with clinical sign

Why are there no column b or column c data in some circumstances?

Table 8b. Females: Summary of clinical signs ~~after 90 days of inhalation~~ **during the inhalation exposure period** (Sham, Freon, DIET)

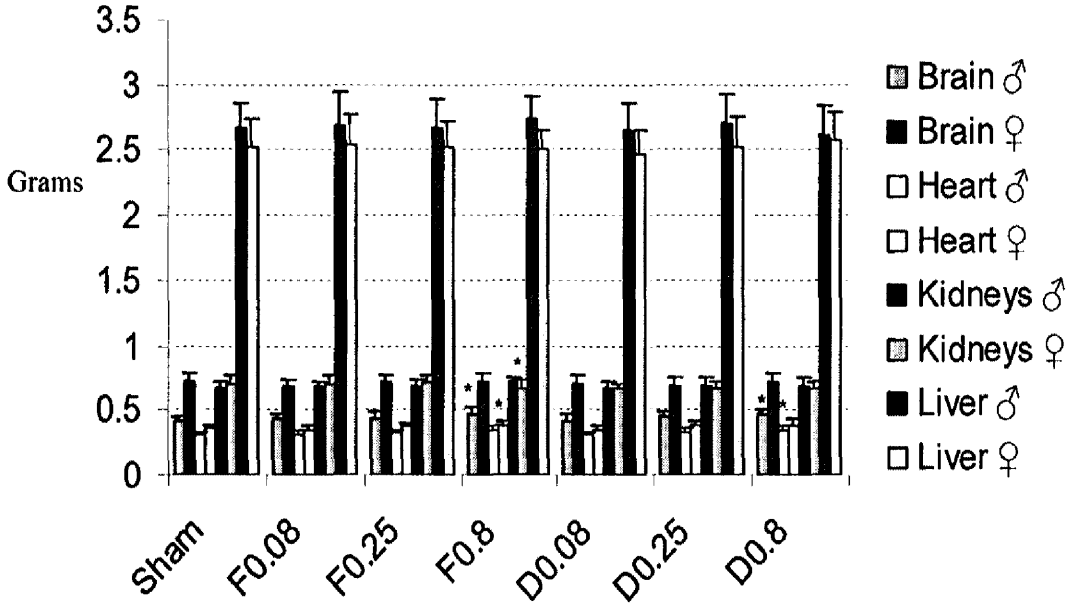
Dosage (mg WTPM/L)	**0**			**0.08**				**0.25**				**0.8**			
Observation	**Sham**			**Freon**		**DIET**		**Freon**		**DIET**		**Freon**		**DIET**	
	a	b	c	a	b	a	c	a	b	a	c	a	b	a	c
Normal (no abnormalities)	25	83.3	12.6	27	90	30	13.1	29	96.7	29	13.1	27	90	29	12.8

Body surface															
Alopecia	13	43.3	12.1	11	36.7	9	5.6	15	50	9	9	14	46.7	8	9.1
Abrasion	0	0	0	1	3.3	1	5	0	0	0	0	2	6.7	0	0
Scabbed area															
Tail abrasion	-	-	0	-	-	0	0	-	-	1	1	-	-	0	0
Eyes															
Area around eye swollen	1	3.3	1	1	3.3	0	0	0	0	0	0	0	0	0	0
Protruding eye	-	-	0	-	-	1	1	-	-	0	0	-	-	0	0
Ear (s)															
Swollen pinna	0	0	0	0	0	1	3	1	3.3	0	0	0	0	1	12
Hematoma	0	0	-	1	3.3	-	-	1	3.3	-	-	0	0	-	-

a = Number of animals affected
b = Percent of animals with positive observation noted at least once during interval
c = Mean number of animal weeks with clinical sign

Why are there no column b or column c data in some circumstances?

Figure 11 shows the 90-day exposure time point differences in **(differences in??)** % organ to body weight ratios. Groups compared were the Sham group, Freon expanded mainstream cigarette smoke groups (F) and DIET cigarette smoke groups (D). Concentrations tested were 0.08 mg WTPM/L (F0.08 and D0.08), 0.25 mg WTPM/L (F0.25 and D0.25), and 0.8 mg WTPM/L (F0.8 and D0.8). Twenty animals per group were analyzed at the 90-day time point and 9-10 animals/group were analyzed at the recovery period time point. Statistically significant differences in several absolute organ weights or organ weight ratios (brain, heart, kidney, lungs, and adrenals) were seen and are indicated by an asterisk where they occur in Figure 11. After the recovery period, these changes disappeared. Because changes were small and inconsistent in relation to exposure concentration, sex, or cigarette type, differences between Freon and DIET cigarettes were not considered biologically significant. **(The y-axis label of this figure is incorrect. Is it expressed as a percentage of body mass? It is not grams. There is something wrong with this figure. A rat brain at necropsy may weigh 1-3 grams (approximately). The liver may weigh 7-11 grams or more. ~~The brain and liver ratios to body weight can not be so similar~~! Further examination has led me to realize that the color scheme is so similar that I could not tell the difference in the blue or blue-gray colors. The color scheme has to be more distinguishable!)**



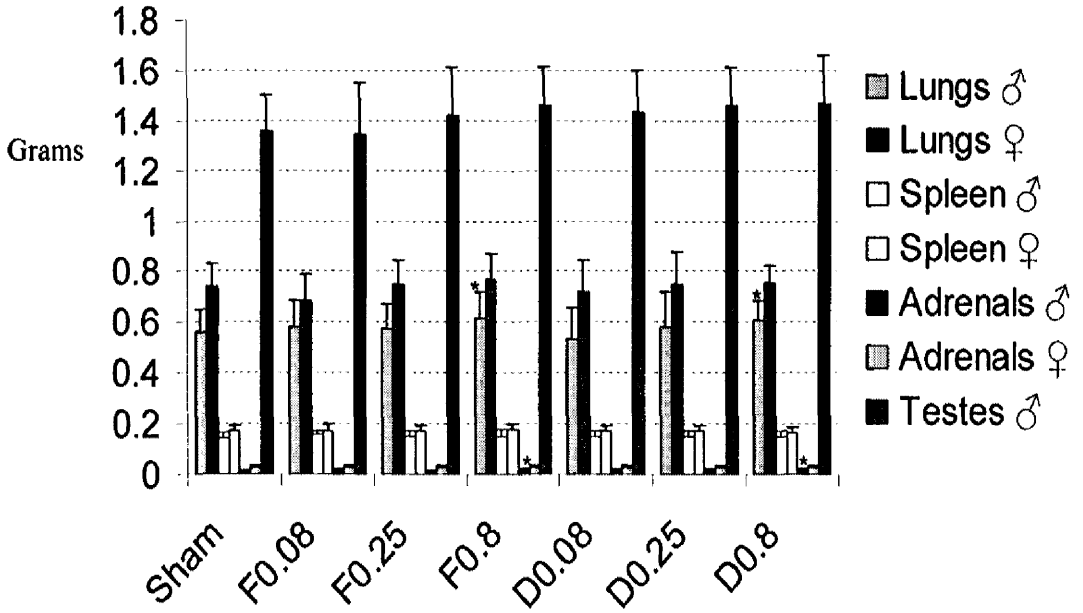


Figure 11. Percent organ to body weight ratios. Statistically significant differences compared to sham are denoted with an asterisk ($p<0.05$). F = Freon, D=DIET. Data represent averages and standard errors. Cigarettes containing Freon expanded tobacco were tested at the following levels (mg WTPM/L): 0.08 (F0.08), 0.25 (F0.25), and 0.8 (F0.8). Likewise, cigarettes containing DIET expanded tobacco were tested at the following levels (mg WTPM/L): 0.08 (D0.08), 0.25 (D0.25), and 0.8 (D0.8).

(The y-axis is wrong as mentioned above. It is not grams! These should be labeled as figures 11a and 11b. Distinguish that some organ to body weight ratios are found in figure 11a and some are found in figure 11b.)

Tables 9a and 9b show that there were no DIET exposure-related changes **(That is incorrect!. There were DIET exposure-related changes. If they were different from sham, by definition, they would be exposure related changes. Did you mean to say that there were no differences observed between the smoke related effects of DIET and Freon?)** and no novel histopathological effects. In this study, the changes related to the Freon cigarette and the DIET cigarettes were comparable in type, frequency, and severity.

Table 9a. Males: Incidence summary of microscopic observations **at the end of the inhalation exposure period** (Sham, Freon, DIET)

Stain	Tisue	Exposure Concentration (mg WTPM/L)	0.00	0.08		0.25		0.8	
		Microscopic Change	Sham	Freon	DIET	Freon	DIET	Freon	DIET
H&E	*Nose*	• Inflammation, chronic active	20	19	20*	20*	20*‡	20*	20*
		• Hyperplasia, epithelial	0	2	5	18*	13*	20*	20*

* Statistical significant difference from sham at the 0.05 level using the Kolmogorov-Smirnov two tailed test. Two numbers with the same value can be different because this test takes into account both incidence *and* severity, even though only severity is shown in the table. **(Severity is not shown in this table. This is incidence.)** Severity Grading Scale: (1) minimal, (2) mild, (3) moderate, (4) marked, and (5) severe. **(What does the symbol by the nose, inflammation, chronic active, of DIET 0.25 indicate?)**

Stain	Tisue	Exposure Concentration (mg WTPM/L)	0.00	0.08		0.25		0.8	
		Microscopic Change	Sham	Freon	DIET	Freon	DIET	Freon	DIET
	Lung lobe	*Left*							
		• NM[4]	20	20	20	20*	20*	20*	20*
		• Individual BGM's[3]	0	20*	20*	20*	20*	20*	20*
		• Aggregate BGM's	0	11*	10*	10*	7	14*	9*
		Intermediate							
		• NM	20	20	20*	20*	20*	20*	20*[1]
		• BGM's, individual	0	20*	20*	20*	20*	20*	20*
		• BGM's, aggregate	0	6	10*	4	9*	14*	10*
		Apical							
		• NM	20	20	20	20*	20*	20*	20*
		• Individual BGM's	0	20*	20*	20*	20*	20*	20*
		• Aggregate BGM's	0	8*	9*	4	3	8*	6
		Right Cardiac							
		• NM	20	20*	20	20*	20*	20*	20*
		• Individual BGM's	0	20*	20*	20*	20*	20*	20*
		• Aggregate BGM's	0	5	4	3	6	12*	10*
		Right Diaphragmatic							
		• NM	20	20	20*	20*	20*	20*	20*
		• Individual BGM's	0	20*	20*	20*	20*	20*	20*
		• Aggregate BGM's	0	7	6	5	5	5	9*
PAS/ AB		*Left*							
		• Goblet cells	17	20	20	20	20	20*	20*
		Intermediate							
		• Goblet cells	17	19	18	19	19	20	20
		Apical							
		• Goblet cells	15	19	20	20	19	20	20
		Right Cardiac							
		• Goblet cells	15	20	19	20	20	20	20
		Right Diaphragmatic							
		• Goblet cells	18	20	20	20	20	20*	20
H&E	*Larynx*	• Ventral - squamous metaplasia	0	20*	18*	17*	19*	19*	20*
		• Chronic active inflammation	20	20*	20*	20*	20*	20*	20*

Table 9b. Females: Incidence summary of microscopic observations **at the end of the inhalation exposure period** (Sham, Freon, DIET)

Stain	Tisue	Exposure Concentration (mg WTPM/L)	0.00	0.08		0.25		0.8	
		Microscopic Change	Sham	Freon	DIET	Freon	DIET	Freon	DIET
H&E	*Nose*	• Inflammation, chronic active	20	20	20	20*	20*	20*	20*
		• Hyperplasia, epithelial	1	0	0	13*	14*	20*	20*

[4] NM= Nonpigmented Macrophages; [3]BGM = Brown Gold Macrophage

* Statistical significant difference from sham at the 0.05 level using the Kolmogorov-Smirnov two tailed test. Two numbers with the same value can be different because this test takes into account both incidence *and* severity, even though only incidence is shown in the table. Severity grading scale range as follows: (1) minimal, (2) mild, (3) moderate, (4) marked, and (5) severe.

Stain	Tisue	Exposure Concentration (mg WTPM/L)	0.00	0.08		0.25		0.8	
		Microscopic Change	**Sham**	**Freon**	**DIET**	**Freon**	**DIET**	**Freon**	**DIET**
	Lung lobe	*Left*							
		• NM[♣]	20	20	20	20	20*ǂ	20*	20*
		• Individual BGM's[♪]	0	20*	20*	20*	20*ǂ	20*	20*
		• Aggregate BGM's	0	5	10*	5	5	3	5
		Intermediate							
		• NM	20	20*	20	20*	20*	20*	20*
		• BGM's, individual	0	20*	20*	20*	20*	20*	20*
		• BGM's, aggregate	0	5	6	8*	5	6	7
		Apical							
		• NM	20	20	20	20*	20*	20*	20*
		• Individual BGM's	0	20*	20*	20*	20*	19*	20*
		• Aggregate BGM's	0	4	5	1	6	4	9*
		Right Cardiac							
		• NM	20	20*	20	20*	20*ǂ	20*	19*
		• Individual BGM's	0	20*	20*	20*	20*ǂ	20*	20*
		• Aggregate BGM's	0	4	3	7	3	8*	8*
		Right Diaphragmatic							
		• NM	20	20*	20	20*	20*ǂ	20*	20*
		• Individual BGM's	0	20*	20*	20*	20*ǂ	20*	20*
		• Aggregate BGM's	0	5	3	5	2	0	4
PAS/ AB		*Left*							
		• Goblet cells	20	20	20	20	20	20*	20
		Intermediate							
		• Goblet cells	16	19	19	20	19	20*	20ǂ
		Apical							
		• Goblet cells	16	19	19	19	20	20	20
		Right Cardiac							
		• Goblet cells	17	19	20	18	20	20	20
		Right Diaphragmatic							
		• Goblet cells	17	20	18	20	20	20*	20ǂ
H&E	*Larynx*	• Ventral - squamous metaplasia	0	20*	18*	19*	20*	19*	20*
		• Chronic active inflammation	19	20*	20*	20*	20*	20*	20*

(I strongly suggest that average severity data, if available, be used instead of incidence data. If average severity tables are not within the report, average severity can be calculated from expanded incidence tables. Typically, a report would only include average severity of smoke related effects. Expanded incidence tables usually exist for only statistically significant differences between comparisons of smoke related effects between cigarette types.. You may find a complete average severity table or a complete expanded incidence table. Incidence with average severity data would be another approach.) The sporadic differences noted in Tables 9a and 9b for the 90 day exposure time point are not considered biologically significant because they are within the biologic variability range, do

[♣] NM= Nonpigmented Macrophages; [♪]BGM = Brown Gold Macrophage

ǂ ~~Statistially~~ **Statistically** significantly different from Freon

not follow dose-response patterns, and/or were not seen consistently in both sexes. In addition, most sporadic changes noted resolved after the 90 day recovery period.

Consequently, inhalation exposure to mainstream cigarette smoke from cigarettes containing DIET produced a toxicological response profile similar to that of mainstream cigarette smoke from cigarettes containing Freon expanded tobacco. DIET processing did not induce biologically significant or unexpected effects.

Dermal Tumor Promotion Study

Study Design

RJRT conducted a study to compare the potential tumor promoting capacity of mainstream cigarette smoke condensates (CSC) from cigarettes containing expanded tobacco (Meckley *et al.*, 2000). The two-stage carcinogenesis model used, is the classical model involving an initiation phase, in which normal cells become neoplastic and a promotion phase, in which latent neoplastic cells can develop into a tumor. **(I don't agree with this sentence. I don't consider that a cell can be deemed neoplastic if it is not associated with a neoplasm. I don't consider that a cell is neoplastic if evidence of neoplasia is not yet determined. My view is that initiation yields genetic damage not neoplastic cells. The promotion phase promotes the development of a neoplasm from that genetic damage. My understanding is that a pathologist would not submit a finding of neoplastic cells at the end of an initiation treatment. Neoplastic and neoplasm are terms related to new growth. Don't confuse biochemical or molecular changes within an initiated cell as "neoplastic". Neoplasia is a histopathology term. All biochemical or cytological methods that could be used to identify "neoplastic" cells would have to be correlated with a confirmed histopathologic finding of neoplasia for a pathologist to accept that method as appropriate. I may be wrong about this but I don't think so.)** The SENCAR mouse is sensitive to initiation with 7, 12-dimethylbenz[a]anthracene (DMBA). **DMBA was used as an initiator. The promotion phase consists of applications of "tar" from cigarettes.** The ~~test~~ cigarettes were the same as those used in the inhalation study.

Cigarettes were smoked on AMESA Type 1300C smoke generators operated under the Federal Trade Commission standard conditions of 2 second puff, 35 ml/puff, taken 1 time/minute to a mean butt length of 3 mm above the filter overwrap. Condensate was collected via cold trap and stored as a concentrated solution in acetone. **Dosing solutions were prepared in an acetone/water vehicle after removal of excess acetone. The final prepared dosing solutions, at the various concentrations, contain 8 percent water.** The d and, in dosing the condensate, **(The cigarette smoke condensate dosing application)** is expressed as mg "tar"/ application. The study used the treatment groups outlined in Table 10 (40 female SENCAR mice/group).

Table 10. Experimental groups

Group No.	Description	Mg/ application
Controls		
1	Acetone/Acetone	
2	DMBA*/Acetone	
Treatment cigarette smoke condensates		
3	DMBA/ Freon	9
4		18
5		36
6	DMBA/DIET	9

7		18
8		36
12	Acetone/Freon	36
13	Acetone/DIET	36

*DMBA = 75 µg

Condensate **or vehicle control** was administered dermally, at 200 µl/ dose, 3 times/ week, for 29 weeks. Treatment groups received a single initiating dose of either 7, 12-dimethylbenz[a]anthracene (DMBA) or acetone on the first day of the study. During the promotion phase of the study, **beginning one week after initiation,** the groups received doses of 9, 18, or 36 mg "tar"/ application. Endpoints monitored included mortality, body and organ weights, clinical observations, tumors, necropsy and histopathology. Tissues collected at necropsy are enumerated in Table 11.

Table 11. Tissues collected and grossly examined at necropsy

Adrenals	Pancreas
Brain	Parathyroids
Carcass	Pituitary
Clitoral glands	Salivary gland
Esophagus	Sciatic nerve
Eyes	Skin (treated and untreated)
Gall bladder	Small intestine (duodenum, jejunum, ileum)
Gross Lesions	Spinal cord (thoracic)
Harderian glands	Spleen
Heart and aorta	Sternebrae, femur and vertebrae (including marrow)
Kidneys	Stomach
Large intestine (cecum, colon, rectum)	Tail
Liver	Thigh muscle
Lungs and bronchi	Thymus
Mammary glands with adjacent skin	Thyroid
Mandibular and mesenteric lymph nodes	Tissue masses or suspect tumors and regional lymph nodes
Nasal cavity and nasal turbinates	Trachea
Oral cavity, larynx and pharynx	Urinary bladder
Organ/tissue	Uterus
Ovaries	Zymbal glands (auditory sebaceous glands)

Most data were analyzed using statistical tests available through the Xybion/**Path-Tox** computer system. Statistical procedures included: one-way analysis of variance, Bartlett's test of homogeneity of variance, Dunnett's t-test, Fisher's least significant difference test, Cochran and Cox's modified t-test, Kolmogorov-Smirnov test, cumulative percentage of tumor bearing animals, and median time to tumor. The one-way analysis of variance was given only on data comparing dose groups. Bartlett's test of homogeneity of variance was performed whenever group comparisons were made. When the data were homogenous, Dunnett's test was performed at p 0.05. When the data were nonhomogenous, Cochran and Cox's modified t-test was performed at p 0.05. Tumor data were analyzed using Fisher's exact test and pathology data were analyzed using the Kolmogorov-Smirnov test. Tests were carried out at 5% two-sided level.

Results

Several relevant clinical signs are noted in the Table 12. Since incidence of clinical signs observed did not follow a dose-response pattern, these signs are not considered biologically significant.

Table 12. Summary of clinical signs for skin disorders

Group Interval: 1-208 days	Acetone/ Acetone		DMBA/ Acetone		DMBA/ Freon						DMBA/ DIET						Acetone/ Freon		Acetone/ DIET	
Dose (mg/ application)	158		158		9		18		36		9		18		36		36		36	
# Animals alive at start of interval	40		40		40		40		40		40		40		40		40		40	
Observation Dorsal skin	a	b	a	b	a	b	a	b	a	B	a	b	a	b	a	b	a	b	a	b
Erythema	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	1	5
Desquamation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3
Peeling skin	0	0	1	5	1	1	1	3	3	7.3	1	5	0	0	9	7.1	21	7.5	19	6.4
Abrasion	0	0	0	0	1	187	0	0	2	4.5	1	8	3	4.3	1	10	2	4	1	10
Laceration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	5	0	0	0	0
Sore	0	0	0	0	0	0	0	0	1	131	0	0	2	33.5	1	78	4	65	5	37.8
Discoloration	0	0	0	0	0	0	0	0	1	131	0	0	1	96	0	0	0	0	0	0

a = Number of animals affected
b = Mean number of animal days with clinical sign during interval

(Look for the uppercase "B" in the above table.) Body weights were measured throughout the study and at the end of the experiment, when organ weights were also determined. Administration of treatment and control articles produced no biologically significant adverse effect on group mean body weights. There were instances of statistically significant differences but these were not obvious trends and did not follow a dose-response. In the acetone/high dose DIET and acetone/high dose Freon groups, there were statistically significant differences in liver to body weight and liver to brain weight **ratios**. However, because there were no discernable histopathological ~~lesions~~ **findings** correlated with these changes, they were not considered biologically relevant.

Tumor data are presented in Table 13, 14, and 15. Tumorigenicity promotion potential was evaluated in terms of number and % of tumor bearing animals, number and types of tumors produced, and **median?** time to tumor onset. Freon and DIET treatment groups were similar in their tumor promoting potential. DIET did not adversely affect the tumor promoting characteristics of the cigarette mainstream smoke condensate.

Table 13. Gross tumor summary at week 31

Group	Group	Number of TBA‡	Total # Tumors	Tumors/TBA
1	Acetone/Acetone	0	0	0
2	DMBA*/Acetone	0	0	0
3	DMBA/Freon (9 mg)	2	6	3
4	DMBA/Freon (18 mg)	4	7	1.75
5	DMBA/Freon (36 mg)	17	99	5.82
6	DMBA/DIET (9 mg)	0	0	0
7	DMBA/DIET (18 mg)	6	29	4.83
8	DMBA/DIET (36 mg)	20	83	4.15
12	Acetone/Freon (36 mg)	2	3	1.5

13	Acetone/DIET (36 mg)	11	15	1.36

‡TBA = Tumor Bearing Animals; *DMBA = 7, 12-dimethylbenz[a]anthracene (75 µg)

Table 14. Group median time to onset of masses

Group #	Group description	Median Time Of Onset (weeks)
1	Acetone/Acetone	-
2	DMBA/Acetone	-
3	DMBA/Freon (9 mg)	12 [a, c]
4	DMBA/Freon (18 mg)	18 [a]
5	DMBA/Freon (36 mg)	20 [a]
6	DMBA/DIET (9 mg)	-
7	DMBA/DIET (18 mg)	17 [a, b]
8	DMBA/DIET (36 mg)	21 [a, b]
12	Acetone/Freon (36 mg)	18 [d]
13	Acetone/DIET (36 mg)	24 [d]

[a] = Significantly greater than DMBA/Acetone control, p>0.05

[b] = Significantly greater than low dose of same cigarette, p>0.05

[c] = Significantly greater than DIET low dose, p>0.05

[d] = Significantly greater than Acetone/Acetone, p>0.05

Table 15. Microscopically confirmed tumor summary

Group	Group	TBA[a]	Total # Tumors	Tumors/ TBA	Benign Growths	Malignant Growths
1	Acetone/Acetone	2	2	1	0	2
2	DMBA/Acetone	0	0	0	0	0
3	DMBA/Freon (9 mg)	2	7 [f]	3.5	7 [f]	0
4	DMBA/Freon (18 mg)	5	7 [c]	1.4	7 [c]	0
5	DMBA/Freon (36 mg)	16 [c, g]	86 [c, g]	5.4	85 [c, g]	1
6	DMBA/DIET (9 mg)	0	0	0	0	0
7	DMBA/DIET (18 mg)	7 [c, h]	32 [d, h]	4.6	32 [d, h]	0
8	DMBA/DIET (36 mg)	20 [c, g]	84 [c, g]	4.2	73 [c, g]	11 [b, g]
12	Acetone/Freon (36 mg)	3	7	2.3	7 [e]	0
13	Acetone/DIET (36 mg)	10	13 [e]	1.3	13 [e]	0

[a] = Tumor bearing animals

[b] = Significantly greater than DMBA/Acetone and DMBA/Freon (36 mg), p<0.05

[c] = Significantly greater than DMBA/Acetone, p<0.05

[d] = Significantly greater than DMBA/Acetone and DMBA/Freon (18 mg), p<0.05

[e] = Significantly greater than Acetone/Acetone, p<0.05

[f] = Significantly greater than DMBA/Acetone and DMBA/DIET (9 mg), p<0.05

[g] = Significantly greater than low and mid dose of same cigarette, p<0.05

[h] = Significantly greater than low dose of same cigarette, p<0.05

There were no biologically significant non-neoplastic changes of the internal organs between DIET and Freon. Several neoplastic changes of internal organs were observed, however, the incidences were low, and were not observed in any discernible pattern and thus, these changes were considered incidental. There were no biologically significant differences noted between DIET and Freon in their ability to induce non-neoplastic changes of the dosed dorsal skin of DMBA-initiated animals (e.g., hyperkeratosis). DIET produced statistically significantly less chronic hyperkeratosis of the inguinal skin in the acetone-initiated, high dose-treated groups compared to the acetone-initiated, high dose Freon group.

Neoplasms of the dosed dorsal skin were produced in all treated groups except the DMBA/low dose DIET group. DIET produced ~~lesser~~ **fewer** or comparable responses as measured by all parameters, except one. There was a statistically significant increase in the number of combined malignant growths for the high dose DIET group compared to the high dose Freon group. However, when Freon was previously tested in the same assay **(at RJRT?)**, the application of 20 mg "tar", three times per week produced a comparable tumor number. **(comparable to what?) comparable to DIET? A different study?**

Collectively these studies, in conjunction with the original NCI data, demonstrate that the activity of CO_2 expanded tobacco is comparable to the activity of unexpanded tobacco. **(Was a comparison made to an unexpanded tobacco cigarette? That is why the types of cigarettes used in the various assays is so important.)** As indicated, there is no evidence which indicates that expansion via CO_2 results in a measurable increase in biological activity relative to Freon expanded tobacco, and presumably therefore to unexpanded tobacco. **(This looks like a concluding paragraph for all studies. Could it be, "Collectively these inhalation and dermal studies ....".)**

In Vitro Studies

In vitro test batteries were conducted and data are presented below **that** compare the effects of cigarette smoke condensates from cigarettes containing DIET, Freon, or unexpanded tobacco. These data indicate that there are no statistically significant differences among the different cigarette smoke condensates compared.

Ames Assay

The objective for running the Ames assay is to compare test cigarettes with reference cigarettes and determine their relative mutagenic potency. This assay was run several times for different purposes and results were consistent. The Ames assay uses the *Salmonella typhimurium* histidine reversion system, which is a microbial assay that measures histidine reversion induced by chemicals causing base changes or frameshift mutations in the genome of the organism. A reverse mutation assay in *S. typhimurium* detects a mutation in a gene of a histidine-requiring bacterial strain to produce a histidine-independent strain.

Study Design

Each cigarette was made of flue-cured and burley tobacco. The control cigarette consisted of 100% unexpanded tobacco and the test cigarettes consisted of 50% expanded and 50% unexpanded tobacco from the pilot and the full scale facilities. The levels of inclusion of expanded tobacco are relevant marketing levels (upper range). The composition of the cigarettes is show in Figure 12.

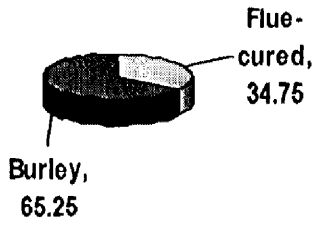


Figure 12. Test cigarette composition (%)

Condensates were prepared according to a method previously described (Doolittle *et al.*, 1990; Steele *et al.*, 1995). Briefly, cigarettes were smoked according to FTC conditions (35 ml puff volume, 2s/puff, 1 puff/minute) using 20-port automatic smoking machines. Mainstream particulate matter was collected on Cambridge filter pads and then the pads were extracted with DMSO by shaking for 20 minutes.

Cigarette smoke condensates ("tar") were assessed for mutagenicity in *Salmonella typhimurium*, as previously described (Maron and Ames, 1984; Yahagi *et al.*, 1975; Doolittle *et al.*, 1990). Briefly, in the preincubation assay, the S9 liver fraction from male Sprague-Dawley rats (induced by the administration of a single injection of Aroclor 1254) was combined with the test bacteria and the test condensate, and allowed to preincubate at 37°C for 20 minutes. Molten top agar supplemented with 0.5 mM histidine/biotin was then added and this mixture was poured onto plates containing minimal glucose agar, incubated at 37°C for 48 hours **after administration of cigarette smoke condensates or vehicle control**, and **colonies** counted. Triplicate plates were involved at each concentration.

Doses added to triplicate plates were 0, 25, 50, 75, 100, 125, and 250 µg **(ug "tar" or ug "CSC")**/plate. TA98 and TA100 were used since, of the strains typically used, they provide the most sensitive consistent response to mainstream cigarette smoke condensate. TA98 detects frameshift mutations and TA100 detects base-pair substitutions. S9 was added because it creates the best conditions for detection of a positive response from cigarette smoke condensate. Triplicate counts were averaged, and the dose responses, e.g., slope values, were analyzed by a linear point rejection model (Bernstein, et al. **year**). Data **(what data? Colony counts at the various dose levels?)** were then analyzed by a test of means, standard deviation, and one-way ANOVA.

Results

As mentioned, the assay was run several times and results were consistent. In one test, statistical comparisons between test groups (DIET-expanded in the full production facility and the pilot) and controls (unexpanded) and between test groups (full production DIET vs. pilot DIET) were conducted for revertants/mg "tar" and revertants/cigarette (Avalos, 2000). Treatment groups are shown in Figure 13. **(In my view, figure 13 does not show "treatment groups". Figure 13 only shows tar yields of 3 cigarette types.)** There were no statistically significant differences at $p<0.05$ (Table 16).

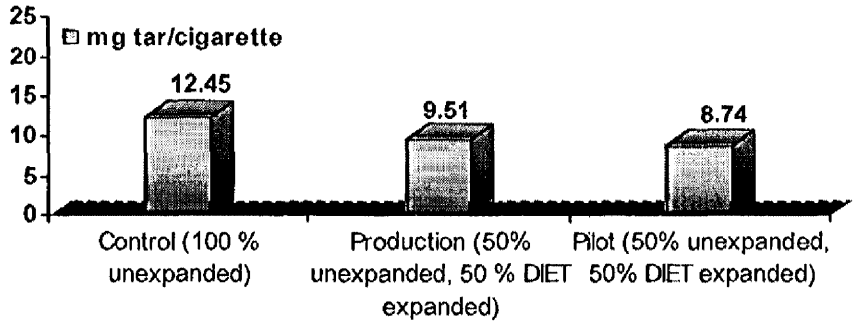


Figure 13. Treatments groups and corresponding "tar" levels (Avalos, 2000)

Table 16. Ames data (Avalos, 2000)

Treatment group	TA98		TA100	
	Revertants/ mg "tar"	Revertants/ cigarette	Revertants/ mg "tar"	Revertants/ cigarette
Control: 100% unexpanded	1542	19198	886	11031
Production: 50% unexpanded 50% DIET expanded	1558	14817	774	7361
Pilot: 50% unexpanded 50% DIET expanded	1953	17069	890	7779

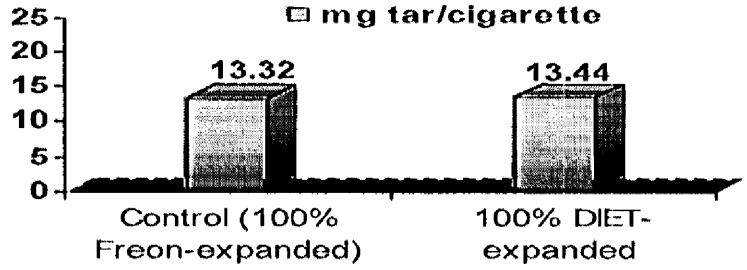


Figure 14. Treatments groups and corresponding "tar" levels (Avalos *et al.*, 1994)

In another test, mainstream cigarette smoke condensates from cigarette prototypes, comprised of 100% expanded tobacco using DIET was compared to Freon control (Avalos *et al.*, 1994). Treatments are shown in Figure 14. **(Figure 14 does not show treatments. Figure 14 only shows tar yields for 2 cigarette types.)** Reverent/mg "tar" and revertant/cigarette from DIET-expanded tobacco condensate were statistically compared to Freon expanded tobacco condensate. No significant differences were detected (Table 17).

Table 17. Ames data (Avalos *et al.*, 1994)

Treatment group	TA98		TA100	
	Revertants/ mg "tar"	Revertants/ cigarette	Revertants/ mg "tar"	Revertants/ cigarette
Freon (100% expanded)	857	11415	624	8312
DIET (100% expanded)	775	10416	907	12190

Additional tests were run and all results are consistent and reproducible. One more example is given showing that there are no statistically significant differences in specific Ames activity (compared on revertants/mg "tar" basis) when tobacco from the DIET process is compared with that from the Freon process and the

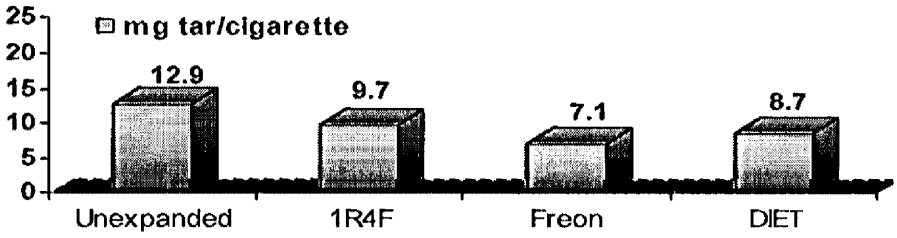


Figure 15. Treatment groups and corresponding "tar" levels (Fulp, 1990)

reference 1R4F (Fulp, 1990; Figure 15 and Table 18) **(Figure 15 only shows tar yields per cigarette. Treatment groups are particular cigarette type and applications ug "tar"/plate).**

Table 18. Ames data (Fulp, 1990)

Treatment group	TA98		TA100	
	Revertants/ mg "tar"	Revertants/ cigarette	Revertants/ mg "tar"	Revertants/ cigarette
Unexpanded	1642	21182	840	10836
Freon	1271	9024	626	4445
1R4F	1477	14327	573	5558
DIET	1314	11432	905	7874

Sister Chromatid Exchange Assay

The objective of the Sister Chromatid Exchange assay (SCE) in CHO cells is to rank the potency of two or more mainstream cigarette smoke condensates to induce sister chromatid exchanges in CHO cells. Sister chromatids are defined as the two copies of a gene that exist side by side in a chromosome. SCE is the interchange of DNA between these two chromatids. The SCE assay measures the ability of test substances to cause an increase in the exchange of genetic material between sister chromatids of a chromosome (increase above normal background levels). It detects reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome. This assay can be considered complementary to Ames, since it measures chromosome breaking, another measure of genotoxicity. This complementary testing approach enables a more rigorous characterization of the genotoxic potential of mainstream cigarette smoke condensates.

Study Design

Similar groups as tested in the Ames assay were used except that one of the SCE assays included an extra reference cigarette, 1R4F described in more detail below.

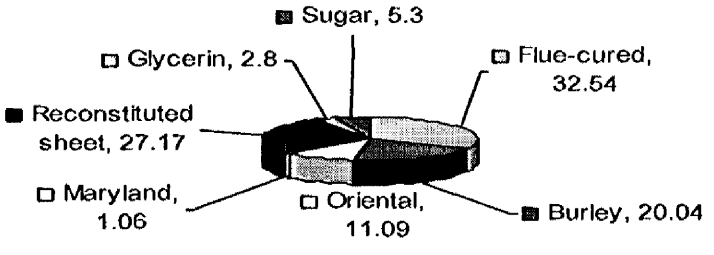


Figure 16. Kentuky 1R4F composition

The 1R4F reference cigarette was developed by the US National Cancer Institute, the Agricultural Research Service of the US Department of Agriculture, **and** the University of Kentucky Tobacco~~,~~——and Health Research Institute to serve as a reference cigarette for experimental purposes. The contribution (%) of various constituents is shown in the pie chart (Figure 16). Based on its tobacco blend, "tar", nicotine, and carbon monoxide yields the 1R4F is viewed as being representative of the average US full-flavored, low "tar" cigarette market (Steele *et al.*, 1995). 1R4F contains no expanded tobacco.

Chinese Hamster Ovary cells were used in all SCE assays (Perry and Wolff, 1974). In the assay without metabolic activation, cell cultures containing approximately 10^6 cells were treated with the test condensate for approximately 2 hours at 37^0C before the addition of 10 µM 5-bromo-2'-deoxyuridine (BrdU) followed by incubation for an additional 22-30 hours (test concentrations between 0 and 60 µg). The exposure period was terminated by washing the cells with saline. Cultures were then reincubated in fresh medium containing 10 µm BrdU and 0.1 µg/ml Colcemid for 2.5 hours. Metaphase cells were collected by mitotic shake-off, swollen in hypotonic KCl solution, dropped onto slides, air-dried, and stained. In the assay with metabolic activation, cells were initially treated with condensate concentrations between 0 and 300 µg for 2 hours in the presence of a S9 metabolic activation (containing liver homogenate from Aroclor 1254 pre-treated rats), washed, and then treated as described for the nonactivation assay. The positive control in the nonactivation assay was mitomycin C, while in the activation assay the positive control was cyclophosphamide.

Data were analyzed by using the Bonferroni method for pair-wise comparisons of cigarettes or student t tests if increases in SCE's were found. Statistically significant differences ($p<0.05$) were noted.

Results

In one study (duplicate assays), although all samples were positive in the SCE assay, there were no statistically significant differences in slope values among the samples in the presence or absence of S9 metabolic activation. Results are presented in the Table 19a and 19b (Bombick *et al.*, 2001; Bowman *et al.*, 2001). Treatment groups are shown in Figure 17. **(Again, in my view, this figure is simply tar yields of various cigarette types.)**

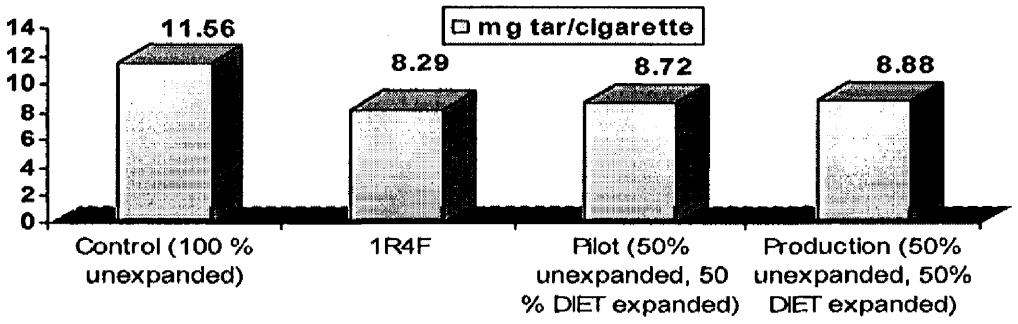


Figure 17. Treatments groups and corresponding "tar" levels (Bombick *et al.*, 2001)

Table 19a. SCE data (Bombick *et al.*, 2001) **(What is unit of measure ? slope ?)**

Sample	+S9 ± standard error	-S9 ± standard error
Control: 100% unexpanded	0.0039 ± 0.00069	0.0435 ± 0.0036
1R4F	0.0042 ± 0.00069	0.0378 ± 0.0036
Production: 50% unexpanded 50% DIET expanded	0.0037 ± 0.00069	0.037 ± 0.0036
Pilot:	0.0042 ± 0.00069	0.0474 ± 0.0036

50% unexpanded 50% DIET expanded		

Table 19b. SCE data (Bowman *et al.*, 2001) **(Unit of measure ?)**

Sample	**+S9 ± standard error**	**-S9 ± standard error**
Control: 100% unexpanded	0.0045 ± 0.00066	0.0401 ± 0.0027
Production: 50% unexpanded 50% DIET expanded	0.0046 ± 0.00066	0.0386 ± 0.0027
Pilot: 50% unexpanded 50% DIET expanded	0.0046 ± 0.00066	0.0415 ± 0.0027

In summary, SCE induction by cigarette smoke condensate from DIET-expanded tobacco was not statistically significantly different than that from unexpanded tobacco.

In two other studies, mainstream cigarette smoke condensates from cigarettes made of 100% expanded tobacco using DIET or Freon, were tested for their ability to induce sister chromatid exchange in CHO cells with and without metabolic activation (Murli, 1992, 1994). While the expanded tobacco cigarette condensates were considered positive for inducing sister chromatid exchange in CHO cells, based on a comparison of the regression slopes of the dose and frequency of sister chromatid exchange observed, they were not statistically different from each other, either in the presence or absence of metabolic activation, under the conditions of this assay (data not shown). The repeat experiments ensure that results are reproducible.

Chromosome Aberration Assay

The objective of the chromosome aberration assay in CHO cells is to compare the potential of the test samples to induce chromosome aberrations. The assays reported here measured the ability of cigarette mainstream smoke condensate to produce gross lesions or changes in chromosome numbers. A chromosome-type aberration results from damage expressed in both sister chromatids at the same site while a chromatid-type aberration results from breakage of single chromatids or breakage and reunion between chromatids. The majority of chromosome aberrations are the chromatid type, but chromosome-type aberrations occur as well. The inclusion of this additional, complementary assay in the test battery provides for an even more comprehensive comparison and evaluation of the genotoxic potential of mainstream cigarette smoke condensate.

Study Design

Mainstream cigarette smoke condensates from cigarette prototypes comprised of 100% expanded tobacco using DIET or Freon were tested for their ability to induce chromosomal aberrations in CHO cells with and without metabolic activation (Murli, 1995; Bombick, 1996).

The nonactivation assay (-S9) was conducted twice. Cell cultures were treated with levels up to 200 µg condensate/ml in the first experiment and 200 to 300 µg condensate/ml in the second experiment. Since the 100% expanded tobacco cigarette prototypes are exaggerations of actual market brands, an additional cigarette, the 1R4F was included as control. This was tested at levels between 200 and 300 µg/ml.

Incubation with test articles lasted for 2 hours. The medium was replaced and incubation continued for 20 more hours. Colcemid (0.1 μg/ml) was added to the medium for the last 2 hours of incubation. Metaphase cells were collected by mitotic shake-off, swollen in hypotonic KCl solution, dropped onto slides, air-dried, and stained. The positive control was mitomycin C.

The assay with metabolic activation (+S9) was similar to the –S9 except S9 fraction was added and concentrations were between 200 and 300 μg condensate/ml. The positive control was cyclophosphamide. At the end, cells were selected for good morphology and 100 cells from replicate cultures were analyzed for chromosomal aberrations.

Initial statistical analysis involved ANOVA and Fisher's exact test with adjustment for multiple comparisons. Significant difference was established for $p<0.01$. A secondary statistical analysis using linear regression was done comparing the slopes of the dose-response curves.

Results

Treatment groups tested are shown in Figure 18 and data in Figure 19. **(Same comment about what figure represents.)**

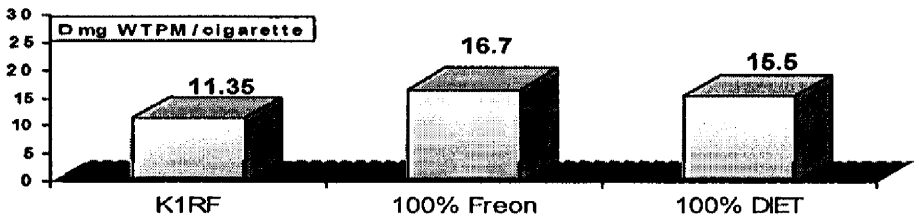


Figure 18. Treatments groups and corresponding "tar" levels (Murli, 1995)

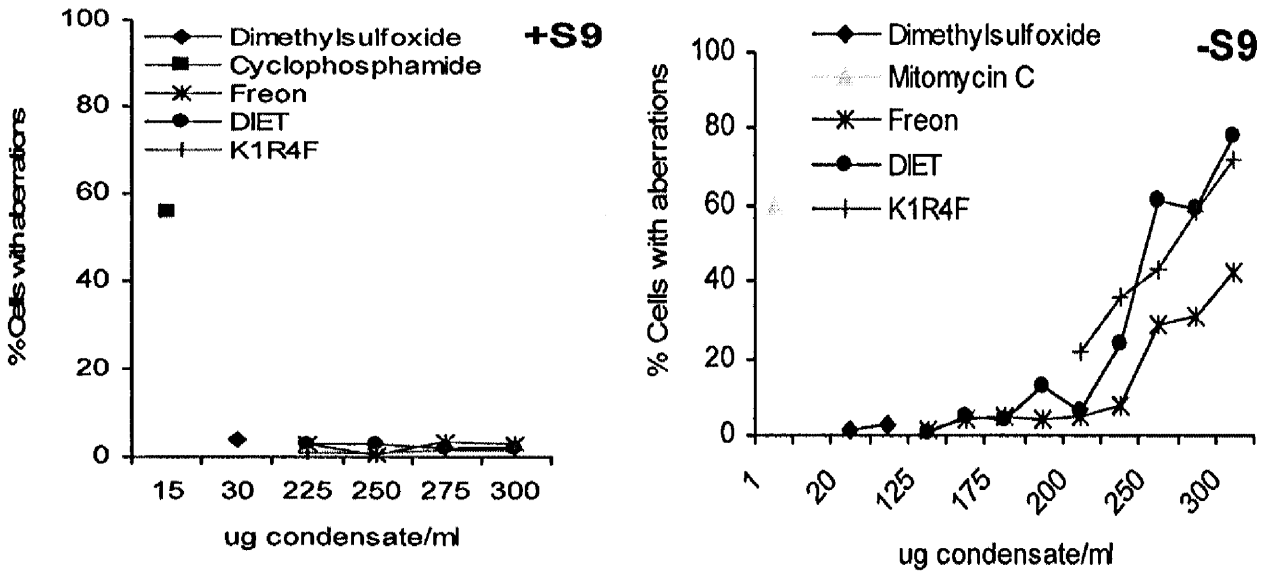


Figure 19. Chromosomal aberration data (Murli, 1995) **(on my screen, the y axis label of the +S9 treatment can not be totally seen.)**

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Cigarette mainstream smoke condensates were negative in the +S9 assay. In the -S9 assay conducted with higher concentrations, all test articles displayed chromosomal aberrations above solvent and negative controls. At levels above 200 µg/ml used in the second experiment, **(a +S9 study or –S9 study? This is not clear.)** the slope for DIET was significantly greater than for Freon using linear regression and point-by-point pairwise comparisons. At 200 µg/ml 1R4F condensate induced a higher response than DIET and Freon. Statistical differences varied with the analysis method and the relevance of this difference **(in the –S9 assay?)** is not fully known. A drawback of this assay **(the chromosomal aberration assay? The +s9 component? The –S9 component?)** is that a general method for positive response comparisons has not been established. In addition, chromosomal aberration assays with cigarettes smoke condensates +S9 have historically given equivocal results that were not always consistent and ranged from positive to negative. Microscopic observations of cells indicated that, at higher concentrations, about 15-30% cytotoxicity occurs. While these data are enclosed for completeness, it must be understood that because cytotoxicity can be a confounder, the relevance of the effects seen *in vitro* with cytotoxic concentrations is diminished.

Conclusion

In vivo and *in vitro* bioassays were conducted to evaluate the biologic activity ~~of samples~~ of mainstream cigarette smoke or smoke condensate from cigarettes containing 100% DIET. DIET samples were compared to control, mainstream cigarette smoke or smoke condensate from cigarettes containing 100% Freon expanded tobacco. In selected tests, additional controls included mainstream smoke from an established, unexpanded experimental reference cigarette, 1R4F as well as mainstream smoke from cigarettes containing unexpanded tobacco before DIET processing. For mainstream smoke chemistry results, comparisons were made among these cigarettes and additionally included US market survey results from commercial cigarettes. Taken collectively, all these studies indicate that the use of CO_2 as an expansion agent for cigarette tobacco does not increase the biological activity inherent to tobacco. As measured by these tests, the CO_2 expansion process produces expanded tobacco with similar activity to previously used and established expansion processes and does not increase biological activity relative to unexpanded tobacco. This conclusion is consistent with the general presumption that tobacco expansion does not increase activity, but may actually lower activity (Dontenwill *et al.*, 1977; Hoffman and Hecht, 1990; NCI, 1976).

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