Experimental Tobacco Carcinogenesis

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In 1912, Adler, in a monograph on lung cancer (*1*), apologized for writing so exhaustively on such an uncommon disease. Today it is the most frequent cause of death from cancer among American men. In respect to the pathogenesis of lung cancer, the Surgeon General's Report of 1964 found that "the magnitude of the effect of cigarette smoking far outweighs all other factors" (*2*).

The epidemiologic evidence that contributed to the conclusions of various review committees (*2–4*) in respect to the association of cigarette smoking with cancer of the lung, oral cavity, larynx, esophagus, and bladder has been supported by extensive experimental data (*5*). Such data do not purport to establish incontrovertibly a causal relationship between smoking and cancer in man, since the final proof can at present be obtained only from epidemiologic findings. The objective of the laboratory studies is, rather, to contribute to an understanding of the factors and mechanisms leading to the disease and thereby to eliminate decisive factors. It is this area which we review here.

Some Characteristics of Tobacco Smoke

Basically, tobacco smoke may be described as an aerosol made up of gases, organic vapors, and particulate matter. It is divided into mainstream and sidestream smoke. The latter emerges between puffs from the burning cone and from the mouthpiece, while the former travels through the column of unlit tobacco before being inhaled. The inhaled smoke may stay in the mouth for a few seconds; this results in partial adsorption, particularly of hydrophilic volatiles. Upon deeper inhalation, the remainder of the smoke reaches the lung. Depending on the degree of inhalation and the condition of the respiratory system, varying amounts, ranging up to more than 90 percent of the aerosol particles, may be retained in the smoker's lungs (*5*).

The general and individual smoking pattern in man may vary significantly, and thus any claim that human smoking patterns have been reproduced in the laboratory must be regarded as unscientific. However, experimental studies can simulate "average smoking patterns." The most widely used standard is the 35-milliliter puff of 2 seconds' duration, drawn once per minute. Seven to twelve such puffs leave a butt of 23 to 40 millimeters.

There is a high concentration of particles in tobacco smoke. One milliliter of smoke from a nonfilter cigarette contains about 5×10^9 particles (*6*); thus, tobacco smoke is an extremely dense respiratory environment (*7*) as compared with the highest reported aerosol concentration for urban pollutants: 10^5 parts per milliliter. Its particles vary in diameter from 0.15 to 1.0 micron and thus fall into the category of particles that are potentially lung-damaging (*5*).

The smoke produced by an average cigarette weighs about 500 milligrams. From 5 to 10 percent of this is moist particulate matter; from 12 to 15 percent, CO_2; from 3 to 6 percent, CO; and the remainder, mainly nitrogen, oxygen, and water (*8*). Over 1200 components have been identified in tobacco smoke, more than 95 percent residing in the particulate matter, or smoke condensate, as it is often called (*9, 10*).

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Respiratory and Skin Carcinogenesis

Complete carcinogenic activity. Tobacco smoke condensate is carcinogenic and sarcogenic to a variety of animal species and tissues (Table 1). Whether from cigarettes, cigars, or pipes, it acts as a complete carcinogen in that it can by itself induce squamous-cell cancer in animals (*5*).

That cigarette smoke is a cause of squamous-cell lung cancer has yet to be experimentally established. Passive inhalation of cigarette smoke by laboratory animals has not yet led to proven bronchogenic squamous-cell carcinoma. Some inhalations in mice, however, have indicated exacerbation of pulmonary adenomas and increased production of alveologenic carcinomas (*5*). These types of tumors may have little relation to the most frequently observed histologic types of human lung cancer. Forced inhalation through an incision in the trachea, as carried out with dogs by Rockey and by Auerbach and their associates, has produced hyperplasia, metaplasia, and, in some instances, carcinoma *in situ* (*11, 12*). The problems associated with such inhalation experiments are discussed below.

Initiators and promoters of tumorigenic activity. We regard tobacco-smoke carcinogens and their effects on mouse skin as an example of the two-step mechanism of carcinogenesis. In carcinogenesis from tobacco the tumor initiators are mostly carcinogenic polynuclear aromatic hydrocarbons (referred to below as "polynuclear hydrocarbons"). The most commonly held concept is that initiators irreversibly affect the genetic apparatus of the cell, leading to a "dormant" tumor cell. The tumor promoters are inactive by themselves, and their effects are reversible. They can, however, "evoke" the initiated cell, thus contributing to tumor induction and proliferation. The mode of action of tumor promoters is not well understood; at least some of them appear to adversely affect mitochondrial respiration (*13*).

The polynuclear hydrocarbon fractions (B and BI; see Figs. 1 and 2) act as relatively strong tumor initiators though they lack tumor-promoting activity. We have recently tested 90 subfractions of BI for tumor-initiating activity on the skin of more than 2000 mice (strain Swiss ICR/Ha Mil), with 0.5 percent croton oil (three applications each week) as promoter. The results indicate tumor-initiating activity

for fractions rich in five-ring as well as in four-ring aromatic hydrocarbons. While tumor initiation by five-ring hydrocarbons is, of course, well known, the tumorigenic and initiating activity of the four-ring hydrocarbons is less familiar. Alkyl derivatives of some four- and five-ring hydrocarbons appear to have especially strong carcinogenic activity. In fact, a study by Lacassagne *et al.* (*14*) demonstrated that methylation (introduction of up to two methyl groups) increased the sarcogenic activity of the benzo[*a*]pyrene ring system. We have shown tumor-initiating activity for the hexacyclic aromatic hydrocarbon indenopyrene, though this component is not a complete carcinogen. Furthermore, several four-ring hydrocarbons were found to potentiate the carcinogenic activity of benzo[*a*]pyrene (*6*). Thus, both four- and five-ring aromatic hydrocarbons, especially when alkylated, are essential for the experimental production of cancer with tobacco "tar." This conclusion is also borne out by the finding that, of the various "tar" fractions, essentially only the neutral portion is a complete carcinogen. Lazsnitzki has demonstrated that the neutral fraction of cigarette "tar," and especially its "hydrocarbon-enriched fraction," can produce hyperplasia and metaplasia in the epithelium of fetal lungs (*15*).

Carcinogenic polynuclear hydrocarbons in the concentrations present in tobacco "tar" clearly do not, by themselves, account for the observed carcinogenic activity. Nevertheless, it has been experimentally well established that minute amounts of initiators can convert a cell into a "dormant" tumor cell. As little as 1 microgram of a carcinogenic polynuclear hydrocarbon may suffice to initiate a cell. Studies have also shown a significant correlation between the benzo[*a*]pyrene content of a given tobacco "tar" and its carcinogenic activity (Fig. 3). It is important to stress the fact that benzo[*a*]pyrene can be regarded merely as an "indicator" for the pyrosynthesized tumor initiators of the carcinogenic hydrocarbons in the tobacco "tar" in that it points to the degree of concentration of other carcinogenic hydrocarbons with four and five rings.

The total yield of polynuclear hydrocarbons in tobacco smoke is largely the result of the burning of tobacco, only insignificant concentrations appearing in extracts of whole tobacco. It is therefore not surprising that such extracts have relatively little activity as complete carcinogens on mouse skin (*5*). On the other hand, a dimethylsulfoxide extract of Turkish tobacco with 7,12-dimethylbenz[*a*]anthracene (DMBA) as a tumor initiator has as much cocarcinogenic activity on mouse skin as smoke condensate obtained from a blended cigarette has (Fig. 4). Both Bock and Van Duuren have demonstrated tumor-promoting activity for extracts of unburned tobacco (*16*).

Table 1. Established tumorigenic activity of tobacco smoke and tobacco smoke condensate, by animal and type of tissue.*

Mode of application	Tissue	Type of tumor
	Mouse	
Topical	Skin (back)	Papilloma, carcinoma
Topical	Cervix	Carcinoma *in situ*, invasive carcinoma
	Rat	
Injection	Subcutaneous	Sarcoma
Injection	Hilum of lung	Carcinoma
	Rabbit	
Topical	Skin (ear)	Papilloma, carcinoma
Topical	Skin (back)	Papilloma
	Hamster	
Inhalation	Trachea, bronchi	Papilloma
	Dog	
Topical	Trachea	Carcinoma *in situ*

* This listing is limited to laboratory studies; thus, pathological evidence for man is not included.

An important question is this: Are the tumor promoters in the extract similar to those in the smoke condensate? Here we are pursuing the hypothesis that the chemical configuration of promoters in tobacco "tar" is comparable to, or dependent upon, that of promoters in the extract, and that some of them are distilled into the smoke without basic structural alteration.

Tumor-promoting activity has been shown for the acidic, phenolic, and neutral fractions of the condensate, indicating that tobacco "tar" contains tumor promoters of various structures. The tumor-initiating activity, on the other hand, is predominantly provided by carcinogenic hydrocarbons. The concentrations of such hydrocarbons and, concurrently, of other tumor initiators in the smoke can be reduced through the addition of nitrates without diminution of the tumor-promoting activity of the resulting "tar." This suggests that the structural configuration of the tumor promoters is not affected by reactions involving free radicals.

Although the "tars" from flue-cured tobaccos were found to have greater

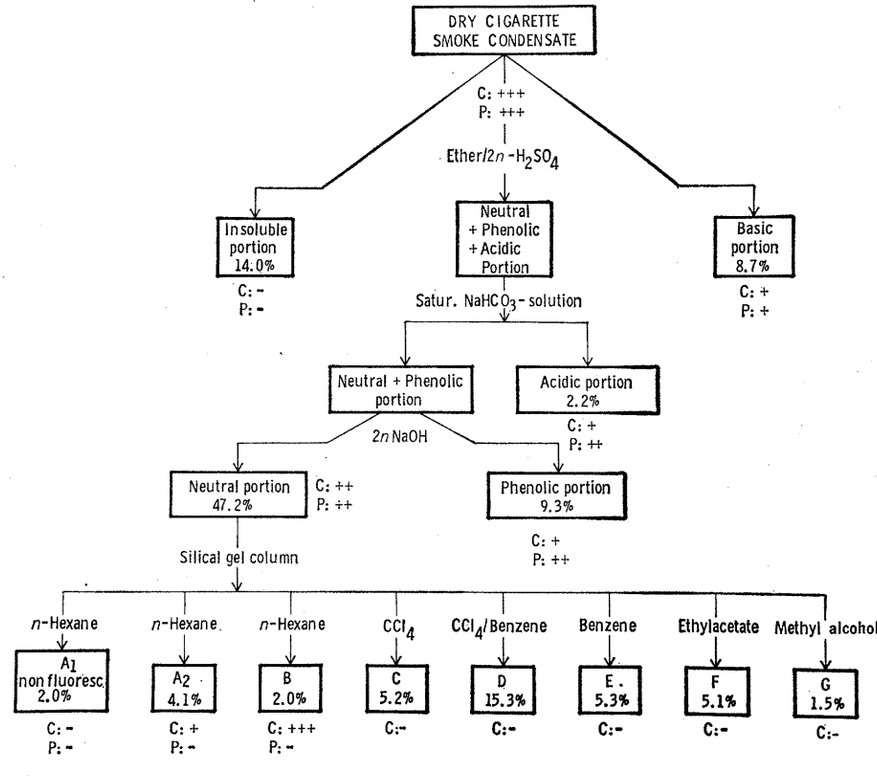


Fig. 1. Fractionation of cigarette smoke condensate; (*C*) relative carcinogenic activity; (*P*) relative tumor-promoting activity.

complete carcinogenic activity than those from air-cured tobaccos (*5*), the tumor-promoting activity was comparable. In general, the concentration of carcinogenic hydrocarbons is higher in low-nitrate flue-cured tobaccos than in high-nitrate air-cured tobaccos (*5*), again supporting the view that the polynuclear hydrocarbon content of tobacco "tars" does not reflect their tumor-promoting activity (*17*).

"Tars" from cigarettes made of hay or of tobacco stems—materials which have high cellulose content—have relatively low tumor-promoting activity. Our finding suggests that the tobacco plant contains specific tumor promoters, or precursors for promoters, which may not be found in other species.

Volatile phenols represent one type of tumor promoter in tobacco smoke. In mouse-skin carcinogenesis, however, they evidently do not play an essential role as such, since a significant reduction of phenols in the smoke condensate is not accompanied by a similar reduction in carcinogenic activity of the "tar" (*5*). However, the presence of phenols and phenolic smoke constituents clearly accelerates the development of tumors on mouse skin, initiated with DMBA or with benzo[*a*]pyrene, and some phenols have been shown to accelerate the development of pulmonary adenomas in mice (*18*). Similarly, volatile aldehydes and acids, such as those found in tobacco smoke, can accelerate papilloma formation in the respiratory tract of hamsters sensitized with diethylnitrosamine (DENA) (*5*). An increase in basal cell hyperplasia and metaplasia has been shown in the trachea and bronchi of strain C3H mice exposed to formaldehyde vapors (*19*). These changes may, at least in part, be secondary to infection, which is more prevalent in mice exposed to respiratory irritants than in those not so exposed.

Ciliatoxic agents. The study of respiratory carcinogenesis must include factors that may indirectly influence the induction of bronchiogenic cancer. Thus, in attempts to produce lung cancer experimentally, the effects of the inhalant on the natural defense mechanism of the respiratory system cannot be disregarded.

Normally, the bronchial tissue is covered by a mucus layer which is propelled upward by ciliary currents. Cigarette smoke as a whole impairs the movement of cilia and may adversely affect the consistency of the mucus

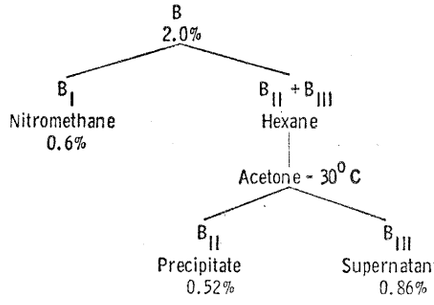


Fig. 2. Subfractionation of fraction B.

layer and may facilitate the deposition and retention of smoke particles on the respiratory mucosa. Assays in vivo as well as on isolated ciliated tissues from a large variety of animals have demonstrated that both the gas phase and the particulate phase of tobacco smoke contain ciliatoxic and mucolytic agents. Therefore it is possible that the mechanism of respiratory carcinogenesis involves three steps, the first being impairment of the normal defensive barrier against inhaled tumorigenic compounds. The gas phase of tobacco smoke is not likely to contain carcinogens. The extent to which the gaseous constituents may directly contribute to pulmonary carcinogenesis remains to be established.

Bladder Carcinogenesis

Epidemiologic data have also suggested an association between cigarette smoking and cancer of the urinary bladder in men (*2, 4, 20*). The limited experimental investigations that have been made on such a correlation have centered around three approaches: induction of tumors in the urinary tract of laboratory animals; identification of known bladder carcinogens; and study of the higher excretory rate of *o*-aminophenols and phenoxazones in the urine of cigarette smokers.

Tobacco "tar" injected into the bladder of mice causes perivesical inflammation and mucosal hyperplasia. The feeding of "tar," or painting of the oral cavity of mice with "tar," led to an anaplastic sarcoma of the bladder in only one case. Neither passive inhalation of tobacco smoke by mice, rats, and hamsters, nor painting of mouse skin with tobacco "tar," nor subcutaneous application of "tar" in rats has so far induced bladder tumors. Recently, one papilloma and one carcinoma were found in the renal pelvis of two of a large number of mice whose skin had been painted with an extract of tobacco "tar" (*21*). Although

Table 2. Chemical constituents of subfraction BI of tobacco smoke condensate.

Constituents	Number of compounds		Percent of subfraction BI
	Non-carcinogens	Carcinogens	
Aromatic hydrocarbons			
Naphthalenes	5		2.07
Anthracenes, phenanthrenes, fluorene	7		0.46
Four-ring hydrocarbons	7	3	.21
Five-ring hydrocarbons	4	5	.036
Six-ring hydrocarbons	2	3	.007
N-Heterocyclics			
Indole + derivatives	6		10.80
Carbazole + derivatives	6		0.50
9,9-dimethylacridane	1		.12
Insecticides and pyrolysis products			
2,2-Bis(*p*-chlorophenyl)-1,1-dichloroethane (DDD)	1		.54
2,2-Bis(*p*-chloropenyl)-1-chloroethylene (DDM)	1		.03
Trans-4,4′-dichlorostilbene	1*		.51
2,2-Bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT)	1		.13
2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-1-dichloroethane(*o,p′*-DDD)		1	.01
2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1,1-trichloroethane(*o,p′*-DDT)	1		.01
Esters			
Di-isooctyl- and *n*-alkyl-phthalates			
Saturated and unsaturated C_{19}–C_{28} fatty acid esters of solanesol, phytol, and long-chain alcohols			
Paraffins C_{18}–C_{33}			< 1.0
Olefins			
Terpenes			
Cyclic and acylic carbonyl components			
Quinones			

* Possible tumor accelerator.

this tumor incidence is very low, future investigations should include examination of the kidneys of animals treated with tobacco "tars." Such studies may even eventually provide a test model for investigation of the tumor-inducing properties of certain chemicals in the urinary tract—studies which would appear to be very pertinent in view of suggestions, from epidemiologic data, that link cigarette smoking with cancer of the kidney, particularly of the renal pelvis (*22*).

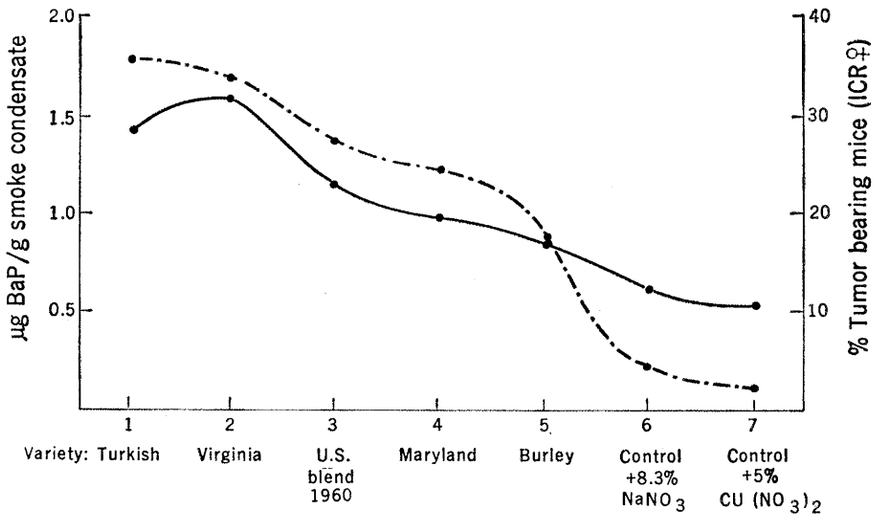


Fig. 3. Benzo[*a*]pyrene (BaP) content and tumorigenicity of cigarette smoke condensates. (Broken line) Percentages of tumor-bearing mice (strain ICR, females); (solid line) amount (in micrograms) of benzo[*a*]pyrene per gram of dry smoke condensate. Number of mice in each test group, 50 or 100.

Chemical Data

The term *experimental tobacco carcinogenesis* is used to describe work on tobacco smoke, its condensate, and its fractions and on extracts of fresh and cured tobacco. Chemical approaches to experimental tobacco carcinogenesis have dealt mostly with the particulate-phase components of the smoke.

Compounds with potential as carcinogens which may possibly be constituents of the volatile phase of tobacco smoke include arsine, nickel carbonyl, nitroolefins, *N*-nitrosamines, and, perhaps, volatile epoxides and peroxides. It is unlikely that the last two would be formed in the reducing atmosphere of the hot zones of a burning tobacco product, and none of these compounds has yet been identified in tobacco smoke. Nevertheless, it is conceivable that *N*-nitrosamines do exist there, even though they are difficult to detect (*23*). Nickel carbonyl is thermically too unstable to persist throughout the burning of tobacco.

The presence of unstable or metastable organic radicals in tobacco smoke has been considered. Theoretically, the concept that radicals present in tobacco smoke are alkylating carcinogens appears sound. However, there are no data from human subjects to support such a concept, nor do reported experiments substantiate it (*24*).

Nitrogen oxides are present in cigarette smoke, especially in that from Burley and Maryland "high-nitrate" tobaccos. While it is possible that these oxides, when inhaled, may react with secondary amino groups, or with secondary amides, of proteins of lung tissue to form carcinogenic *N*-nitrosamines or amides, again neither human nor experimental data support such a concept (*25*).

Tumor initiators. The fractionation of the particulate matter of cigarette smoke by classical methods results in neutral, acidic, and basic portions (Fig. 1). Since only the neutral portion is carcinogenic, the search for tumor initiators was directed toward this fraction, and particularly toward its carcinogenic fraction B, which was obtained by adsorption chromatography. The subfraction BI was found to be active both as a complete carcinogen and as a tumor initiator (*5*). This subfraction contains polynuclear aromatic hydrocarbons as well as a variety of other neutral and weakly polar components (Table 2). Subfraction BI was further separated into 90 subfractions; after 11 months of testing, tumor-initiating activity was found essentially only for those subfractions which contain four- and five-ring aromatic hydrocarbons.

The data now available warrant the conclusion that cigarette smoke contains more types of polynuclear aromatic hydrocarbons than have so far been identified (*5*, *9*). A significant part of the initiating activity of tobacco "tar" is certainly due to the presence

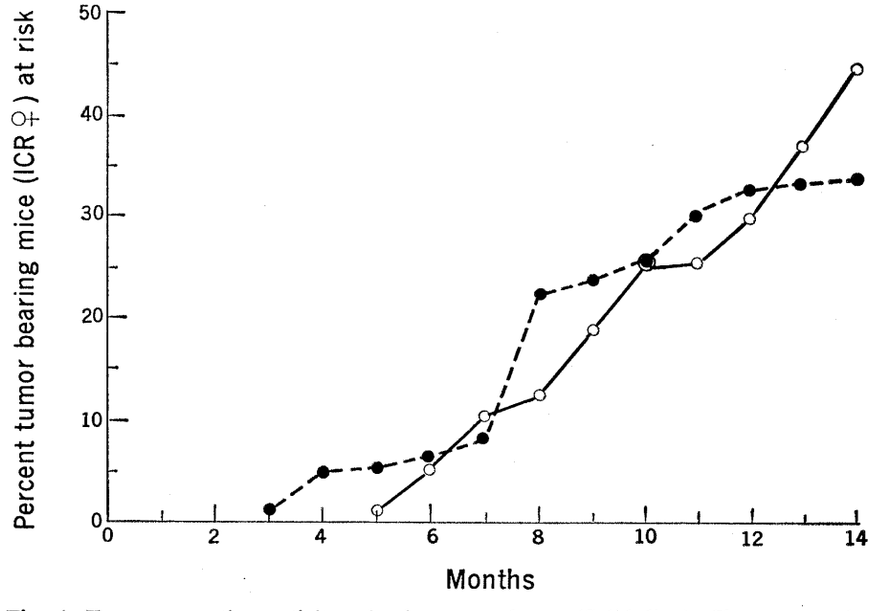


Fig. 4. Tumor-promoting activity of tobacco products. (Solid line) Cigarette smoke condensate (50-percent acetone suspension); (dashed line) dimethylsulfoxide extract of Turkish tobacco (applied as a 50-percent suspension of residue in dimethylsulfoxide). There were 60 mice in each test group.

of such hydrocarbons. Four-ring hydrocarbons, mainly of the chrysene type, and derivatives thereof appear to account for a significant proportion of the activity.

An important conclusion relates to the significance of benzo[*a*]pyrene in tobacco smoke. Benzo[*a*]pyrene and practically all other polynuclear aromatic hydrocarbons are formed in the burning cone of a tobacco product in at least two consecutive steps. In the reducing atmosphere of the glowing cone, radicals consisting of carbon and hydrogen are produced by pyrolysis. These radicals combine partially with each other, thus forming new compounds (through pyrosynthesis), some of them four- to six-ring aromatic hydrocarbons. Inhibition of pyrosynthesis from radicals or increase in its rate is indeed reflected in a similar change in the formation of most polynuclear aromatic hydrocarbons.

If the complexity of such pyrosynthesis during the smoking process is disregarded and if only different concentrations of benzo[*a*]pyrene are considered, it is found that addition of this carcinogenic hydrocarbon to a suspension of tobacco "tar" will not increase the tumorigenic activity (*26, 27*). However, adding the 17 polynuclear hydrocarbons predominant in tobacco "tar," thus bringing into account at least some of the actual occurrences of the pyrosynthesis, accelerates the tumorigenicity of such "tar" (*17*).

Tumor-promoting agents. As discussed above, bioassays on mouse skin have given evidence that both the acidic and the neutral fractions of cigarette smoke condensate have tumor-promoting activity.

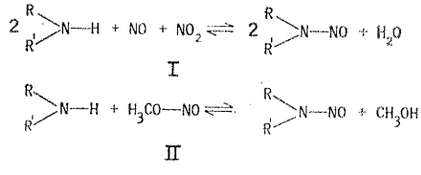
While certain acids, as well as phenols, have been shown to have such cocarcinogenic activity, the major neutral promoters in tobacco and in its smoke are yet to be identified. These compounds can be altered by hydrolysis and photooxidation, and some of them are volatilized at temperatures above 300°C (the peak temperature during smoking is 880°C). Some of these compounds may also be tobacco-specific.

Ciliatoxic constituents of tobacco smoke. Most of the chemical studies and in vitro assays for cilia toxicity have been directed toward identifying and then reducing volatile and semivolatile constituents of smoke (*5, 27–30*). Relatively high cilia toxicities were found in vitro for hydrogen cyanide, acrolein, formaldehyde, and formic acid, and lower activities for acetaldehyde, acetic acid, benzoic acid, volatile phenols, and others.

Assays of cigarette smoke, particularly those performed in vitro with short-term exposure, revealed that certain filter materials, especially water and various forms of charcoal, can reduce the cilia toxicity. Chemical analysis revealed that these filters can effect a reduction of up to 80 percent in hydrogen cyanide and acrolein (*31*). Recent assays, however, have shown that the differences in cilia toxicity between various types of cigarette smoke disappear when the smoke is passed through a short, moistened tube (*32*). This effect was to have been expected, since most of the identified ciliatoxic agents are strongly hydrophilic.

In general, smokers retain cigarette smoke in their mouth for a few seconds before inhaling it. During this time, up to 80 percent of the volatile ciliatoxic agents may be absorbed by the moist surfaces (*30*). In view of this, it remains to be established that filters which selectively remove volatile ciliatoxic agents have much value, especially when the "tar" reduction that they effect is relatively low.

Suspected carcinogens in tobacco smoke. Certain *N*-nitrosamines are strong animal carcinogens (*33*). Since tobacco smoke contains secondary amines (*5, 9*), and since most tobaccos, in particular Burley and Maryland varieties, contain alkali nitrates, the gas phase of tobacco smoke has been considered a potential environment for the formation of carcinogenic *N*-nitrosamines (*4*). Theoretically, nitrosamines can be formed in several ways; the most important are the following:



I

II

Extensive formation of nitrosamines in tobacco smoke according to mechanism I seems a remote possibility, because only traces of NO_2 are found, and HNO_2 formation depends on the presence of both NO and NO_2. This mechanism must be considered, however, when secondary amines as well as alkali nitrates occur in tobacco in unusually high concentrations, or when tobacco smoke is "aged" and contains significant amounts of NO_2 due to oxidation. The second mode of formation of nitrosamines (mechanism II) appears more likely, since up to 400 micrograms of methylnitrite are found in the smoke of one cigarette.

While nitrosamines have not yet been identified in fresh smoke of commercial cigarettes, more sensitive analytical methods may lead to the detection of trace amounts ($< 10^{-7}$ gram per cigarette). Should such data be obtained and confirmed, the selective reduction of secondary amines from the gas phase would obviously be indicated.

Bioassays have not indicated that nitrosamines play a role in experimental tobacco carcinogenesis. However, the occasional occurrence of papillary growths in the trachea of hamsters exposed in inhalation chambers to "aged" smoke in which the NO_2 content has been increased by NO oxidation (*5, 34*) indicates the potential of tobacco smoke for forming carcinogenic nitrosamines.

The mainstream smoke of cigarettes contains trace amounts of primary and secondary nitroalkanes (*35*). Since their concentrations depend on the nitrate content of the tobacco, it may be assumed that alkyl radicals and NO_2 react in the hot zones of a cigarette and form nitroparaffins. Thus it is possible that some nitroolefins may be formed in tobacco smoke from alkenyl radicals and NO_2. Since several nitroolefins are known to be carcinogenic to the respiratory tract of laboratory animals (*36*), analytical investigations in this area are needed.

Several investigators found traces of polonium-210 in domestic and foreign tobacco, as well as in cigarette smoke (*4, 5*). The reported concentrations range from 1 picocurie to 50 picocuries per gram. From 30 to 50 percent of the ^{210}Po was recovered in the mainstream smoke of nonfilter cigarettes.

Analyses of human tissues demonstrated that the lung, blood, and liver of smokers contain a higher concentration of ^{210}Po than the corresponding organs of nonsmokers (*37, 38*). The average daily ^{210}Po inhalation rate for individuals who smoked 20 cigarettes a day was estimated to be about 2 picocuries (*37*). Autopsy studies indicated a yearly alpha-radiation dose of 41 millirem for the basal cells of the subsegmental bronchi and of 75 millirem for the basal cells of the terminal bronchi. These values are small compared with the dose rate of 1 to 2 rem per year originating from the decay of naturally occurring radium and thorium and their short-lived daughter products. On the basis of these and similar data it appears unlikely that the inhalation of

^{210}Po from tobacco has a role in tobacco carcinogenesis (*37, 39*).

However, if experimental evidence or data from human subjects should cast doubt on these estimates, then specific filters are available which can remove up to 90 percent of the ^{210}Po from the mainstream smoke (*40*). Phosphate fertilizers are one major source of ^{210}Po in U.S. varieties of tobacco (*41*), and thus the use of fertilizers free of this substance should significantly reduce the concentration of this radioactive nuclide in tobacco.

The progressive development of organic pesticides as well as the efforts of public health organizations has led to a steady decline in the application of arsenical sprays to tobacco. During the past 10 years the average arsenic content of cigarette tobaccos has declined from 40–60 parts per million to values below 10 parts per million (*42*). Since tobacco burns in a reducing atmosphere having a hydrogen content of up to 8 percent, it is possible that the carcinogenic arsine (AsH_3) is formed, although this compound has not yet been identified in tobacco smoke.

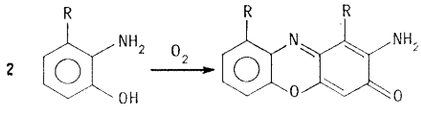
1,1-Dichloro-2-(*o*-chlorophenyl)-2-(*p'*-chlorophenyl)-ethane (or *o,p'*-DDD) induces necrosis in the adrenals of dogs and testical tumors in rats (*43*). This agent is a technical by-product of the major tobacco insecticide *p,p'*-DDD and thus appears also in tobacco. About 0.5 microgram of *o,p'*-DDD is found in the mainstream smoke of a nonfilter cigarette (*44*). Should these analytic and biologic data be further substantiated, a reduction in the use of this insecticide, and even total elimination of its use, should be encouraged.

Recently the mutagen maleic hydrazide was shown to be carcinogenic to the experimental animal (*45*). This hydrazide is widely used to inhibit the growth of tobacco-plant suckers and is found in tobacco and in tobacco smoke (*46*). It should be replaced by a less harmful sucker inhibitor (*45*).

Suspected Bladder Carcinogens

Several aromatic amines, such as β-naphthylamine and benzidine and its derivatives, are known to have a role in occupation-linked cancer of the bladder (*47*). Recently β-naphthylamine was identified in trace amounts as a pyrolysis product of certain α-amino acids (*48*). Unfortunately, in the reported chemical-analytic studies on certain carcinogenic aromatic amines, the high reactivity of these amines was disregarded and methods of only limited sensitivity were used (*49, 49a*).

Biochemical studies involving methods of average sensitivity have demonstrated that the urine of smokers contains, on the average, 50 percent more of the normal intermediate metabolites of tryptophan, 3-hydroxyanthranilic acid, and 3-hydroxykynurenine than the urine of nonsmokers does (*50*). These *o*-aminophenols are carcinogenic when implanted into mouse bladders. Since they also appear in the urine of patients with non-occupation-linked cancer of the bladder, the findings suggest that tobacco smoke may inhibit the enzyme system, which then metabolizes these *o*-aminophenols, causing them to form methylnicotin amide. The urine of patients with bladder cancer contains *o*-aminophenol oxidation products with phenoxazone structure:



This suggests that the urine of cigarette smokers should also be analyzed for phenoxazones.

Chemical Indicators for Carcinogenicity

Table 3 presents several, but not all, identified and suspected tumorigenic agents of tobacco smoke. It does not include carcinogenic mytoxins or additives, although such agents may occasionally be found.

We have observed a correlation between the concentration of certain tumorigenic agents in the smoke condensate and the tumorigenicity of the condensate to mouse skin. Such a correlation for benzo[*a*]pyrene and "tar" tumorigenicity is shown in Fig. 3. On the basis of this observation and of the fact that most, if not all, other polynuclear aromatic hydrocarbons are concurrently pyrosynthesized in the burning cigarette by similar mechanisms, benzo[*a*]pyrene is chosen as an "indicator" of the presence of tumor-initiating polynuclear hydrocarbons. A significant reduction ($>$ 25 percent) in the benzo[*a*]pyrene concentration in the "tar" has so far always been found to relate to a significant reduction in the tumorigenicity of the "tar." Similarly, a reduction in the concentration of phenol itself has always been paralleled by a reduction in the concentration of other volatile phenols. Since polyphenols and polysaccharides in the tobacco are considered major precursors of volatile phenols (*51*), such a correlation is to be expected.

We have taken several other "indicators" to be representative of "tar" yield, alkaloid toxicity, and ciliatoxicity. To the extent to which these indicators predict a given biologic activity of a tobacco smoke condensate, they permit better planning of bioassays and thus save time and effort.

Since our knowledge of other known or suspected tumorigenic agents in tobacco smoke is rather limited, other individual agents have not yet been tried as indicators for different groups of tobacco carcinogens. Furthermore, it appears unlikely that we will always find an individual compound as representative of a group of carcinogenic,

Table 3. Identified or suspected tumorigenic agents in cigarette smoke.

Type of components	Estimated concentrations in 100 cigarettes (85 mm, nonfilter)	Relative importance in experimental tobacco carcinogenesis
Carcinogens and tumor initiators		
Polynuclear aromatic hydrocarbons*	10– 30 μg	Major tumor initiators
N-Heterocyclic hydrocarbons	1– 2 μg	Minor importance as initiators
N-Nitrosamines	$<$ 10 μg	Suspected carcinogens of some importance
Nitroolefines	$<$ 1 μg	Suspected carcinogens of minor importance
o,p'-DDD	10–100 μg	No essential contribution
Maleic hydrazide	10–100 μg	No real importance suspected
β-Naphthylamine	2– 3 μg	Suspected bladder carcinogen
Other aromatic amines	10– 50 μg	Carcinogens of some importance
Polonium-210	1– 50 pc	Of some importance only in case of relatively high concentration
Promoting agents		
Neutral promoters [unknown structure(s)]	?	Of essential importance
Volatile phenols	20– 30 mg	Of some importance
Nonvolatile phenols	?	Possibly of some importance
Nonvolatile fatty acids	20–100 mg	Of minor importance

* Four- and five-ring aromatic hydrocarbons.

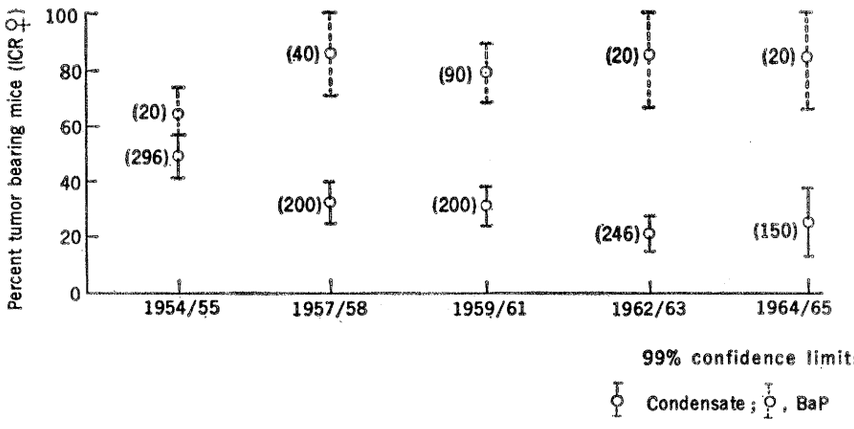


Fig. 5. Decline of tumorigenicity, on mouse skin, of cigarette smoke condensates as tested during the period 1954–65 compared with the response to a 0.005-percent benzo[*a*]pyrene solution. Numbers in parentheses are numbers of mice per group.

or cocarcinogenic, smoke constituents as the indicators mentioned above. For one thing, *N*-nitrosamines, nitroolefins, and groups such as aromatic amines have specific precursors for individual compounds. Furthermore, it is necessary to consider the complexity of biologic responses to structurally similar coexistent compounds.

Reduction of Tumorigenicity

One major objective of experimental tobacco carcinogenesis is the reduction of the tumorigenicity of cigarette smoke and other tobacco products. As we have stated, it is possible, on the basis of chemical indicators, to predict a given biologic activity, and in general we have found a meaningful correlation between such indicators and biologic findings.

Among feasible methods for reducing the tumorigenic and ciliatoxic properties of cigarette smoke are modification of breeding, culturing, and curing of tobacco and selection of tobacco according to its chemical composition (for example, high nitrate content, low nicotine content). The use of additives, choice of optimum tobacco cut and paper of optimum porosity, the use of stems, and the addition to cigarette tobaccos of shredded "reconstituted tobacco sheet" (a material prepared from tobacco dust and fines combined with adhesives) are other measures effective in reducing the yield of particulate matter in the smoke and/or diminishing the tumorigenicity of the smoke condensate.

Mechanical and selective smoke filtration is effective in reducing the yield of particulate matter from the smoke, and selective filtration can suppress the ciliatoxicity of the smoke.

Nonselective reduction. Various studies have established a dose response for the tumorigenicity of tobacco "tar" (*5, 52*). Thus reduction of the "tar" and nicotine yield through modification of agricultural practices, selection of "low-tar-yielding" tobaccos, use of reconstituted tobacco, and effective mechanical filtration of the aerosol particles diminishes exposure to "tar" and nicotine and thus benefits the smoker, provided he does not smoke more cigarettes.

Some of these measures have been applied in increasing degrees during the past 15 years, with the result that "tar" and nicotine yields in the smoke of domestic cigarettes have been gradually lowered.

Selective reduction. Most stimulating to academic research are attempts to selectively reduce the carcinogenicity and ciliatoxicity of tobacco smoke. Since the formation of initiating carcinogens in tobacco smoke is probably a result of the degree of combustion and possibly of the presence of certain specific precursors, a modification of either of these factors may lead to a reduction in the concentration of initiating carcinogens.

In recent years the choice of "low-tar-yield" tobaccos may have contributed to the selective reduction of the carcinogenicity of "tars." Several studies demonstrated that cigarettes of comparable weight deliver significantly varying amounts of "tar" when they differ significantly in the degree of combustion, as indicated by the number of puffs required to reach a given butt length. We might therefore expect that cigarettes made of "low-tar-yield" tobaccos would show a selective reduction of those substances that are specifically a consequence of incomplete combustion. Benzo[*a*]pyrene is such a substance, and we find that the significant reduction in the particulate matter and the tumorigenic activity of the condensate of smoke from American cigarettes has been paralleled by a reduction in benzo[*a*]pyrene concentration over the past 15 years (the average benzo[*a*]pyrene content of 1 gram of "tar" changed from 1.2 micrograms to 0.9 microgram). Thus, a general modification of the composition of American cigarettes has led to a reduction not only in the overall "tar" yield but apparently also in carcinogenic activity (Fig. 5).

The type of polynuclear aromatic hydrocarbon pyrosynthesized is determined by the temperature profile of the burning tobacco. Modification of this determinant, however, is difficult (*5*). Comprehensive studies made in our laboratory have shown that adding nitrates (especially alkali nitrates), at a concentration of 5 to 8 percent, to tobacco or selecting nitrate-rich tobaccos will significantly reduce the yield of polynuclear aromatic hydrocarbons and the tumorigenic activity of the resulting "tars" (*17*) (Fig. 3). Since most such hydrocarbons in smoke are synthesized from the pyrolytically formed radicals consisting of carbon and hydrogen, we considered the possibility that nitrogen oxides (originating from alkali nitrates) may react in the hot zones with these radicals and thus inhibit the pyrosynthesis of polynuclear hydrocarbons. This hypothesis is supported by the identification of nitroalkanes in tobacco smoke and the dependence of their concentrations on the nitrate content of the tobacco (Fig. 6).

The addition of nitrates does not, however, significantly affect the tumor-promoting activity of a given tobacco "tar"—a finding that underscores the differences in the formation of tumor initiators and promoters in cigarette smoke.

Perhaps, owing to the presence of precursors, flue-cured and sun-cured tobaccos tend to yield more benzo[*a*]pyrene and more phenols in the smoke than air-cured tobaccos and also tend to produce "tars" that yield more tumors in the mouse-skin assay (*5*). Qualitative differences in the types of tobacco and differences in the enzymatic processes that occur during the curing of these tobaccos obviously influence smoke constituents. For one thing, flue-

cured tobacco contains more sugar than air-cured tobacco does. Moreover, the nitrate content of air-cured tobacco (up to 5 percent) is considerably higher than that of flue-cured tobacco (less than 1 percent). The differential biological activity of flue-cured and air-cured tobaccos that is reflected in differences in chemical composition must still be explored epidemiologically. In this respect it is of interest to note that the French, who have a significantly lower rate of lung cancer than the British do, smoke predominantly air-cured tobaccos with a relatively high nitrate content, while the English smoke mostly flue-cured tobaccos with a low nitrate content. Obviously other factors need to be considered in comparing the incidences of lung cancer in the two countries.

Experiments have indicated that hydrolysis of tobacco may reduce the tumor-promoting activity of tobacco smoke condensate. "Tar" from cigarettes made wholly from tobacco stem has less tumor-promoting activity than "tars" from standard tobaccos. This may indicate that the tumor-promoting agents are located mainly in the leaf, an inference consonant with the view that terpene esters contribute to the promoting activity.

Modification of filters can range from the use of cellulose acetate with specific additives for removing phenols to the use of charcoal and water for reducing gaseous components (especially hydrogen cyanide, volatile acids and aldehydes, and the vaporized nicotine) (*31, 53*). Selective filtration applies only to smoke constituents that are at least partially vaporized.

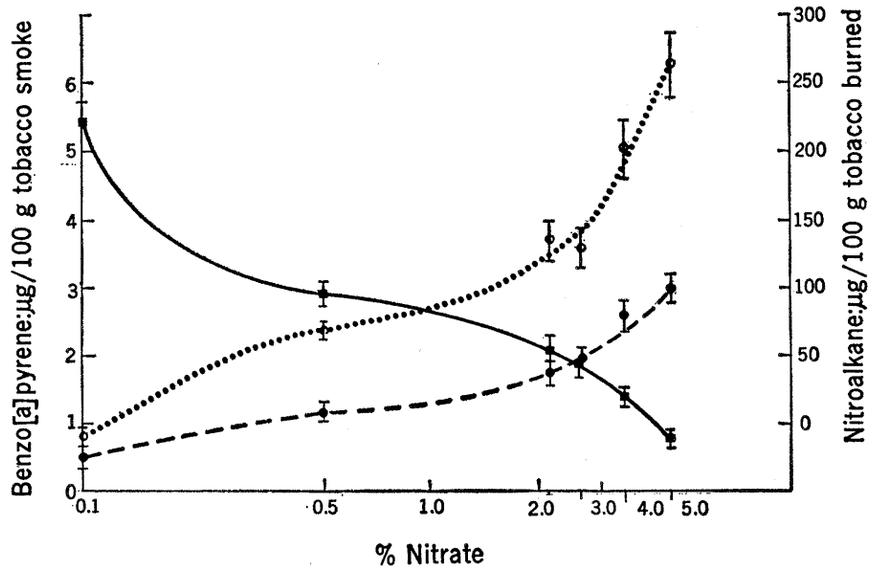


Fig. 6. Benzo[*a*]pyrene, nitromethane, and nitroethane concentrations in the mainstream smoke of cigarette tobacco. (Solid line) Benzo[*a*]pyrene (see scale at left); (dashed line) nitromethane (see scale at right); (dotted line) nitroethane (see scale at right).

Problems of Experimental Tobacco Carcinogenesis

In planning experiments in environmental carcinogenesis it is important to consider carefully not only the choice of methods of chemical analysis but the choice of the bioassay systems, their duration and cost, and of course the meaningfulness of the results to man.

Ideally, the experimental system should approximate the human setting, and the suspected human carcinogen should be tested on the same type of tissue in various animal species and strains. In studies of the relationship of cigarette smoke to lung cancer it would therefore be desirable to investigate the effect of inhaled smoke on the bronchial tree of laboratory animals. The immediate problem is that it appears difficult, if not impossible, to have animals inhale cigarette smoke directly through the mouth. The rhesus monkey can seemingly be trained to puff on cigarettes, as Jarvik has shown (*54*), but cannot readily be made to inhale the smoke. Even if it could be, the cost of the monkeys required and of an experiment of the necessary duration would be prohibitive. Smaller laboratory animals breathe obligatorily through the nose and have intricately developed nasal passages which, particularly under stress, can absorb and trap much of the smoke in the respiratory environment, especially the particulate components. At a time when we believe we are close to comprehending the complexity of the genetic code, we tend to overlook simple Darwinian logic which probably accounts for the differently developed nasal passages in animals and in man. Factors other than the defensive system of the nasal passages also contribute to the improbability that passive inhalation experiments will lead to the development of squamous-cell lung cancer. Major factors are the obviously weak carcinogenicity of tobacco smoke, its toxicity due to the presence of nicotine and CO, and the dosage problem related to these factors. We must also recognize that the respiratory epithelium itself has well-developed defensive systems—ciliary activity and mucus formation—which are probably adequate to withstand the addition of weakly carcinogenic factors, particularly at low dosages. This may be deduced from studies by Saffiotti *et al.* (*55*), who demonstrated that the amount of benzo[*a*]pyrene required to produce squamous-cell cancer in the respiratory tract of hamsters is relatively larger than the dose shown to produce skin cancer in mice. At that, these investigators had to mix the benzo[*a*]pyrene with iron dust in order to assure its localization in the respiratory epithelium.

Studies by Auerbach on dogs "smoking" through an incision in the trachea bring the tobacco smoke directly into the respiratory system and facilitate its administration in larger doses. The problem is that the dogs tend to succumb to emphysema and pulmonary emboli relatively early in the experiment, apparently as a consequence of their exposure to smoke (*12*).

Since tobacco smoke is a relatively weak carcinogen, in such experiments rather large amounts of "tar" and "tar"-fractions must be applied for a long time, and this makes most bioassays both costly and time-consuming. The high toxicity of tobacco "tar," primarily related to its alkaloid content, adds to the difficulty of biologic experiments, particularly those dealing with organ, tissue, or cell cultures. Various "tar" components, tumor initiators, tumor promoters, and substances that may enhance or decrease absorption or may inhibit the action of tumor initiators and promoters should be considered. Furthermore, the interaction of simultaneously applied components may pro-

duce effects different from those expected when single components are tested, or when fractionated materials are applied following an initiation phase. In view of the multitude of components in tobacco smoke condensate, this interrelation of different substances will probably never be fully understood.

The fact remains, of course, that tobacco smoke condensate has been shown to be carcinogenic to a variety of animal tissues when placed in direct contact with them. We have chosen mouse epidermis as our primary bioassay system. Respiratory epithelium would have been preferable, but, for the reasons indicated, we think that it is not a suitable experimental system in routine studies.

It may be asked, to what extent is the mouse's skin representative of its respiratory epithelium? We need to consider the fact that squamous epithelium is a very primitive type of epithelium, and that, before columnar ciliated respiratory epithelium undergoes malignant transformation, it reverts to the squamous type. Thus, histologically, the squamous epithelium of mouse skin closely resembles the area in the respiratory epithelium where metaplasia has occurred (*56*). Of course, the respiratory epithelium lacks hair follicles and sebaceous glands, while in the skin these might specifically localize carcinogens and perhaps facilitate their metabolism. However, tobacco "tar" has also been shown to produce cancer of the mouse cervix, an organ without such structures (*57*). When the polynuclear aromatic hydrocarbons, which we regard as the main tumor initiators in tobacco smoke condensate, were applied at sufficiently high dosage, they produced squamous-cell cancer of the respiratory epithelium of hamsters (*55*).

It is thus well established that certain hydrocarbons known to be carcinogenic to the skin of certain animals are also carcinogenic to their respiratory epithelium. As we state above, the main problem in experimental tobacco carcinogenesis is that toxicologic and anatomic factors make it unlikely that sufficient doses reach the bronchial epithelium.

In summary, we feel that mouse epidermis adequately represents various epithelial surfaces susceptible to carcinogens on contact.

A major question concerns the contributory effects of the gaseous constituents of smoke to the carcinogenic activity of whole cigarette smoke. As discussed above, we have no evidence that the gaseous components are in themselves carcinogenic. The gas phase can, of course, like the particulate phase, lead to the impairment of cilia (*29*). Destruction of the ciliary defenses is likely to facilitate the absorption of carcinogenic constituents of smoke. The relative ease with which the ciliated columnar epithelium of the bronchial tree can be transformed has been shown by Tipton and Crocker (*58*). Within days after tobacco "tar" was applied to the bronchial epithelium of dogs by means of a bronchoscope, metaplasia had occurred. Auerbach and his co-workers have demonstrated the same changes in dogs exposed to cigarette smoke introduced through an incision in the trachea (*12*). Metaplasia appears to be a necessary step toward malignant transformation of respiratory epithelium, but its occurrence does not of itself prove that transformation, such as can be caused by various irritants from tobacco smoke, has taken place. In view of man's manner of smoking and of his long-term exposure, it appears that the oral cavity is far more selective in removing the components of the gas phase than in removing those of the particulate phase. Also, the physical characteristics of the two phases suggest that, in respect to ciliary activity, removal of the particulate matter may be the more pertinent consideration. Nevertheless, no one would object to removing as many potential irritants from cigarette smoke as possible as long as this does not significantly alter the *p*H. An important remaining task is a more precise elucidation of the relative roles of the particulate and the gas phases as inhibitors of ciliary activity in man.

Future studies. The identification of components that contribute to the tumor-initiating, tumor-promoting, and ciliatoxic activities of tobacco smoke should be continued, with special emphasis on the isolation and biochemistry of the neutral tumor promoters. Reproducible data on *N*-nitrosamines and aromatic amines in tobacco smoke must be obtained.

Both empirical and planned attempts to reduce the tumorigenic and toxic activity of tobacco smoke should be expanded. Results indicating that such a reduction has occurred should be checked by bioassays and by studies of changes in the chemical constituents of the smoke.

Mouse skin is likely to remain our most useful bioassay system. In inhalation studies we need to explore the extent to which different components actually reach the lower respiratory tract. Short- and long-term studies with larger animals, involving the direct application of smoke to the lower respiratory tract, though costly, should be useful.

In analytical techniques for the determination of constituents in fresh (that is, unchanged) smoke, more extensive use will be made of gas chromatography, perhaps in combination with the use of computers (*59*). It appears, however, that gas chromatography is not applicable to the direct analysis of high-boiling smoke constituents. The actual analysis of fresh smoke can be accomplished either directly by gas chromatography or indirectly by trapping smoke constituents, prior to gas chromatography, in such a way that they remain unaltered.

The biochemical and biochemical-analytical aspects of tobacco carcinogenesis require more intensive study. For example, biochemical tests for tumor promoters may be helpful in isolating these cocarcinogens from tobacco products. Studies on isolated mitochondria, for instance, have indicated that promoters significantly impair oxidative phosphorylation. Biochemical-analytic techniques may be especially helpful in comparing urinary excretion patterns for certain metabolites, especially *o*-aminophenols, and their oxidation products.

The independent laboratory scientist is primarily concerned with the academic aspects of tobacco carcinogenesis. The tobacco industry, professional organizations, and certain governmental agencies are also concerned with this research area. Publication by such groups of their vast scientific studies not directly related to the commercial aspects of tobacco would be a welcome contribution. Indeed, a unique aspect of experimental tobacco carcinogenesis is the fact that it encompasses so many scientific interests, which should be further coordinated.

Conclusion

In the final analysis, the laboratory experience needs to be evaluated in the light of human data. Does a particular animal carcinogen, especially one affecting tissue similar to that in man, also have a carcinogenic effect on man? The epidemiologist will have to investigate the cancer risk among groups smoking different types of cigarettes and different blends of tobaccos. Where

the laboratory data support epidemiological experience, certainly both data and experience assume greater significance. In this respect it may be asked to what extent alteration of tobacco smoke to reduce its tumorigenic potential, as expressed by chemical indicators and reflected in bioassays, can be of importance to man. We reason from dose-response studies on man and on the experimental animal that a reduction in exposure to total smoke will be associated with a reduction in the risk of contracting those diseases associated with cigarette smoking.

We may assume, but only assume, that a reduction in specific toxic and tumorigenic agents which causes a reduction of toxicity and tumorigenicity in assays with animals will similarly affect man.

We will continue to direct our studies toward improving our understanding of the mechanism of tobacco carcinogenesis. Through such studies we expect to learn more about the basic mechanism of chemical carcinogenesis in general, and also hope to contribute to a decrease in the incidence of a major group of human cancers.

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49a. While this article was in preparation, we, together with Y. Masuda, developed in our laboratory a sensitive method for quantitative determination of amines in cigarette smoke. The carcinogenic β-naphthylamine in the smoke of an 85-millimeter cigarette of blended U.S. tobaccos was found to be 2.2 $\times 10^{-8}$ gram.

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56. We are reminded of a statement made by E. V. Cowdry some years ago to the effect that, if the mechanism of carcinogenesis in mouse epidermis were understood completely, this would probably elucidate the mechanism of carcinogenesis in the epithelial surfaces of most species. Dr. Cowdry probably based this statement on the fact that squamous epithelium is one of the most primitive forms of cells in biological systems and thus would be more comparable from one species to another than tissues and organs composed of more highly differentiated cells.
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60. This study was supported by American Cancer Society grant E-231 and, in part, by National Cancer Institute grant CA-08748.