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Received for review May 4, 1977. Accepted August 30, 1977.

Chemical Studies on Tobacco Smoke. 53. Use of Radioactive Tobacco Isolates for Studying the Formation of Smoke Components

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Tobacco plants exposed to $^{14}CO_2$ during growth served as sources for radioactive phytosterols, neophytadiene, alkaloids, fatty acids and others. Techniques for the isolation and identification of these compounds included solvent distribution, gel-permeation chromatography, thin-layer chromatography in conjunction with radioscanning, and gas chromatography–mass spectrometry. The radioactive isolates were then incorporated into cigarettes, and their distribution patterns in the smoke were determined. A substantial portion of the labeled isolates was combusted to $^{14}CO_2$, in addition to being transferred intact into the smoke "tars", and degraded to volatile organic compounds. Measurable quantities (<1%) were also converted to polynuclear aromatic hydrocarbons. The techniques utilized can serve as models for additional studies of this kind and lead to practical approaches for modifying smoke composition.

The biological activity and organoleptic and other properties of cigarette smoke are a function of the chemical compounds that comprise the smoke. These compounds originate from the leaf, by direct transfer (e.g., distillation, sublimation), by thermally degradative processes, and/or by pyrosynthesis. By identifying precursors in the leaf that give rise to undesirable smoke constituents, one could ultimately modify the levels of these precursors in the leaf and consequently control the composition of the smoke.

Previously, precursor–product relationships were established by pyrolysis studies. In these, selected leaf components or extracts were subjected to appropriate high temperatures, and the products thus generated were identified (Higman et al., 1970; Schmeltz et al., 1972; Wakeham, 1972; Schlotzhauer et al., 1976). However, such studies were models of only one process in an ignited cigarette, i.e., pyrolysis, and did not reflect the complex phenomena occurring there.

In order to understand better the pathway of individual tobacco constituents from the leaf into the smoke, a number of workers have used cigarettes containing isotopically labeled compounds (Jenkins et al., 1976; Wakeham, 1972; Houseman, 1973). By such techniques, these workers have studied the distribution of certain leaf constituents and their thermally altered products in the particulate and vapor phase of smoke. However, studies such as these have limitations because many tobacco constituents (e.g., terpenoids) containing label are hardly accessible. Moreover, those that are available are usually not of sufficient activity for tracer studies or are labeled in only one position.

To overcome these difficulties, we isolated various ^{14}C-tobacco constituents from tobacco plants grown in a $^{14}CO_2$ atmosphere. These constituents naturally formed in the plant should theoretically contain random labeling in all C atoms and be especially suitable for extensive tracer studies. The ^{14}C isolated materials were used in smoke formation studies, not only to determine how they distribute in the smoke, but also to identify, with some quantitation, the compounds they give rise to in the process. The techniques used and some of the data obtained are presented in this paper.

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EXPERIMENTAL SECTION

Apparatus. The syringe-type applicator for impregnating cigarettes with radioactive materials was developed at Oak Ridge National Laboratories, Oak Ridge, Tenn. Its use is described (Hoffmann et al., 1977). Cigarettes were smoked on a Phipps and Bird Single Port Smoking Machine. Both mainstream and sidestream smoke were collected utilizing a specially designed smoking chamber (Liu et al., 1974) and trapping system (Marmor and Minnemeyer, 1975).

Radioactive (^{14}C) samples, in solution, were counted in a Nuclear-Chicago Isocap 300 Scintillation Counter. A Packard Radiochromatogram Scanner, Model 1201, was used to detect radioactive zones on thin-layer chromatographic (TLC) plates.

Gas chromatography (GC) was performed on a Hewlett-Packard Model 7620A Research Chromatograph equipped with flame-ionization detectors. Gas chromatography–mass spectrometry (GC–MS) was accomplished on a Hewlett-Packard system (5980A mass spectrometer interfaced with a 5710 gas chromatograph and a 5933A data system).

Reagents. All organic solvents were spectrograde, and the other chemicals were reagent grade. Counting solutions were Toluscint I (ICN Pharmaceuticals, Inc.) for materials soluble in organic solvents and Aquascint I (ICN Pharmaceuticals, Inc.) for water-soluble materials. Plant fibers were solubilized with NCS Solubilizer (Amersham Searle) prior to counting. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Inc. GC columns and TLC plates were obtained from various commercial sources.

Gas Chromatography. The GC column conditions used for the various tobacco isolates were as follows: sterols, 3% OV-17, 10 ft × 0.13 in. o.d., 230 °C; fatty acids, 3% OV-17, 10 ft × 0.13 in. o.d., 160 °C; fatty acids, 10% DEGS-PS, 8 ft × 0.13 in. o.d., 165 °C; neophytadiene, 3%

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OV-17, 10 ft × 0.13 in. o.d., 150 °C; alkaloids 3% OV-17, 10 ft × 0.13 in. o.d., 140 °C. All runs were under isothermal conditions; the flow rate was 40 mL/min, and injector and detector temperatures were at 300 °C. The column support was Chromosorb W in all cases. The fatty acids were chromatographed as their methyl esters.

Thin-layer Chromatography (TLC)–Radioscanning. For the identification of radioactive compounds in each of the fractions and the determination of their specific activities, TLC was used in conjunction with radioscanning techniques. Quantitation was achieved by GC of the compounds washed off the TLC adsorbents and confirmation of identities by GC–MS. The following TLC conditions were used (isolate, TLC adsorbent, solvent system): neophytadiene, silica gel G-25, $CHCl_3$; phytosterols, silica gel G-25, $CHCl_3$–ethyl acetate (7:1); palmitic acid, silica gel G-25, $CHCl_3$; stearic acid, silica gel G-25, $CHCl_3$; alkaloids, alumina-alox G-25, $CHCl_3$; polynuclear aromatic hydrocarbons, silica gel G-25, benzene–hexane (5:1). All plates were 5 × 20 cm. The coatings were 0.25 mm thick and contained fluorescent indicator.

Counting Procedure. Radioactive isolates from tobacco or tobacco smoke which were soluble in organic solvents (i.e., $CHCl_3$, hexane) were prepared for counting (in the Nuclear Chicago Instrument) by taking appropriate aliquots (≤1 mL) and adding each of them to 15 mL of scintillation "cocktail" (Toluscint). Water-soluble isolates were prepared in a similar fashion using Aquascint in the counting solution. Cigarette "tar" was dissolved in acetone, and aliquots were treated as above. Cigarette butts and ashes were washed with hexane and acetone, and appropriate aliquots of the washings were taken for counting.

For radioactive plant fibers, about 1 mg of the latter was immersed, until completely wet, in 250 μL of H_2O in a 20-mL scintillation vial. Then 1 mL of NCS Solubilizer (Amersham-Searle) was added; the vial was capped and warmed at 40 °C for 1 h. After digestion of the solid was complete, the solution was counted as above.

In all cases, background was subtracted from the counts, and efficiency was determined by counting known amounts of a ^{14}C standard and preparing and utilizing appropriate quenching curves. Counting efficiency of the Nuclear Chicago instrument was about 85%.

Radioactive "peaks" obtained by use of the TLC-Radioscanner were quantitated by applying ^{14}C standards to the plate and comparing peak areas.

Tobacco Fractionation. Tobacco plants were grown under greenhouse conditions in solution culture. Established plants with 12 leaves and approximately 20 in. tall were placed in a specially constructed chamber (Smith et al., 1962) and were exposed to an atmosphere containing 700 ppm of $^{14}CO_2$ (16.6 mCi) for 38 days, after which they were harvested. The various plant parts (leaves, stems, flowers, suckers, and roots) were then extracted with ethanol in a blender. As an example, one such ethanolic solution (725 mL) contained about 13 g of an extract from two leaves of midstalk position. Sufficient H_2O was added to this solution so that two layers were formed on washing with n-hexane (3 × 500 mL). The residue (732 mg) from the hexane solution was dissolved in 5 mL of $CHCl_3$–MeOH (5:1) and placed on a Sephadex LH-20 column (105 cm × 2 cm) packed with the $CHCl_3$–MeOH solvent system. The column was then eluted with $CHCl_3$–MeOH (5:1) at a flow rate of 0.85 mL/min, and fractions (5–10 mL) were collected. Aliquots of these were counted. Compounds eluting in radioactive fractions were identified by GC–MS. Isolated acids were methylated with "Methyl-8" (Pierce Chemical Co.) prior to GC studies.

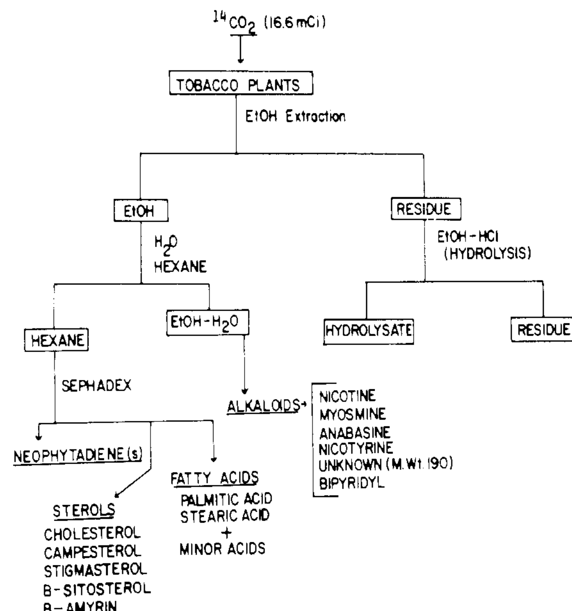

Figure 1. Scheme for isolation of radioactive materials from tobacco plants grown in an atmosphere of $^{14}CO_2$.

Radioactive alkaloids were isolated from the aqueous ethanolic solution by making the latter alkaline with 5 N NaOH and then extracting with $CHCl_3$. GC–MS of the dried $CHCl_3$ concentrate confirmed the presence of nicotine, myosmine, anabasine, nicotyrine, and bipyridyl therein.

Application of the ^{14}C Isolates to Cigarettes. Prior to being smoked, the cigarettes (85 mm, nonfilter, usually ten in number) were impregnated by the syringe technique (Liu et al., 1974; Hoffmann et al., 1977) with 1 000 000 to 3 000 000 dpm of test compound in 10 μL of $CHCl_3$. The radioactivity was distributed throughout the cigarette from the tip (burning end) to a point 23 mm from the butt end of the cigarette. The treated cigarettes were kept at constant temperature–humidity (22 °C, 60% RH) for 24 h.

Smoking Procedure. Cigarettes were smoked one at a time under standard conditions (puff duration: 2 s; interval between puffs: 58 s; puff volume: 35 mL; butt length: 23 mm). Each of the smoke streams (main and side) was diverted through a Cambridge filter (one filter/five cigarettes) and into a series of two traps (bubblers), the first containing toluene and the second an aqueous solution of 1.5 N NaOH. The filters were weighed before and after smoking to determine wet "tar" weights (from each of the smoke streams). The "tars" were then washed off the filters with acetone, and aliquots of the acetone solution were counted. The ashes and butts were washed with hexane and then with acetone, and the resulting solutions were also counted. The NaOH and toluene solutions containing $^{14}CO_2$ and ^{14}C volatile organics, respectively, were counted similarly.

RESULTS AND DISCUSSION

Fractionation of Radioactive Tobacco. Radioactive plant materials were initially extracted with ethanol and subsequently fractionated as shown (Figure 1). The residues remaining after extraction with ethanol were hydrolyzed with ethanolic HCl, resulting in a soluble hydrolyzate and an insoluble fiber. By determining the radioactivity in the ethanolic extract, the hydrolysate, and the fiber, we were able to estimate the degree of incorporation of $^{14}CO_2$ into the various plant parts (Table I). Greatest incorporation was observed to occur in the roots,

Table I. Incorporation of $^{14}CO_2$ into Various Parts of Tobacco Plants

Plant parts[a]	Weight, g	Activity, dpm × 10^{-9}	Sp act., dpm/g × 10^{-9}
Leaves			
1 + 2 (top)	205	4.5	0.022
3 + 4	250	4.0	0.016
5 + 6	205	2.3	0.011
7 + 8	285	2.8	0.010
9 + 10	260	3.0	0.012
10 + 12 (bottom)	280	2.8	0.010
Roots	1260	8.7	0.007
Flowers		8.1	
Suckers	640	5.2	0.008
Stems	525	3.6	0.007
Totals	3910	45.0	

[a] From four plants.

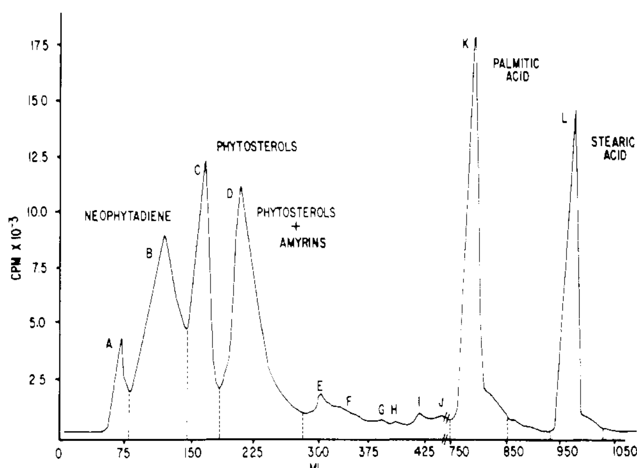

Figure 2. Radiochromatogram of hexane-soluble components off Sephadex LH 20. Major fractions eluted and were collected as follows: B (64 mg; 80–140 mL), C (78 mg; 140–180 mL), D (101 mg; 180–280 mL), K (152 mg; 750–850 mL), L (56 mg; 925–1025 mL).

flowers, and suckers. Among the leaves, greatest incorporation was noted in the upper, younger leaves. This, of course, is not unexpected, inasmuch as the lower leaves were relatively mature when the plants were placed in the $^{14}CO_2$ atmosphere. The correlation between stalk position of leaves and degree of incorporation of $^{14}CO_2$ is somewhat more marked when the data are expressed in terms of specific activity. Correlations between the stalk positions of the leaves and levels of various leaf constituents have been reported before (Wynder and Hoffmann, 1967; Tso, 1972; Rathkamp et al., 1973). The relatively high level of radioactivity in the roots may represent rapid assimilation of incorporated $^{14}CO_2$; expressed in terms of specific activity; however, the level of radioactivity in the roots is rather low and is probably indicative of the large weight of plant material that comprises the roots.

The ethanolic extract from the midstalk leaves was further fractionated into polar (ethanol-soluble) and nonpolar (hexane-soluble) fractions. Chromatography of the latter on Sephadex LH-20 resulted in its resolution into subfractions (Figure 2), containing neophytadiene (B), phytosterols (C), phytosterols plus amyrins (D), palmitic acid (K), and stearic acid (L). Material in A has not yet been characterized. By using thin-layer chromatography (TLC) and radioscanning techniques (Figure 3), in conjunction with gas chromatography–mass spectrometry (GC–MS), we were able to determine the specific activities of the above compounds, in addition to their contribution

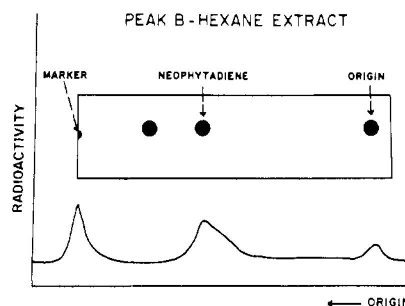

Figure 3. Radioscan of developed TLC plate showing radioactivity associated with neophytadiene isolated from ^{14}C tobacco.

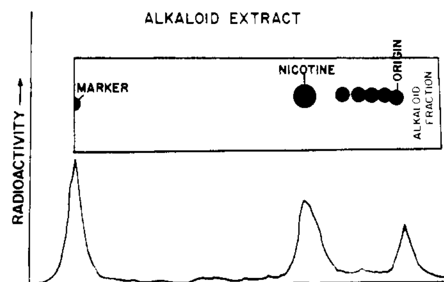

Figure 4. Radioscan of developed TLC plate containing alkaloid fraction, showing radioactivity concentrated in nicotine.

Table II. Isolated Radioactive Tobacco Components

	Activity dpm × 10^{-6}	% of fraction activity	% of total plant activity	Sp act., μCi/μm
Neophytadiene	16.64	81	0.38	0.274
Phytosterols[a]	4.12	26	0.15	0.410
Palmitic acid	17.44	95	0.62	0.205
Stearic acid	10.56	95	0.37	0.088
Nicotine[b]	128.	73	4.5	0.027

[a] In fraction C. Shown to consist of the four major tobacco phytosterols, cholesterol, campesterol, stigmasterol, β-sitosterol (Schmeltz et al., 1975a). Calculated as cholesterol. [b] In an alkaloid fraction.

to the activity of each fraction, and to that of the whole plant (Table II). For the compounds isolated, the specific activities are rather low as are the percentages of total incorporated activity. Previous studies have indicated that in tobacco plants administered $^{14}CO_2$, the bulk of incorporation occurs in the carbohydrates (Tso, 1972); the relatively low incorporation (<3%) into the hexane-soluble materials, i.e., terpenoids, that we encounter is, therefore, not surprising. Nonetheless, the radioactive isolates were sufficiently active for use in smoke formation studies. The specific activity of nicotine, isolated in an alkaloid mixture from the ethanol-soluble material (polar fraction), was determined as above (Figure 4). Although the specific activity of nicotine is also rather low, the percent of the total activity incorporated into nicotine is relatively high (≃5%). This reflects the large amounts of nicotine produced by the plant at this stage, and the utilization of CO_2 in the biosynthesis of nicotine via acetic acid and various amino acids (Leete, 1973; Schmeltz, 1971). Nicotine and other tobacco alkaloids are actively involved in the general metabolic pool (Tso, 1972).

Other material in the alkaloid fraction, in addition to nicotine, was visualized by the TLC–radioscanning technique. A substantial portion of this material remained at the origin (Figure 4) and is probably carbohydrate, or an alkaloid–carbohydrate complex (Kisaki et al., 1976).

Smoke Formation Studies. Cigarettes containing radioactive isolates were smoked, and the entire smoke was

Table III. Percentage Distribution of Radioactivity in Cigarette Smoke from Cigarettes Containing Radioactive Tobacco Constituents

	^{14}C tobacco constituents				
	Phytosterols	Neophytadiene	Fatty acids[a]	Polar fraction[b]	Alkaloids[c]
Mainstream					
"Tar"	19.0	26.0	22.81	10.00	19.20
CO_2	0.58	6.1	1.41	11.40	4.50
Volatiles	1.01	2.6	0.90	1.80	0.14
Sidestream					
"Tar"	17.2	17.9	14.60	14.60	31.80
CO_2	19.9	25.0	16.70	37.00	10.70
Volatiles	5.8	9.2	4.90	15.20	3.20
Butt	6.7	2.7	3.50	0.70	3.10
Ash	1.24	2.4	0.60	0.20	0.20
Total	71.43	91.9	65.53	90.9	72.84

[a] Principally (95%) [^{14}C]palmitic acid. [b] Contains reducing sugars. [c] Principally [^{14}C]nicotine, >73% radioactivity of isolate.

collected. By determining the radioactivity in the "tars" (collected on Cambridge filters), and in the aqueous NaOH and toluene scrubbers through which the smoke streams passed, we were able to account for the distribution of labeled leaf constituents in both mainstream and sidestream smoke, as nonvolatile organics, CO_2, and volatile organics. Radioactivity in the cigarette butts and ashes was also determined. Theoretically, we should have been able to account for all the radioactivity, except for that attributable to very low boiling organic compounds and to ^{14}CO, which we have not yet been able to determine satisfactorily. Disregarding the trapped gas phase compounds, one may generally assume that the radioactivity in the "tar" on the Cambridge filter represents, for the most part, unaltered, or only partially altered, leaf constituents transferred into the smoke. As will be discussed below, only a small portion of the radioactivity in the "tar" could account for pyrosynthesized PAH. Smoke constituents that result from severe degradation of leaf constituents would most likely be diverted to the gas phase and be trapped by the alkaline or toluene scrubbers as CO_2 or organic volatiles. A summary of the distribution patterns obtained with each of the radioactive isolates is given in Table III; additional details are discussed below.

Fate of Phytosterols. A total of about 36% of the radioactivity of the leaf ^{14}C-labeled phytosterols is transferred into the "tars" of mainstream and sidestream smoke (19% into the former, 17% into the latter). Previous studies reported lower transfer rates for phytosterols into mainstream "tar" (Grunwald et al., 1971; Cheng et al., 1973; Schmeltz et al., 1975a), and in the present study, therefore, the major portion of radioactivity in the mainstream "tar" is likely attributable to unaltered phytosterols transferred intact into the smoke, the rest to partially altered phytosterols. Radioactivity was also distributed into the vapor phase of both smoke streams, 20% as CO_2 appearing predominantly in the sidestream, and 7% as volatile organic compounds, again predominantly in the sidestream. It is not surprising that the major portion of the thermal degradation products of the phytosterols appears in the sidestream. Sidestream smoke is formed by the continuous exposure of tobacco to relatively high temperatures, during and between puffs, and therefore, one should find a preponderance of degradation products in the sidestream (Schmeltz et al., 1975b). This is also indicated by the fact that more of the radioactivity of the ^{14}C-labeled phytosterols is found in the sidestream smoke "tar" (19%) than in the mainstream smoke "tar" (17%) compared to the transfer of intact phytosterols (9 and 14.3%; Schmeltz et al., 1975a). On the other hand, mainstream smoke is formed from tobacco that is exposed to a sharp temperature gradient (Schmeltz and Hoffmann, 1976), and only a very small portion of the tobacco experiences very high temperatures. In mainstream smoke, therefore, we find substantial quantities of distillation and sublimation products, plus those carried along by "mechanical" transfer, in addition to pyrolytic and other degradation products.

Fate of Neophytadiene. Neophytadiene, a major terpenoid in tobacco which exists in the smoke in several isomeric forms, eluting in one GC peak (Guerin and Olerich, 1975), distributed in the smoke as follows (Table III): 44% in the "tar" (mainstream plus sidestream); 31% combusted to CO_2, most of this appearing in sidestream; 11% converted to volatile organics, again predominantly in sidestream.

The distribution pattern for nonvolatile leaf constituents seems to recur, with three major pathways from the leaf, basically intact precursor into the "tar", generally equally distributed between mainstream and sidestream, combustion products (CO_2) predominantly into sidestream, and volatile organics (thermal degradation products) also into sidestream, for the most part. The important question of conversion of terpenoids, such as neophytadiene, into PAH during cigarette smoking will be discussed below and, as will be seen, represents in absolute terms a minor process, which nevertheless is highly important in terms of tobacco carcinogenesis (Wynder and Hoffmann, 1967).

Fate of Fatty Acids. Radioactive palmitic acid was observed to distribute in the smoke in a manner similar to that of phytosterols and neophytadiene. Thirty-eight percent went into the "tar" (23% in mainstream, 15% in sidestream). Carbon dioxide accounted for 15% of the leaf acid, virtually all of the CO_2 appearing in the sidestream (Table III).

Fate of a Polar Fraction. An ethanol-soluble ^{14}C fraction from which hexane solubles and alkaloids were removed, and which contained reducing sugars and likely oxygenated acids and pigments, was shown to generate large quantities of $^{14}CO_2$ during cigarette smoking. The CO_2 accounted for nearly 50% of the radioactivity initially in the tobacco isolate (Table III). About 17% of the initial radioactivity was found among the organic volatiles and 24% in the "tars" of both streams. The high yield of $^{14}CO_2$ is consistent with previous data of Gager and co-workers (1971), which showed that cigarettes containing [^{14}C]-glucose or [^{14}C]sucrose give rise to relatively high yields of $^{14}CO_2$ during smoking. In both studies, the $^{14}CO_2$ was found predominantly in the sidestream.

Fate of Nicotine. When cigarettes containing randomly labeled [^{14}C]nicotine were smoked, more than half the radioactivity distributed into the "tar" of both streams,

Table IV. Sidestream to Mainstream (SS/MS) Distribution Ratios of Smoke Constituents Derived from ^{14}C Tracer Studies

Labeled leaf constituent	Labeled smoke constituents			
	CO_2	Volatile organics	Nonvolatile organics[b]	"Tar"
Sterols	28.2	4.5	1.1	$\frac{(24.0)^a}{(25.8)} = 0.9$
Fatty acids	11.3	5.4	1.2	$\frac{(25.0)}{(38.0)} = 0.6$
Polar fraction	3.2	8.4	1.5	$\frac{(23.2)}{(21.6)} = 1.1$
Alkaloids	2.4	22.9	1.7	$\frac{(26.6)}{(24.3)} = 1.1$
Neophytadiene	4.1	3.5	0.7	$\frac{(28.1)}{(29.3)} = 0.9$

[a] (Milligrams of SS "tar"/cigarette)/(milligrams of MS "tar"/cigarette). [b] Assumed to consist primarily of intact leaf constituent plus small amount of degradation products.

but principally in sidestream "tar" (32%). Considered in the light of previous work, virtually all the transferred radioactivity in the mainstream "tar" represents unaltered nicotine (Houseman, 1973; Schmeltz et al., 1964). It is interesting that nearly twice as much nicotine transfers into the sidestream as into the mainstream (Schmeltz et al., 1975b) and that, compared to other precursors looked at, relatively little CO_2 and volatile organics originate from nicotine. This may reflect the higher thermal stability and/or ease of volatility of this compound as compared to other leaf constituents under the conditions prevalent in a burning cigarette. A previous report has shown that nornicotine (a minor tobacco alkaloid, usually) may be a better precursor for alkyl pyridines (and myosmine) than nicotine during smoking (Glock, 1962). However, levels of nornicotine are substantially lower than those of nicotine in the leaf, and the former's contribution to levels of alkyl pyridines in the smoke may not be as significant as is nicotine's. Additional studies utilizing [^{14}C]nornicotine would be helpful here, including the identification of radioactive products it gives rise to in the smoke.

Data obtained from the above smoke formation studies are in line with and substantiate earlier studies (Jenkins et al., 1976; Houseman, 1973; Gager et al., 1971; Cheng, 1973; Grunwald et al., 1971; Bush et al., 1972). As a result, the techniques that were used to arrive at the data are illustrative of techniques that may be readily used in future studies with regard to formation of specific smoke constituents.

In this study, CO_2 was shown to be a major product arising from the precursor compounds, even though the smoking of tobacco is characterized by incomplete combustion which accounts for the numerous compounds (e.g., PAH), in addition to CO_2, obtained in the process. Like many of the smoke constituents, the greater portion of CO_2 was diverted to the sidestream smoke in all cases (Table IV). The radioactivities in the "tars" of the mainstream and sidestream were more or less the same, indicating that a substantial portion of the leaf constituents studied distributed intact, equally into both streams. This trend was also noted in the "tar" weights obtained (Table IV). Overall, most products of pyrolysis or thermal degradation are directed predominantly to the gases and vapors, especially to those of the sidestream, while the portions of leaf constituents that preserve their structures during smoking are diverted equally to the particulates of both streams.

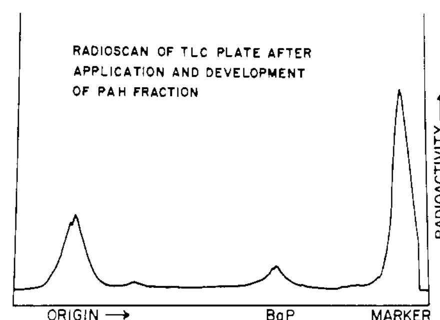

Figure 5. Radioscan of TLC plate containing [^{14}C]PAH fraction.

Table V. Conversion of Leaf Constituents to PAH

Leaf constituent	% conversion[a]
Phytosterols	<1.0
Palmitic acid	<1.0
Neophytadiene	0.1
Polar fraction	0.15
Alkaloids	0.10

[a] Represents radioactivity found in a PAH fraction from cigarette smoke, worked up as described in text.

Formation of Polynuclear Aromatic Hydrocarbons (PAH). The interesting question of PAH formation, although frequently reported on (Jenkins et al., 1973; Schlotzhauer et al., 1976), has not yet been fully resolved. Although pyrolysis studies have conclusively demonstrated the formation of PAH from organic matter exposed to high temperatures (Schmeltz and Hoffmann, 1976), the extent to which leaf constituents generate PAH under actual smoking conditions certainly needs additional study. In this study, the radioactive mainstream "tar" obtained during smoking was fractionated (Schmeltz et al., 1976) and a PAH fraction was further enriched by TLC. The TLC plate was then radioscanned (after development) and label was detected in the PAH region (Figure 5). Mass spectral analysis demonstrated the presence of benzo[*a*]pyrene and trimethylphenanthrene in the radioactive region. The percent conversion to PAH ranged from about 1.0% for the phytosterols to less than 0.1% for neophytadiene (Table V). Although these conversions are rather low, in the case of the phytosterols, for example, the percent conversion to PAH observed could account for a substantial portion of the PAH known to be present in the smoke. However, one cannot make definitive statements until similar tracer studies are carried out with sugars and other leaf constituents that are present in significant concentrations in tobacco and are known to generate PAH at elevated temperatures. Studies along these lines are continuing in our laboratory.

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Received for review May 23, 1977. Accepted August 4, 1977. This study was supported by the U.S. Department of Agriculture, Contract No. 12-14-1001-439. Portions of this study were presented at the 30th Tobacco Chemists' Research Conference, Nashville, Tenn., 1976.

Multiple Enzyme Forms of Tomato Seeds and Seedlings

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Proteins were extracted from dormant seeds, germinating seeds, and 12-day-old seedlings of Chico III and Homestead-24 tomato cultivars. Disc gel electrophoresis of the crude extracts showed that the number of enzyme forms differed with developmental stage of each cultivar. Generally the number of enzyme forms was greater in seedlings than in dormant seeds; exceptions were the numbers of enzyme forms of catalase and malic dehydrogenase, which decreased. In Homestead-24, the number of general protein forms was greater in seedlings than in dormant seeds. In Chico III, the number of protein forms was the same in dormant seeds and seedlings. The number of protein forms was lowest at the germinating seeds stage of both cultivars. Differences between the enzyme activities of the two cultivars were detected.

Seeds contain two types of proteins: metabolic proteins, both enzymatic and structural, which are concerned with cellular activities, and a second type, the storage or reserve proteins. Many of the former exist in multiple molecular forms known as isoenzymes. The latter have no enzymatic activity (Varner, 1965; Atschul et al., 1966). Both types of proteins function after promotion of seed germination. Reserve proteins are hydrolyzed by enzymatic proteins, and the degradation products are a source of nitrogen and carbon for the developing seedling (Oota et al., 1953).

Disc gel electrophoresis has been the most convenient method for the resolution of protein mixtures. Isoenzymes present in seed tissue (Cherry and Ory, 1973; Macko et al., 1967) and in other plant tissues (Kadam et al., 1973; Hall et al., 1969) have also been resolved into distinct patterns by this technique.

Numerous studies of enzyme activities in tomato fruit have been reported (Lee and MacMillan, 1968; Nakagawa et al., 1970; Hobson, 1967, 1974) and most concerned the enzymatic changes associated with ripening. Only few investigations have dealt with the identification of enzyme forms present in tomato seeds and seedlings and the changes those enzyme forms undergo during seed germination. A fundamental knowledge of the enzyme forms present in seeds and seedling would allow a better understanding of the biochemical steps that accompany the development of the plant. Any differences in enzyme distribution among varieties might enable the screening of plant crops for factors such as disease resistance and agronomic performance.

We undertook to identify and compare the enzyme forms of dormant seeds, germinating seeds, and seedlings of a firm (Chico III) and a soft (Homestead-24) variety of tomato, and to determine whether firmness of fruit could be related to the electrophoretic patterns of enzymes from plants at an early stage of development. We investigated the enzyme forms of acid and alkaline phosphatase, esterase, catalase, peroxidase, and malic dehydrogenase (MDH). Of these, only the isoenzymes of catalase, peroxidase, and MDH have well-understood genetics in studies on the physiological role(s) of specific enzymes (Scandalios, 1974).

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