Chemical Studies on Tobacco Smoke

XIII. Inhibition of the Pyrosyntheses of Several Selective Smoke Constituents*

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Experimental data suggest a correlation between the nitrate content of cigarette tobacco and the yield of certain pyrosynthesized compounds in the smoke (4, 11). In Burley tobacco the nitrate content of a leaf is inversely related to its stalk position. The mainstream smoke of cigarettes made from Burley tips with relatively low nitrate content yields significantly more particulate matter (TPM) and polynuclear aromatic hydrocarbons (PAH) than the mainstream smoke of cigarettes made from nitrate rich, lower leaves of the same Burley variety. Furthermore, the reduction of benzo(a)pyrene (BaP) is significantly higher than the reduction of TPM (4). Recent studies by Benner et al. confirmed our findings on the correlation between the nitrate content of the tobacco leaves and the BaP yield in the smoke (1). When 8.3% NaNO_3 was added to a cigarette tobacco blend, we observed a significant reduction in the smoke of TPM, phenol, and nicotine and, again, a selective reduction for BaP (10).

These observations suggest that the formation of PAH in the hot zones of nitrate rich tobaccos is significantly more inhibited than can be explained by the additional availability of oxygen from the thermal degradation of alkali nitrates. We hypothesized that in the hot zones of a glowing tobacco product C,H-radicals are formed by pyrolysis. These radicals can react with each other and form PAH among other compounds. However, the last step, the actual pyrosynthesis, may be partially inhibited by an excess of thermally activated nitrogen oxides. These oxides serve as scavengers for radicals. Our hypothesis is supported by the observation that, with elevated nitrate content of the tobacco, the yield of primary and secondary nitroparaffins increases, whereas the yield of PAH decreases (4). Recently reported data on the yields of nitrobenzenes in cigarette smoke are in line with our hypothesis (6).

In the study reported here, we challenged our working hypothesis on the pyrosynthesis and inhibition of PAH by quantitatively analyzing the mainstream smoke of cigarettes for compounds that are probably formed by different mechanisms. For the first phase of the in-

vestigation, we chose free and N-alkylated indoles, smoke constituents that are formed predominantly from one specific precursor (5). With naphthalene, we found a hydrocarbon which is believed to be mainly formed from one type of precursor (2, 3, 12) and, with the nonalkylated PAH phenanthrene, benz(a)anthracene (BaA) and BaP, we found a group of hydrocarbons that is most likely pyrosynthesized from a wide range of precursors via C,H-radicals. Finally we selected two nitroalkanes and nitrobenzene as smoke compounds that are formed from C,H-radicals and nitrogen oxide(s) in a competitive reaction with the pyrosynthesis of PAH from C,H-radicals (4, 6).

EXPERIMENTAL

Apparatus

For the determination of the total particulate matter, we employed the twenty-port, automated Phipps and Bird machine (13), and for the analysis of individual smoke compounds a 30-channel automatic smoker with vibrating liquid trap (14). A Perkin-Elmer gas chromatograph Model 800 with dual-flame ionization detector and a Cary Model 11 recording spectrophotometer served for the quantitative determinations. The β -radiation of the ^{14}C -labelled internal standards was counted with a Nuclear Chicago Scintillation System 720. The mass spectra were obtained with a Hitachi-Perkin-Elmer RMU-6D by the Morgan-Schaffer Corporation (Montreal, Canada). The laboratories were illuminated by yellow light (Sylvania Electric Tubes F-40 G.O.). Evaporations were completed at reduced pressure (mostly 12 mm Hg) with water bath temperatures below 45°C and, during the naphthalene analysis, below 38°C.

Reagents

All organic solvents were spectrograde, the other chemicals were of analytical grade. Alumina Woelm neutral (activity II, except as indicated) was obtained from Alupharm Chemicals, Gas Chrom P (80–100 mesh), SE-30 and OV-225 from Applied Science Laboratories, and Sephadex LH-20 from Pharmacia Fine Chemicals.

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Reference Compounds

All agents used as references were purified by column chromatography and, in some cases, also by preparative gas chromatography (4–7, 9). The purity of these compounds was ascertained for indoles by gas chromatography and refractometric indexes (5, 7); for PAH by paper chromatography and ultraviolet absorption spectra (9); and for nitroalkanes and nitrobenzenes by gas chromatography using an electron capture detector as well as a flame ionization detector, and by refractometric indexes (4, 6).

Gas Chromatography

The methods used for the determination and separation of indoles and N-alkylindoles (5, 7) as well as for nitroalkanes and nitrobenzenes were reported earlier (4, 6). For the separation of naphthalene from a concentrate, we employed a 3 mm by 6.1 m column filled with 5% SE-30 on Gas Chrom P. The column was operated at 160° C. For the phenanthrene analysis we used the same column but operated it at 180° C.

Internal Standards

For the quantitative analysis, we used indole-2-¹⁴C (4.05 mCi/mM) as internal standard. This was prepared from indole-2-¹⁴C-picrate (Tracerlab) (7), 1-methylindole-2-¹⁴C (4.05 mCi/mM; Amersham-Searle), on alumina purified phenanthrene-9-¹⁴C (5.1 mCi/mM; Tracerlab), BaA-9-¹⁴C (9 mCi/mM; Nuclear Chicago), and BaP-¹⁴C (3.22 mCi/mM; Nuclear Chicago) (8). As an internal standard for the nitroalkanes, we employed nitromethane-¹⁴C (2.7 mCi/mM) and for the nitrobenzenes freshly purified nitrobenzene-U-¹⁴C (3.2 mCi/mM).

Experimental Cigarettes

A Bright tobacco variety low in nitrate content (<0.1%) was cut at 0.8 mm and employed as standard tobacco for the filter-free, 85 mm cigarettes. In order to enrich the nitrate content of the standard tobacco artificially, we placed 45 kg of the cut Bright tobacco repeatedly in a rotating cylinder and sprayed it slowly with 50% KNO₃ solution (45° C), which contained 1.1, 2.3, 3.2, and 3.7 kg of the nitrate. The moisture content of the sprayed tobacco was adjusted to about 11% by blowing an air stream with a relative humidity of 45% through the tobacco in the rotating cylinder. These nitrate enriched tobaccos were then machine-made into 85 mm cigarettes, using the same cigarette paper as for the control cigarettes. We selected 40 cigarettes by weight from 200 cigarettes (moisture content about 11%) of equal nitrate content, removed the paper, and took samples for the nitrate determination according to Broadbuss et al. (15).

Quantitative Analysis

The methods for the quantitative determination of indoles, N-alkylindoles, nitromethane, nitroethane, and nitrobenzenes in the mainstream smoke were recently reported in detail (5, 7, 9).

Naphthalene was determined from the mainstream smoke of 100 cigarettes. The cigarettes were smoked individually on the 30-channel smoker with vibrating liquid trap filled with 1 l of acetone that was cooled by dry ice/acetone from the outside. After the smoking, 2 µg naphthalene-1-¹⁴C in 1 ml toluene were added as internal standard. The trapping solvent with the "tar" plus washings was reduced to dryness by distillation under reduced pressure in a water bath at 35° C. A 30 cm distillation column filled with glass rings was employed in order to reduce the loss of naphthalene to a minimum. The "tar" was dissolved in about 100 ml of methanol/water (4:1) and 100 ml of n-hexane. The n-hexane layer plus the 200 ml n-hexane from two additional extractions of the lower layer was evaporated under reduced pressure to about 50 ml. This n-hexane solution was extracted four times with nitromethane. The combined nitromethane layers were reduced to dryness and resulted in 250–300 mg of a PAH concentrate. This concentrate was dissolved in 10 ml n-hexane/benzene (6:1) and chromatographed with n-hexane on 100 g alumina, Woelm activity I. After about 500 ml forerun, we obtained about 300 ml eluate with β-activity. The oily, white-yellow residue of this eluate (10–20 mg) was dissolved in 0.2 ml of n-hexane and analyzed gas chromatographically. It showed a significant peak with the retention time of naphthalene. However, when the material represented by this peak was collected and analyzed by mass spectrometry, only about 60% of the column effluent was given by naphthalene itself. Peaks were observed in the mass spectrum with m/e 170 up to m/e 278. Therefore, we chromatographed the naphthalene concentrate on 40 grams of Sephadex LH-20 at 32° C (2×70 cm column) for each analysis. As a solvent, we used 2-propanol.

Every hour, one 1.8 ml fraction was collected. In general, we found the β-activity in 6–8 fractions between fractions 40–55. Consequently, we combined these fractions and analyzed the concentrate by gas chromatography. The column effluent with the retention time of naphthalene showed a mass spectrum comparable with that of authentic naphthalene; the impurities were lower than 3%.

Phenanthrene, BaA, and BaP were determined in the mainstream smoke of 300 cigarettes. We employed the 30-channel smoker by Seehofer et al. (12). The actual collection process was the same as employed for the naphthalene analysis. After the smoking, we added 1–2 µg of phenanthrene-¹⁴C, BaA-¹⁴C and BaP-¹⁴C as internal standards. The trapping solvent (acetone) plus the washings were combined and evaporated to dryness. The "tar" was distributed between two solvent pairs, methanol/water (4:1) and cyclohexane, and cyclohexane and nitromethane as reported earlier (15). The concentrate of the nitromethane layers was chromatographed on 300 g of alumina Woelm, activity II. As elution solvents, we used n-hexane and n-hexane/benzene 8:1 and 6:1. This technique enabled us to obtain a radiogram for every

analysis in which the three peaks were clearly separated from one another. The first radioactive group of fractions was rechromatographed on Sephadex LH-20 and the resulting residue of the radioactive fractions was analyzed at 180° C by gas chromatography (SE-30 column, 3 mm by 6.1 m).

The residues of the eluates from the second peak (BaA) and the third peak (BaP) in the radiogram of the alumina column chromatography fractions were separately applied to acetylated paper and chromatographed for 14 hours with methanol/ether/water (4:4:1) as solvent. The fluorescent BaA and BaP bands were extracted from the paper with benzene/methanol (4:1). The extract was filtered through small alumina columns and analyzed by ultraviolet spectrometry (13).

Under the above outlined conditions, we found the following experimental deviations for the selected compounds in the mainstream smoke of cigarettes: naphthalene and phenanthrene $\pm 6\%$, BaA and BaP $\pm 8\%$, indole $\pm 5\%$, 1-methylindole $\pm 7.5\%$, skatole $\pm 6\%$, 1,3-dimethylindole $\pm 8.0\%$, nitromethane and nitroethane $\pm 6\%$, and nitrobenzene $\pm 5\%$.

RESULTS AND DISCUSSION

Table 1 presents the results from the quantitative analysis of the mainstream smoke of experimental and standard

cigarettes for indole, skatole, 1-methylindole (1-MI), 1,3-dimethylindole (1,3-DMI), naphthalene (Naph.), phenanthrene (Ph), benz(a)anthracene (BaA), benzo(a)pyrene (BaP), nitromethane (N.M.), nitroethane (N.E.) and nitrobenzene (φ -NO₂). Since a comparison of the yields of smoke constituents should not be based on the number of cigarettes, but rather on the amount of cigarette tobacco burned, we have expressed the data of Table 1 also for 100 g tobacco (minus added KNO₃) smoked (Table 2 and Figure 1).

The eleven components we selected for this study are practically exclusively pyrosynthesized during the burning of tobacco. We hypothesized, however, that these smoke constituents are formed by different mechanisms. The free and N-alkylated indoles are derived from a specific precursor (tryptophan) by cleavage of the C-2-C- α or C- α -C- β carbon-carbon bonds and/or N-methylation and hydrogen addition. Naphthalene is most likely pyrosynthesized from specific tobacco terpenes (2, 3, 12), the polynuclear aromatic hydrocarbons (PAH) phenanthrene, benz(a)anthracene, and benzo(a)pyrene are formed from C,H-radicals without specific precursors. The nitroalkanes and nitrobenzene are formed from C,H-radicals and nitrogen oxides in a competitive reaction with the pyrosynthesis of PAH.

Despite the fact that we may have changed certain physicochemical parameters of the experimental ciga-

Table 1. Selective cigarette smoke components depending on the nitrate content of tobacco.
(values for 1 cig)

% KNO ₃ added	0	2.46	5.05	6.96	8.07
Naphthalene (μ g)	1.85	1.92	1.84	1.86	1.41
Phenanthrene (μ g)	0.447	0.242	0.251	0.237	0.20
Benzo(a)pyrene (ng)	27.4	24.8	19.2	18.4	17.2
Benz(a)anthracene (ng)	6.5	5.6	5.1	4.3	3.8
Indole (μ g)	17.4	14.4	14.4	13.1	13.0
Skatole (μ g)	12.9	12.2	12.5	12.6	12.3
1-Methylindole (μ g)	0.39	0.35	0.34	0.35	0.36
1,3-Dimethylindole (μ g)	0.25	0.24	0.23	0.24	0.24
Nitromethane (μ g)	0.18	0.66	0.75	0.80	0.90
Nitroethane (μ g)	0.24	1.11	1.14	1.20	1.76
Nitrobenzene (ng)	26.8	36.4	35.6	46.7	83.2

Table 2. Selective cigarette smoke components depending on the nitrate content of tobacco.
(μ g values for 100 g tobacco smoked) *

% KNO ₃ added	0	2.46	5.05	6.96	8.07
Naphthalene	232.0	240.0	231.0	233.0	172.0
Phenanthrene	53.9	34.1	31.4	29.6	24.6
Benzo(a)pyrene	3.31	3.1	2.4	2.3	2.1
Benz(a)anthracene	0.80	0.70	0.64	0.54	0.47
Indole	2098.0	1810.0	1796.0	1642.0	1594.0
Skatole	1540.0	1528.0	1564.0	1570.0	1492.0
1-Methylindole	45.7	43.8	42.4	44.1	44.8
1,3-Dimethylindole	30.1	29.7	28.8	29.4	30.0
Nitromethane	22.6	83.4	94.1	99.4	112.0
Nitroethane	28.4	139.1	142.5	150.0	221.4
Nitrobenzene	3.24	4.56	4.45	5.84	10.4

* Tobacco minus added potassium nitrate burned to 23 mm butt length.

Figure 1. Selective cigarette smoke components depending on the nitrate content of tobacco. (Values for 100 g tobacco smoked)

rettes to some degree by addition of potassium nitrate to tobacco and, despite the fact that we will disregard data from the cigarettes with 8.07% KNO_3 , certain conclusions can be drawn from this study. The formation of naphthalene and the free and N-alkylated indoles in the smoke of cigarettes is inhibited only to a minor extent by the addition of 2–7% of KNO_3 and, with it, the presence of additional oxygen and nitrogen oxides in the burning cone of the cigarettes. Contrary to this observation, we can state that the formation of the three-, four-, and five-ring aromatic hydrocarbons phenanthrene, benz(a)anthracene and benzo(a)pyrene in the smoke of cigarettes enriched with KNO_3 is significantly inhibited and that the formation of nitroalkanes and nitrobenzenes is significantly increased.

The experimental data of this investigation support our working hypothesis that the PAH are at least partially pyrosynthesized from pyrolytically formed C_2H -radicals. An increase of nitrogen oxides in the burning cone of cigarettes leads to an inhibition of the pyrosynthesis of PAH and does this partially by a competitive reaction between C_2H -radicals and nitrogen oxides. Another possible mechanism for the pyrosynthesis of PAH is presently explored. However, so far, we do not know, if an excess of nitrogen oxides will affect this second mechanism of the pyrosynthesis of PAH.

The present study is solely designed to find the mechanism of the pyrosynthesis of carcinogenic aromatic hydrocarbons in tobacco smoke and to develop methods that will lead to a reduction in the tumorigenicity of cigarette smoke when assayed in the experimental animal. We do not suggest that the smoke of these experimental cigarettes is less harm-

ful, yet, we intend to increase our knowledge in experimental tobacco carcinogenesis. We hope to find some answers to why certain natural tobacco types, relatively high in nitrate, deliver a mainstream smoke less tumorigenic to the experimental animal than a mainstream smoke of cigarettes low in nitrate content

SUMMARY

Earlier studies demonstrated that polynuclear aromatic hydrocarbons (PAH) are selectively reduced in the smoke of alkali nitrate rich tobaccos. We hypothesized, therefore, that in the burning cone of a tobacco product, the non-volatized organic compounds are partially pyrolyzed to C_2H -radicals that may combine with each other and form, among others, the thermodynamically favored PAH. Since we have in the burning cone of nitrate rich tobaccos an excess of thermally activated nitrogen oxides, we assumed that these may react as scavengers for C_2H -radicals and, with it, partially inhibit the PAH pyrosynthesis.

The present study was designed to challenge our working hypothesis. For the experiments we employed cigarettes to which we had added various amounts of KNO_3 (0, 2.5, 5.0, 7.0 and 8.0%). As expected, the yields of nitromethane, nitroethane, and nitrobenzene in the smoke increased with the increased of nitrate in the tobacco and the yields of phenanthrene, benz(a)-anthracene, and benz(a)pyrene decreased. The concentration of naphthalene was only to a minor degree reduced with the increase of nitrates in the tobacco. One explanation for this observation could be that naphthalenes are primarily formed from specific tobacco terpenes, as suggested in the literature. As was expected, the smoke yields of N-unsubstituted and N-alkylated

indoles was relatively little affected by the increase in the nitrate content, since these agents are predominantly formed from tryptophan.

It was our objective to contribute to the understanding of the pyrosynthesis of carcinogenic PAH and the selective reduction of PAH in the smoke of nitrate rich tobacco. With the increase of our knowledge in this area we may also find an explanation for the reduced tumorigenicity of condensates from nitrate rich tobaccos.

ZUSAMMENFASSUNG

Frühere Studien haben gezeigt, daß im Rauch von alkalinitratreichen Tabaken polycyclische aromatische Kohlenwasserstoffe (PAH) selektiv reduziert werden. Wir stellten daher die Arbeitshypothese auf, daß gewisse PAH wenigstens teilweise von pyrolytisch gebildeten C₂H-Radikalen gebildet werden. Diese C₂H-Radikale reagieren in der Brennzzone von nitratreichen Tabakprodukten mit Stickstoffoxiden und vermindern dadurch die Bildung von PAH. Unsere Hypothese konnte zum Teil dadurch bewiesen werden, daß die Ausbeute an primären und sekundären Nitroalkanen wie auch Nitrobenzolen von der Nitratkonzentration der Tabake abhängt. Phenanthren, Benz(a)anthracen und Benzo(a)pyren werden im Rauch von nitratreichen Tabaken selektiv reduziert. Die Ausbeute an Naphthalin ist jedoch nur unwesentlich von der Nitratkonzentration in Tabak abhängig. Eine Erklärung für diese letztere Beobachtung mag teilweise dadurch gegeben sein, daß Naphthaline vornehmlich von gewissen Tabak-Terpenen gebildet werden. Wie erwartet, werden die Rauchkonzentrationen von N-nichtsubstituierten und N-alkylierten Indolen nur wenig durch die Nitratkonzentration des Tabaks beeinflußt. Diese Verbindungen werden hauptsächlich von Tryptophan gebildet.

Die vorliegende Studie wurde begonnen, um ein besseres Verstehen der Pyrosynthese von krebserregenden, aromatischen Kohlenwasserstoffen zu gewinnen und die Reduktion von PAH im Rauch von nitratreichen Tabaken zu erklären. Mit dem Anwachsen unserer Kenntnisse auf diesen Gebieten mag möglicherweise auch eine Erklärung gefunden werden für die reduzierte Tumoraktivität von Kondensaten natürlicher, nitratreicher Tabake.

RESUME

La réduction sélective d'hydrocarbures aromatiques polynucléaires dans la fumée de tabac riche en nitrate d'alcalis a été démontrée dans des travaux antérieurs. Nous avons présenté alors l'hypothèse selon laquelle, dans le cône brûlant d'un produit du tabac, les composés organiques non-volatilisés sont partiellement pyrolysés en radicaux C₂H qui pourraient se combiner entre eux et former entre autres un PAH, qui est thermodynamiquement favorisé. Etant donné la présence d'un excès d'oxydes azotés activés thermiquement dans le cône de combustion des tabacs riches en nitrates, nous admettons que ces oxydes puissent agir en tant que capteurs de radicaux C₂H, et inhiber partiellement la pyrosynthèse des PAH.

Ces travaux ont été conçus pour étayer notre hypothèse de travail. Dans la partie expérimentale nous avons ajouté aux cigarettes étudiées du nitrate de potasse en différentes concentrations (0; 2,5; 5,0; 7,0 et 8,0%). Comme prévu, les rendements en nitrométhane, nitroéthane, et en nitrobenzène dans la fumée ont augmenté parallèlement à l'augmentation de nitrate dans le tabac, et le rendement en phénanthrène, benz(a)pyrène, et benz(a)anthracène a diminué. Par l'augmentation de nitrates dans le tabac, on n'a observé qu'une très faible diminution de la concentration de naphthalène. On pourrait expliquer ce résultat en admettant, comme la littérature le suggère par ailleurs, que les naphthalènes sont formés principalement à partir de polyterpènes spécifiques du tabac. Comme prévu également, le rendement en indoles N-alkylés et N-non substitués est relativement peu affecté par l'augmentation de la concentration en nitrates, puisque ces composés dérivent principalement du tryptophane.

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