mass spectrometry. Moreover, the derivatives should give characteristic fragmentation patterns and preferably also an abundant molecular ion.

Our results show that perfluoroacylated derivatives of the catecholamines have excellent properties for mass spectrometric analysis. Because fluorine is monoisotopic, abundant isotope peaks are not observed. This is an advantage for comparison with deuterium and ^{13}C-labelled compounds which only give small increments in the *m/e* value. Furthermore the molecular ion was, in each case, an abundant fragment, usually over 10%, and for HVA-Me and MOPEG it was the base peak (Figure 6).

Our results give the basic information for proceeding with the analysis of the catecholamine metabolites in several directions. First they can be analyzed with great sensitivity using an electron capture detector. For biological work they must, however, be fairly extensively purified prior to the GLC analysis.

Second, studies on the metabolism of the catecholamines using precursors labelled with stable isotopes are facilitated by the development of methods by which the main metabolites can be examined by combined gas chromatography and mass spectrometry (*24*). Identification of small amounts of these compounds in the presence of large amounts of impurities could also be made by the use of the mass spectrometer as a detector, focused on abundant fragments arising from catecholamine metabolites (*25*). Work is in progress to develop techniques along those lines for the analysis of catecholamine metabolites.

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Quantitative Determination of 9-Methylcarbazoles in Cigarette Smoke

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An analytical method for the determination of 9-methylcarbazoles in cigarette smoke is described. The nonvolatile particulate matter of the smoke collected in solvent is distributed between two pairs of solvents and the resulting concentrate is chromatographed on alumina and subsequently analyzed by gas chromatography. 9-Methyl-^{14}C-carbazole was synthesized and served as internal standard. The mainstream smoke of an 85-mm U.S. nonfilter cigarette contained 103 ng of 9-methylcarbazole, 11.8 ng of 1,9-dimethylcarbazole, 19.9 ng of 2,9- and 3,9-dimethylcarbazole, and 5.7 ng of 4,9-dimethylcarbazole. 9-Ethylcarbazole was identified in a concentration of about 6 ng/cigarette. The identification of 9-alkylcarbazoles in cigarette smoke represents their first isolation from a respiratory environment. The lack of biological data at this time does not permit a conclusion as to the possible biological significance of 9-methylcarbazoles in experimental tobacco carcinogenesis.

BIOASSAYS ON MOUSE SKIN with fractions of cigarette smoke condensate have established that the highest carcinogenic and tumor-initiating activity resides in the neutral subfraction BI (*1*, *2*). This subfraction amounts to about 0.6% of dry cigarette "tar" and contains polynuclear aromatic hydrocarbons, chlorinated hydrocarbon insecticides, indoles, and carbazoles (*2*). More recent studies indicated the presence of 9-methylcarbazoles in one of the biologically active subfractions of BI. Until now, 9-alkylcarbazoles have not been identified in the human respiratory environment (*3–5*).

The 9-alkylcarbazoles differ significantly from other carbazoles in their absorption behaviour. In the method reported here, the alkylcarbazoles are enriched by distribution of the cigarette "tar" between two pairs of solvents and by chromatography on alumina. The resulting concentrate is separated into individual components by gas chromatography; 9-methyl-^{14}C-carbazole is employed as internal standard for the quantitative determination.

EXPERIMENTAL

Apparatus. A Perkin-Elmer gas chromatograph Model 800 with dual flame ionization detector was used for the qualitative analysis. The β-radiation was counted with a Nuclear Chicago Scintillation System 720. Ultraviolet absorbance measurements were made with a Cary Model 11 recording spectrophotometer. For the quantitative analysis, we smoked the cigarettes individually with a CSM-10 (Cigarette Components Ltd.) whereas for the isolation of N-alkylcarbazoles we employed a 30-channel automatic smoker with vibrating liquid trap (*4*, *6*). The mass spectra were obtained with a Hitachi-Perkin-Elmer RMU-6D by the Morgan-Schaffer Corporation (Montreal, Canada). The energy of the bombarding electrons was kept at 70 eV. The elemental analyses were carried out by Spang Microanalytical Laboratory, Ann Arbor, Mich. Evaporations were completed at reduced pressure with water bath temperatures below 50 °C. The melting points were determined in sealed capillary tubes.

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Reagents. All solvents were spectrograde. The carbazoles for the synthesis of 9-methylcarbazoles were purified by column chromatography and their purity was ascertained by gas chromatography (*7*). Alumina Woelm neutral (activity II except as indicated) was obtained from Alupharm Chemicals, Sephadex LH-20 from Pharmacia Fine Chemicals, Gas Chrom P (80–100 mesh), OV-1 and OV-225 from Applied Science Laboratories.

Synthesis of 9-Methyl-^{14}C-Carbazoles. 0.55 mg of Dimethylsulfate-^{14}C (22.7 mCi/mM; Amersham/Searle) and 1.03 mg of carbazole were dissolved in 1.5 ml of acetone. Under magnetic stirring at room temperature 0.4 ml of a 25% NaOH solution was slowly added. Twenty minutes later, three ether extractions were made and the resulting solution was dried (Na_2SO_4) and evaporated. The yellow-white semi-crystalline residue (40.9% of the β-activity of the dimethylsulfate-^{14}C) was chromatographed on 5 grams of alumina with *n*-hexane. The radioactive fractions were combined (38.5% of β-activity) and rechromatographed on 5 grams of alumina. The fractions now contained 0.45 mg of 9-methyl-^{14}C-carbazole with a specific activity of 11.2 mCi/mM (yield 76%). The chemical and radiochemical purity of the labelled compound was ascertained by repeated column chromatography and gas chromatography. The counting efficiency for unquenched 9-methyl-^{14}C-carbazole in toluene with 0.4% PPO (2.5-diphenyloxazole) and 0.005% POPOP (*p*-bis-[2(5-phenyloxazole)]benzene) was 72.3%.

Reference Compounds. 9-Methylcarbazole (N-methylcarbazole) was synthesized according to Stevens and Tucker (*8*) and purified by repeated column chromatography on alumina and by recrystallization from ethanol; mp 89 °C cor. λ(cyclohexane) max. 342 mμ (ϵ 7430), 328 (5110), 294 (31000), 263 (28200), 254 (28100), 244 sh (34600), 236 (58600), 231 sh (49300).

2,9-Dimethylcarbazole. 120 mg of 2-methylcarbazole dissolved in 5 ml of acetone was added to 2.5 ml of dimethylsulfate. Under magnetic stirring the solution was mixed with 5 ml of a saturated NaOH solution. The flask was tightly stoppered, covered with aluminum foil and stirred at ambient temperature for 14 hours. The slightly yellow reaction mixture was poured into water, extracted with ether, dried (Na_2SO_4) and evaporated. The residue was dissolved in a few milliliters of *n*-hexane/benzene (6:1) and chromatographed on 10 grams of alumina (activity I) with hexane as the eluent. The early fractions containing most of the dimethylcarbazole were combined, evaporated, and the residue recrystallized from *n*-hexane (yield 70 mg; 54%), colorless cubes, mp 88–88.5 °C cor. The purity of 2,9-dimethylcarbazole was proven by gas chromatography. Calcd. for $C_{14}H_{13}N$: C, 86.12; H, 6.71; N, 7.17. Found: C, 86.14; H, 6.76; N, 7.14. λ(cyclohexane) max: 343 mμ (ϵ 4190), 327 (3860), 297 (19700), 265 (18600), 238 (49700), 233 sh (37800).

The other three isomeric dimethylcarbazoles were synthesized by the same method and gave comparable yields. 1,9-Dimethylcarbazole, mp 114.5–115 °C cor. Calcd. for $C_{14}H_{13}N$: C, 86.12; H, 6.71; N, 7.17. Found: C, 86.15; H, 6.59; N, 7.09. λ(cyclohexane) max. 344 mμ (ϵ 4780), 328 (3800), 294 (19500), 263 (21400), 250 sh (26100), 240 (41300), 233 sh (32200). 1,9-Dimethylcarbazole was recently synthesized by an alternate method: mp 117 °C (*9*). 3,9-Dimethylcarbazole, mp 83–83.5 °C cor. [Lit. (*10*), 81 °C] λ(cyclohexane) max. 348 mμ (ϵ 4000), 333 (2970), 297 (19800), 263 (14500), 238 (34200), 233 sh (32000). 4,9-Dimethylcarbazole, mp 105–105.5 °C cor. Calcd. for $C_{14}H_{13}N$: C, 86.12; H, 6.70; N, 7.08. Found: C, 86.04; H, 6.70; N, 7.08. λ(cyclohexane) max. 343 mμ (ϵ 6090), 328 (4100), 291 (23100), 264 (24000), 240 (47300), 233 sh (36600).

Table I. 9-Alkylcarbazoles: Partition Coefficients and Retention Times

Partition coefficients (20 °C)			Retention time (min)	
C_C/C_M	C_N/C_C		I[a]	II[b]
4.2	2.8	9-Methylcarbazole	7.9	5.4
4.7	2.6	1,9-Dimethylcarbazole	13.6	8.6
5.0	2.2	2,9-Dimethylcarbazole	10.6	7.5
5.0	2.2	3,9-Dimethylcarbazole	10.6	7.5
5.1	2.2	4,9-Dimethylcarbazole	11.4	7.6
4.3	2.7	9-Ethylcarbazole	7.9	5.9

C_C = Concentration in cyclohexane.
C_M = Concentration in methanol/water (8:1).
C_N = Concentration in nitromethane.

[a] Condition I-3mm × 2m stainless steel 5% OV 225-210 °C isotherm.

[b] Condition II-3mm × 2m stainless steel 10% OV-1-210 °C isotherm.

9-Ethylcarbazole. The commercial sample was purified by column chromatography; mp 69–70 °C cor. (UV-spectrum (*11*). DISTRIBUTION: Distribution between methanol/water (8:1), cyclohexane, and cyclohexane, nitromethane leads to a significant enrichment of N-alkylcarbazoles from cigarette "tar." The distribution coefficients were determined by ultraviolet absorption spectra (Table I).

Gas Chromatography. The most satisfactory separation of 9-methylcarbazoles was obtained at 210 °C on a 3-mm by 2-m column filled with 5% OV-225 on Gas Chrom P. This column separates also carbazole from 9-methylcarbazoles. The separation of 9-methylcarbazole from 9-ethylcarbazole, however, was best achieved on a 3-mm by 2-m column with 10% OV-1 on Gas Chrom P. The retention times are given in Table I. About 1 μg of 9-methylcarbazole reaches the full scale of a 1-mV recorder with an attenuation of 100. For the gas chromatographic isolation of the N-alkylcarbazoles from a concentrate from cigarette smoke a 1:4 glass splitter was installed so that 80% of the effluent was collected in a glass capillary. The effluent was rechromatographed for mass spectral analysis.

Procedures. A. Isolation of 9-Alkylcarbazoles. 1,000 Cigarettes without filter tips (85mm) were smoked with a 30-port automatic smoking machine at a rate of one puff per minute of 2 seconds duration and a puff volume of 35 ml; butt length 23mm. The mainstream smoke was drawn through 500 ml of acetone in a collecting vessel with vibrator. The filtrate of the acetone, smoke condensate suspension, and the acetone washings were evaporated to dryness and yielded 30 grams of residue which was dissolved in 250 ml of methanol/water (8:1) and extracted three times with 250 ml of cyclohexane. The combined cyclohexane layers were concentrated to 150 ml (residue 8.14 grams) and extracted three times with nitromethane. The combined nitromethane layers were evaporated and dried. The residue (2.71 grams) was dissolved in 30 ml of *n*-hexane/benzene (6:1) and chromatographed with *n*-hexane on 400 grams of alumina (column 3 × 75 cm). After 400 ml of forerun, the eluate was collected in 100 ml fractions, evaporated, and analyzed by gas chromatography. (Non-N-substituted carbazoles are not eluted with *n*-hexane). Fractions 5–7 contained component(s) with the retention time of 9-methylcarbazole. The combined

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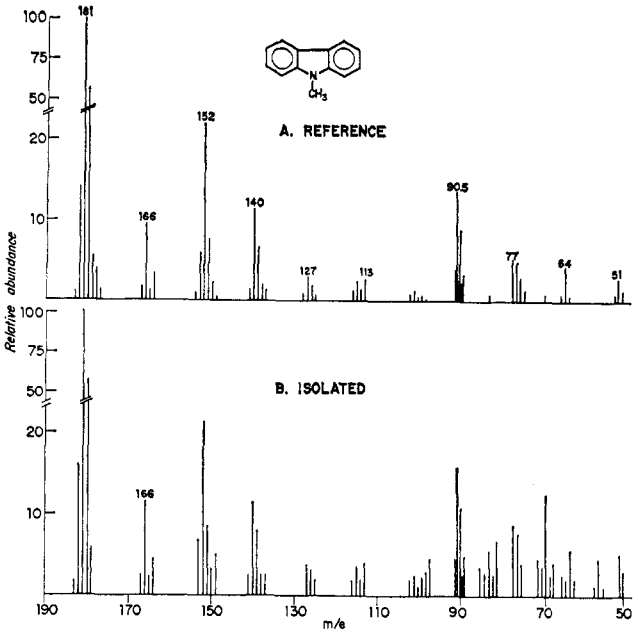

Figure 1. Mass spectra of 9-methylcarbazole

fractions (19.4 mg) were dissolved and chromatographed with 2-propanol on 40 grams of Sephadex LH-20 (column 2 × 70 cm) at a column temperature of 32 °C. Every hour a 1.8-ml fraction was collected. The combined fractions 63–75 (2.1 mg) and 76–82 (3.2 mg) were analyzed by gas chromatography for 9-methyl and 9-ethylcarbazole, respectively. The column effluents corresponding to the retention times of 9-methylcarbazoles were collected and rechromatographed for mass spectral analysis. The material indicated by the peak corresponding to the retention time of 2,9- and 3,9-dimethylcarbazoles was also analyzed by ultraviolet spectrometry.

B. Quantitative Analysis. 300 Cigarettes were smoked under standard conditions with CSM-10. The mainstream smoke was directed through a series of 3 gas wash bottles filled with *n*-hexane. The smoke suspensions and washings

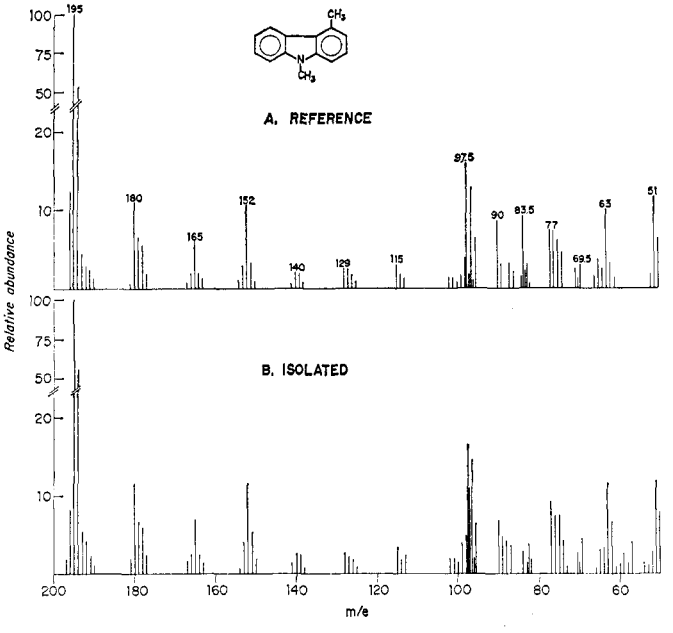

Figure 2. Mass spectra of 4,9-dimethylcarbazole

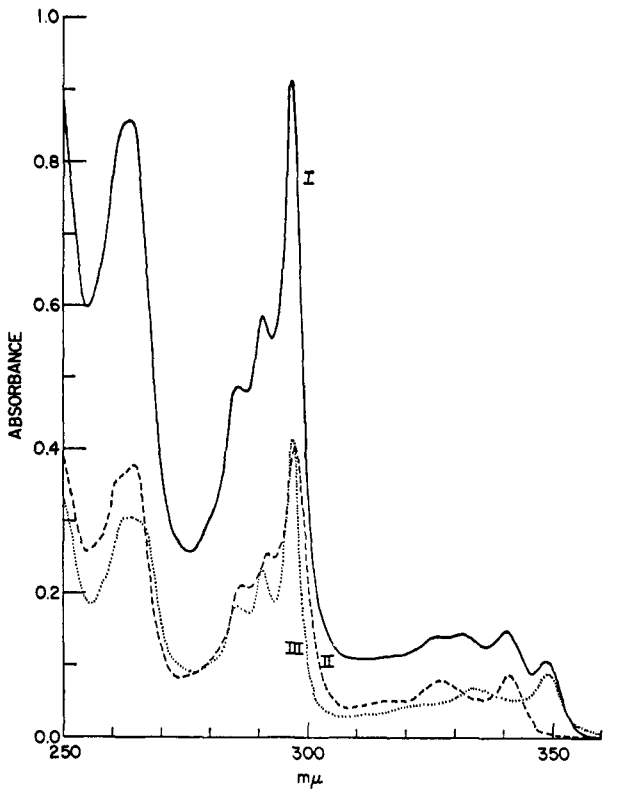

Figure 3. Ultraviolet absorption spectra of an admixture of 2,9- and 3,9-dimethylcarbazole isolated from cigarette smoke (I); 2,9-dimethylcarbazole (II; 0.4 mg/l); and 3,9-dimethylcarbazole (III; 0.4 mg/l).

Solvent: cyclohexane; cell length: 10 cm

were combined and 0.8 μg of 9-methyl-^{14}C-carbazole added as internal standard. The "tar" was dissolved in 100 ml of methanol/water (4:1) and extracted three times with 100 ml of cyclohexane. The cyclohexane solutions were combined and evaporated to dryness. The residue was dissolved in 100 ml of cyclohexane, three times extracted with 100 ml of nitromethane, and evaporated. The residue (0.6 gram) was dissolved in 10 ml of *n*-hexane/benzene (6:1) and chromatographed on 120 grams of alumina. Aliquots of the *n*-hexane fractions (100 ml each) were counted. Generally, the β-activity was found in fractions 6–10. These were combined, evaporated to dryness (residue 4–7 mg) and aliquots were taken for gas chromatographic analysis and liquid scintillation counting.

RESULTS AND DISCUSSION

Figures 1 and 2 compare mass spectra of synthetic and isolated 9-methylcarbazoles. The ultraviolet spectral analysis of the isolated admixture of 2,9- and 3,9-dimethylcarbazole indicated that both components are present in cigarette smoke in a ratio of 1:1 (Figure 3). The differences in the fragmentation patterns and in the relative intensities in the mass spectra of these four isomeric dimethylcarbazoles are only minor and thus do not suffice as the sole method for their differentiation. However, because the fragmentation patterns are characteristic for these dimethylcarbazoles they served for their identification in cigarette smoke together with the retention times from two gas chromatographic systems. 9-Ethylcarbazole could be identified by retention times and mass spectrum (Figure 4). It is estimated to be in the smoke in a concentration of about 6 ng/cigarette. For the quantitative analysis, five times 300 cigarettes were smoked and 9-

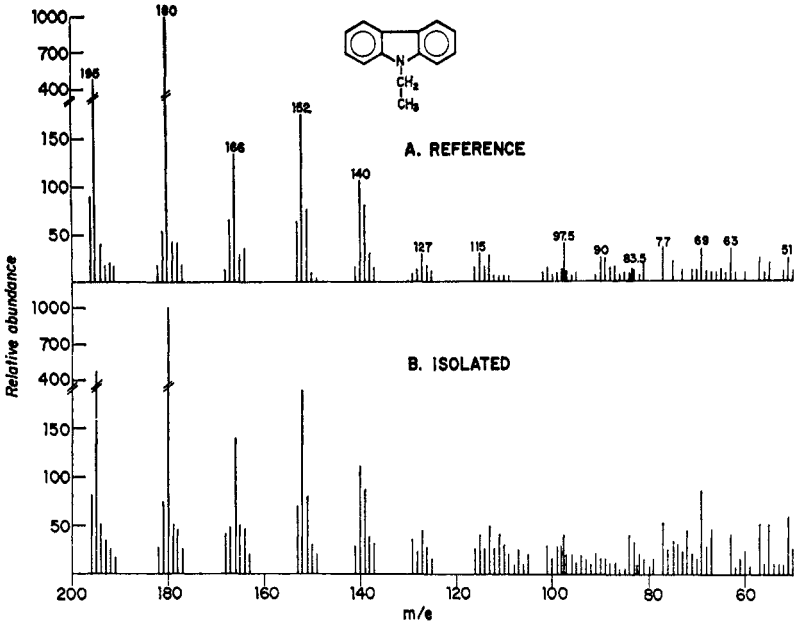

Figure 4. Mass spectra of 9-ethylcarbazole

Table II. 9-Methylcarbazoles in Cigarette Smoke[a]

Number of analyses	9-Methyl-carbazole (ng/cig.)	1,9-Di-methyl-carbazole (ng/cig.)	2,9- + 3,9-Dimethyl-carbazole (ng/cig.)	4,9-Dimethyl-carbazole (ng/cig.)
I	99.8	11.4	18.8	6.4
II	104.8	11.6	20.4	5.2
III	100.6	12.4	18.7	5.4
IV	100.4	12.6	20.6	6.2
V	109.4	11.2	21.2	5.2
Average	103.0	11.8	19.9	5.7
Standard deviation	4.1	0.63	1.13	0.57
Deviation coefficient	3.9%	5.3%	5.6%	10.0%

[a] Calculated with the isotope dilution method using 9-methyl-^{14}C-carbazole as internal standard.

methyl-^{14}C-carbazole was used as internal standard. From the final gas chromatograms (Figure 5), it was calculated that the mainstream smoke from an 85-mm U.S. blended cigarette without filter tip contains 103 ng of 9-methylcarbazole, 11.8 ng of 1,9-dimethylcarbazole, 19.9 ng of 2,9- and 3,9-dimethylcarbazole, and 5.7 ng of 4,9-dimethylcarbazole (Table II). The experimental deviation varied between 4–10% depending on the concentration of the N-methylcarbazole. The recovery for 9-methylcarbazole was better than 90%. An analysis showed that the carcinogenic subfraction BI contains more than 90% of the 9-alkylcarbazoles of the mainstream smoke.

Biological data are needed to evaluate the possible contribution of 9-methylcarbazoles to the experimental carcinogenicity of the particulate matter of cigarette smoke and its most active subfraction BI.

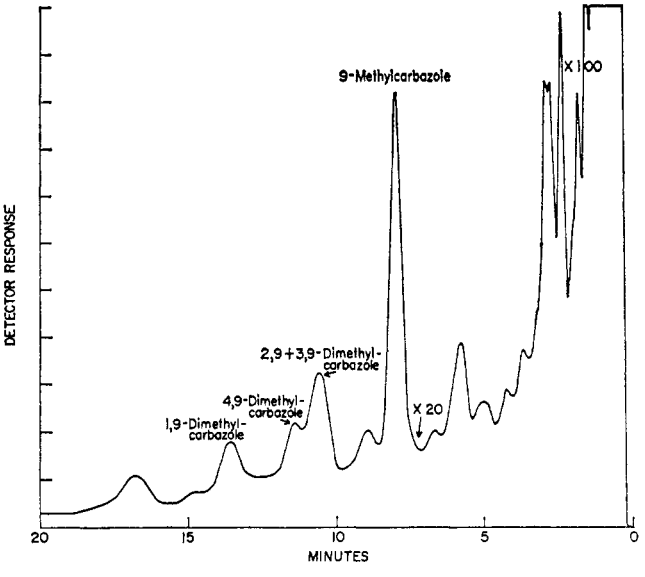

Figure 5. Gas chromatogram of concentrates of 9-methylcarbazoles from cigarette smoke

An *in vitro* test alkylating agents (*12*) was negative for all five 9-methylcarbazoles. This, however, does not exclude the possibility that *in vivo* 9-methylcarbazoles may react as alkylating agents after biochemical activation.

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