TABLE II

FRACTIONATION OF CIGARETTE SMOKE ALKALOIDS BY GAS CHROMATOGRAPHY

Separation Performed:	Pre-nicotine	Immediate Post-nicotine	Late Post-nicotine
Column[a]	1 m. × 6 mm.	1 m. × 10 mm.	1 m. × 6 mm.
Temp., °	145	190	190
He rate, ml./min.	47	75	60
Retention time of nicotine, min.	24[b]	18	5.5
Retention time of other peaks	4.9, 6.2, 7.9, 11.3, 14.4, 17.0, 20	23, 28, 33, 39, 51, 60	27, 31, 49

[a] Packed with polypropylene glycol, mol. wt. 1025, on Firebrick, 1:4. [b] On separately run sample.

about 50 μl. with a stream of nitrogen. This solution was then chromatographed under the same conditions used in collection of the original sample. The chromatogram is shown as Fig. 4.

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Alkaloids of Tobacco Smoke. II. Identification of Some of the Alkaloids in Burley Cigarette Smoke[1]

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By combinations of gas and paper chromatography and ultraviolet spectroscopy, myosmine, nornicotine, anabasine, anatabine, 2,3′-dipyridyl, and cotinine have been conclusively identified in Burley tobacco cigarette smoke. Rough determinations were made for the quantities of these substances and of nicotine in the smoke.

In the preceding paper,[2] a gas chromatographic method for the separation of an alkaloid extract obtained from Burley tobacco cigarette smoke was described. A number of the compounds producing the observed chromatographic peaks have been identified, some for the first time in cigarette smoke. Approximate figures for the amounts of the identified alkaloids were also obtained by the gas chromatographic method, providing the first published data on the secondary alkaloid content of tobacco smoke.

The gas chromatographic technique of separation was advantageous in that it easily provided crude samples of the individual alkaloids from a mixture by simply condensing them from the gas stream as they were eluted. These samples can then be purified by re-chromatography to remove impurities eluted in close proximity and collected simultaneously. Only when compounds are eluted at essentially the same time does this procedure fail to provide a pure specimen. This procedure avoided the time consuming and tedious operations of fractional crystallization, extraction, or distillation employed in the past for alkaloid separations. The specimens obtained generally contained a trace of the material used as the stationary liquid phase in the column, but this did not interfere with the subsequent work. This impurity could probably be removed if necessary.

The retention time of each gas chromatographic fraction from the alkaloid extract of cigarette smoke was observed on columns of polypropylene glycol (mol. wt. 1025) and of polyethylene glycol (mol. wt. 20,000). These were compared with the retention times of known alkaloids obtained under identical conditions. Checks on both columns between a known and a compound of smoke origin provided a tentative identification. The different retentive ability of the two columns for the various alkaloids[3] made this a reliable cross-checking procedure. In one case, this technique failed to provide distinction between two compounds; myosmine and nornicotine were eluted more or less together on

(1) Supported by a grant from the Damon Runyon Memorial Fund. Presented at the Twelfth Tobacco Chemists' Research Conference, October 23, 1958, Durham, N. C.

(2) L. D. Quin, *J. Org. Chem.*, **24**, 911 (1959).

(3) See Table I, ref. 2.

both columns, and a smoke alkaloid fraction having the same retention time could not be identified as one or the other of these two alkaloids. Paper chromatography, however, later proved the presence of both alkaloids in this fraction, In another case, a fraction thought to be homogeneous and indicated to be anabasine on the polypropylene glycol column actually was found to be a mixture when it gave two peaks on the polyethylene glycol column. Paper chromatography also indicated the presence of anabasine and another alkaloid in this fraction.

For final identification, the purified smoke alkaloid fraction was generally chromatographed on paper together with the known(s) suggested by gas chromatography. If the R_f values checked in the two solvent systems used, it was considered that conclusive identification of the smoke alkaloid was obtained. In two cases, ultraviolet absorption spectra were recorded for the alkaloids to assist in the identification work.

By the above techniques, it was shown that myosmine, nornicotine, anabasine, anatabine, 2,3′-dipyridyl, and cotinine are present in Burley tobacco cigarette smoke. The experimental data are summarized in Table I. The first two of these compounds constitute fraction 10 and the others, fractions 11, 12, 13, and 16, respectively, appearing on the gas chromatograms reproduced in the preceding paper.[2] Among these compounds, cotinine has never been reported as a constituent of tobacco smoke, although it has recently been found in tobacco.[4] Nornicotine, anabasine, 2,3′-dipyridyl, and anatabine have not been identified previously in cigarette smoke, but have been detected recently in cigar smoke[5] and are well known alkaloids of tobacco itself. Myosmine has been known for some time to be a constituent of cigar smoke;[6] it has recently been detected in the smoke of high nornicotine-content cigarettes.[7]

The other chromatographic peaks have not yet been associated with specific compounds. None correspond with some of the available knowns studied, namely, nicotyrine, metanicotine, *N*-methyl nicotinamide, and nornicotyrine. There is preliminary evidence that pyridyl alkyl ketones may be present; however, adequate proof of these has not yet been obtained.

The failure to detect nicotyrine in the smoke extract is interesting, since this compound has been reported to be a constituent of tobacco smoke.[5,8] It was considered possible that it may have been present in the smoke but was not recovered by the isolation procedure. That this may be true was found by the failure to detect nicotyrine in a synthetic mixture of alkaloids including this substance which had been subjected to the isolation procedure used in obtaining the smoke sample. Metanicotine also failed to survive the isolation procedure. The conclusion, therefore, cannot be drawn that a substance is absent from smoke unless it is known that the substance is recoverable under the experimental conditions.

The amount of cotinine in the smoke extract was determined by comparing the area of its gas chromatographic peak with the area from a sample of known, and approximately the same, concentration. A value of 57 micrograms of cotinine per cigarette smoked was thus obtained. A nicotine analysis performed similarly gave a value of 5.08 mg. per cigarette. The validity of this figure was demonstrated when a closely checking value (5.18 mg. per cigarette) was obtained for the extract by the established method for nicotine analysis of Cundiff and Markunas.[9]

Insufficient quantities of the pure individuals prevented a similar direct determination of the other identified alkaloids. However, a rough determination was made by relating the peak areas due to these compounds on a single chromatogram to the area obtained from chromatographing a known amount of myosmine under the same conditions. This procedure does not make allowance for any differences in area-weight relationships among the alkaloids, and the values must be considered as approximate for the present. The following values for micrograms per cigarette smoked were obtained: myosmine–nornicotine, 88; anabasine, 11;[10] anatabine, 14; 2,3′-dipyridyl, 7. It is hoped these values will be refined in future work, and that individual figures for myosmine, nornicotine, and anabasine can be obtained.

EXPERIMENTAL

Isolation and purification of smoke alkaloids. As described previously,[2] desired fractions were collected from the gas chromatographic separation of the alkaloid extract of Burley tobacco cigarette smoke and purified by re-chromatography. Generally, three 50 μl. aliquots, representing about 15 cigarettes smoked, were chromatographed to obtain sufficient material for further study. The final products of the isolation procedure were 50–100 μl. of benzene solutions of the alkaloids of interest.

Identification by comparative gas chromatography. Ten to 20 μl. aliquots of the above solutions were chromatographed on each of 1 m. by 6 mm. columns containing polypropylene glycol, mol. wt. 1025 and polypropylene glycol, mol. wt. 20,000, on alkali-washed Firebrick in the weight ratio of 1 to 4. Experimental conditions varied from one identification run to another, but the temperature was generally about 190° and helium flow about 40–55 ml. per min. Selected

(4) W. G. Frankenburg and A. A. Vaitekunas, *J. Am. Chem. Soc.*, **79**, 149 (1957).

(5) F. Kuffner, K. Schick, and H. Bühn, *Monatsh.*, **87**, 749 (1956).

(6) A. Wenusch and R. Schöller, *Fachl. Mitt. österr. Tabak-Regie*, **2**, 2 (1933).

(7) J. M. Moseley and C. H. Rayburn, Abstracts of Papers, Eleventh Tobacco Chemists' Research Conference, October 10–11, 1957, New Haven, Conn.

(8) A. Wenusch, *Der Tabakrauch*, A. Geist, Bremen, 1939.

(9) R. H. Cundiff and P. C. Markunas, *Anal. Chem.*, **27**, 1650 (1955).

(10) Including another alkaloid, unidentified, which is present in this fraction.

TABLE I

IDENTIFICATION OF SMOKE ALKALOIDS

	Retention Time, Min.		R_f Values		λ_{max}, mμ	
	I[a]	II[b]	I[c]	II[d]	First	Second
Fraction 10	13.5	13.9	18, 88	33, 61		
Nornicotine	13.3	13.2	18	33		
Myosmine	13.5	14.0	87	60		
Fraction 11	17.4	11.8, 13.6	18, 97	42, 90		
Anabasine	17.4	13.6	17	43		
Fraction 12	23.2	20.9	27	35		
Anatabine	23.0	20.8	27	35		
Fraction 13	20.0	23.2	[e]	[e]	274	238
2,3′-Dipyridyl	20.1	23.0	[e]	[e]	274[f]	238[f]
Fraction 16	49	52	88	70	260	
Cotinine	49	51	88	72	260	

[a] On polypropylene glycol. [b] On polyethylene glycol. [c] 1-Butanol-pyridine-water system. [d] 1-Butanol-benzene-acetate buffer system. [e] At solvent front. [f] See also R. L. Frank and J. V. Crawford, *Bull. soc. chim. France*, 1958, 419.

known alkaloids were run individually at the same time as smoke fraction. The retention times of the smoke alkaloids and of the knowns indicated to be identical with these appear in Table I.

Identification by paper chromatography. Two solvent systems of Kuffner, Schick, and Bühn[5] were used: 1-butanol-pyridine-water, 3 to 1 to 3, v./v., and 1 butanol-benzene-acetate buffer,[11] 85 to 5 to 30, v./v. In each case the lower layer was placed in the bottom of a chromatographic chamber lined with paper, and the upper was retained for placement in troughs for descending chromatography. Whatman's No. 1 paper was sprayed lightly with 0.2*M* ammonium tartrate and with 0.2*M* ammonium chloride for use in the two systems, respectively. A 10–20 μl. aliquot of the benzene solution of the smoke alkaloid and of a known alkaloid thought to be identical were spotted on the paper and left 4 hr. in the chamber for equilibration. The solvent was then placed in the trough and the chromatograms developed overnight. After air-drying, the strips were sprayed with a 0.5% solution of benzidine in absolute ethanol and exposed to cyanogen bromide vapor. Spots were generally round with little streaking, except in the case of myosmine and cotinine in the second-named system. R_f values are recorded in Table I.

Identification by ultraviolet spectroscopy. Two purified smoke alkaloids (cotinine and 2,3′-dipyridyl) were dissolved in absolute ethanol and ultraviolet spectral measurements with a Warren Spectracord made. Results appear in Table I, along with data for the known compounds obtained similarly.

Quantitative analysis by gas chromatography. A. Cotinine. Conditions: 1 m. by 6 mm. column of polypropylene glycol, mol. wt. 1025, on Firebrick, 1: 4; 190°; helium flow, 55 ml. per min.; sensitivity, 1; recorder range, 1 mv. Samples: 50 μl. of smoke alkaloid extract of 1.70 ml. (from 150 cigarettes) and 20 μl. of a benzene solution containing 8.9 mg. cotinine per ml. Peak areas were measured with a planimeter. The smoke cotinine area was 1.4 times the known's area; the smoke extract thus contained 5.0 mg. cotinine per ml.

B. Nicotine. Conditions: 1 m. × 10 mm. column of polypropylene glycol, mol. wt. 1025, on Firebrick 1:4; 190°; helium flow, 75 ml. per min.; sensitivity, 1/32; recorder range, 1 mv. Samples: 20 μl. of the same smoke extract as above and 20 μl. of a benzene solution containing 0.397 g. of nicotine per ml. The smoke nicotine peak was 1.13 times the known's area; the former thus contained 0.449 g. per ml.

C. Other identified alkaloids. Twenty μl. of the same smoke sample was chromatographed as in the nicotine analysis, except at a sensitivity of 1. Twenty μl. of a benzene solution containing 10 mg. myosmine per ml. was also run. The myosmine-nornicotine peak of the former was 0.78 times the myosmine peak of the latter; the smoke extract thus contained 7.8 mg. of myosmine-nornicotine per ml. From chromatographing a 50 μl. sample of the smoke extract, the following peak area relationships were established: myosmine-nornicotine, 1; anabasine, 0.13[10]; anatabine, 0.16; 2,3′-dipyridyl, 0.075. The content of the latter three compounds is therefore 1.0, 1.2, and 0.59 mg. per ml., respectively.

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(11) Prepared from 0.2 M acetic acid and 0.2 M sodium acetate, 1 to 10 v./v.