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COMPOSITION STUDIES ON TOBACCO—XXV.

MOIETIES IN A HIGH MOLECULAR WEIGHT SMOKE PIGMENT: ALKALOIDS AND A SILICONE

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Abstract—A high molecular weight pigment from cigarette smoke was fractionated by precipitation, solvent extraction and dialysis. The major fraction was a nondialyzable, weakly acidic substance of molecular weight ≥ 100,000. This fraction contained the following: iron; a silicone; hydrolyzable amino acids and quinic acid in small amounts; and several alkaloids including nicotine, nornicotine, metanicotine and cotinine. In addition to these alkaloids evidence was obtained for the presence of several other bases including pyridine derivatives which are known to be pyrolytic products of nicotine or are previously identified cigarette smoke constituents. This is the first report of nicotine and related alkaloids occurring in tobacco leaf or smoke as moieties in high molecular weight substances.

INTRODUCTION

THE presence of a high molecular weight brown polymeric pigment in cigarette smoke was reported recently.[1,2] Although some superficial similarities between this pigment and known tobacco leaf pigments[3–6] were observed, many significant differences were noted.[1] Both leaf and smoke pigments contain iron, chlorogenic acid and amino acids, but the smoke constituent gives much lower yields of caffeic, quinic and amino acids on acidic and alkaline hydrolyses. Less than one-sixth of the total chlorogenic acid in the smoke pigment can be hydrolyzed under such conditions, indicating the presence of resistant linkages which are apparently absent or greatly reduced in number in the leaf pigment. Rutin is present in the higher molecular weight fractions of the leaf pigment but absent in the smoke pigment. The nitrogen content is higher and the iron content is lower in the smoke pigment. The major component of the smoke pigment has an approximate molecular weight of ≥ 100,000, but the comparable fraction of the leaf pigment is 16,000–30,000.

The presence of this pigment in smoke is of interest both academically and pragmatically. The occurrence of brown acidic substances in tobacco leaf was observed one hundred years ago,[7] and subfractions of such substances were given names[8,9] such as "Tabakensäure (α, β, γ)", "Kentuckinsäure", "Kentuckylinsäure", "Resin acids", etc. Other substances

[1] R. L. STEDMAN, W. J. CHAMBERLAIN and R. L. MILLER, *Chem. Ind.* 1560 (1966).
[2] W. J. CHAMBERLAIN and R. L. STEDMAN, *Tobacco Sci.* **10**, 162 (1966).
[3] H. E. WRIGHT, JR., W. W. BURTON and R. C. BERRY, JR., *Arch. Biochem. Biophys.* **86**, 94 (1960).
[4] H. E. WRIGHT, JR., W. W. BURTON and R. C. BERRY, JR., *Phytochem.* **3**, 525 (1964).
[5] J. S. JACOBSON, *Arch. Biochem. Biophys.* **93**, 580 (1961).
[6] O. T. CHORTYK, W. S. SCHLOTZHAUER and R. L. STEDMAN, *Beitr. Tabakforsch.* **3**, 422 (1966).
[7] A. HAID's work cited in references 8 and 9.
[8] J. VON DEGRAZIA, *Fachliche Mitt. Österr. Tabakregie* **3**, 109 (1913); from H. BRÜCKNER, *Die Biochemie des Tabaks*, p. 226. Paul Parey Verlag, Berlin (1936).
[9] A. WENUSCH, *Z. Lebensm. Untersuch. Forsch.* **69**, 81 (1935).

with some similar properties were also reported in cigarette smoke.[9] As far as we are aware, no reports of the isolation or structure of these historically interesting "brown acids" have appeared in modern literature. From the earlier descriptions, it is apparent that the solubilities, physical appearance and chemical analyses (high C:H ratios) of some of these substances are quite similar to the recently isolated leaf and smoke pigments.

Of pragmatic interest are several reports describing the presence in cigarette smoke of high molecular weight brown, polymeric substances which have cocarcinogenic activity.[10–12] Such substances occur in the basic, acidic and neutral fractions of smoke, and it was postulated that they may arise through copyrolysis of carbonyls and heterocyclic nitrogen compounds. However, no structural information was presented. Again, some similarity is apparent among these substances, the historically interesting components and the brown pigment described in the present report.

The characterization of high molecular weight natural products is difficult since, in most cases, isolation of a "pure compound" cannot be assured. However, much valid information can be gained by such studies, although complete structural elucidation may not be accomplished, e.g. recent studies on animal and plant melanins.[13] Within these limitations, further information on the structure of the smoke pigment has now been obtained.

RESULTS AND DISCUSSION

By partitioning smoke condensate from U.S. commercial cigarettes between ether and aqueous alkali, the pigment was obtained in the latter layer in yields of 8–12 per cent of the weight of the condensate (Fig. 1). Adjustment of the pH of the alkaline extract to 6·1 gave a weakly acidic precipitate (*A*) in yields of about 7–11 per cent of the original condensate. By lowering the pH of the filtrate from this precipitation to pH 1·0, a strongly acidic precipitate (*B*) was obtained in yields $<1{\cdot}0$ per cent of the original condensate. Purification of *A* and *B* by reprecipitation from dilute alkaline solution gave dark brown, amorphous solids in both cases.

Effort was concentrated on the further fractionation of the purified, weakly acidic precipitate. Continuous ether extraction removed about 30 per cent of purified *A*. The ether-solubles contained some material giving a positive Liebermann–Burchard test and other properties suggestive of steroidal glycosides known to be in smoke.[14] The ether-insoluble substance (*C*) was dialyzed against pH 10·0 phosphate buffer. The dialysis removed about one-half of *C* and the nondialyzable fraction was a black, shiny, amorphous material resembling coal.

The i.r. spectrum of this nondialyzable, weakly acidic fraction (NWA) was nondescript with broad —OH or —NH— absorption at 2·7–3·3 μ and a wide band at 5·6–6·5 μ indicative of —CO— and —NH— groups; this spectrum was generally similar to those of the unfractionated smoke pigment and of the leaf pigment. The fraction revealed no pertinent structural details by NMR spectrometry and melted with decomposition at about 300°. Elemental analyses, molecular weight approximations and other determinations are given in Table 1 for NWA and other fractions of the smoke pigment. Molecular weights were approximated

[10] S. NEUKOMM, J. BONNET and M. DE TREY, *Bull. Soc. Vaudoise Sci. Nat.* **67**, 433 (1961).
[11] J. BONNET and S. NEUKOMM, *Helv. Chim. Acta* **39**, 1724 (1956).
[12] J. BONNET and S. NEUKOMM, *Acta Union Intern. Contra Cancrum* **15**, 561 (1959).
[13] M. PIATTELLI, E. FATTORUSSO, R. A. NICOLAUS and S. MAGNO, *Tetrahedron* **21**, 3229 (1965).
[14] A. G. KALLIANOS, F. A. SHELBURNE, R. E. MEANS, R. K. STEVENS, R. E. LAX and J. D. MOLD, *Biochem. J.* **87**, 596 (1963).

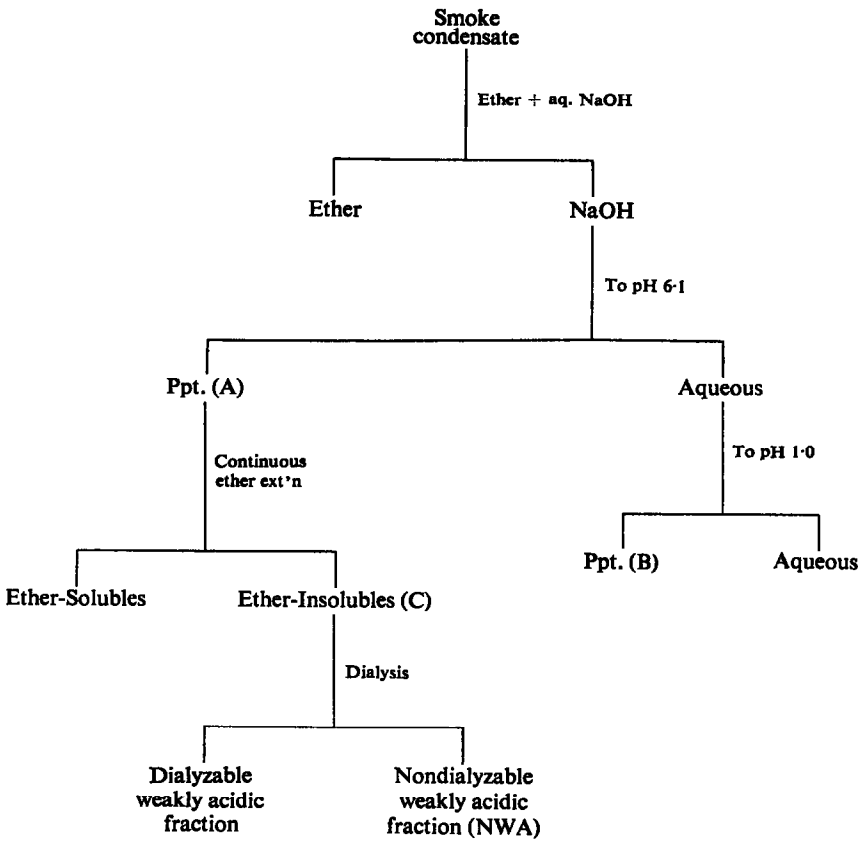


FIG. 1. ISOLATION AND FRACTIONATION OF CIGARETTE SMOKE PIGMENT.

TABLE 1. APPROXIMATE MOLECULAR WEIGHTS AND OTHER ANALYTICAL DATA ON VARIOUS FRACTIONS OF THE PIGMENT

	Weakly acidic pigment		Strongly acidic pigment
	Dialyzable	Nondialyzable (NWA)	
Mol. wts.			
Major	5,000–10,000	⩾100,000	20,000–50,000
Minor	⩽1,000	None	⩾100,000
Analyses (%)			
Carbon	—	66·39	61·07
Hydrogen	—	6·94	5·96
Nitrogen	—	3·44	5·36
Sulfur	—	1·00	0
Ash	—	0·32	—
Silicon	—	0·11	—
Iron	—	0·16	—
Equiv. wt.	—	330	—

by gel filtration on polyacrylamide of different molecular exclusion ranges. In most cases, only bands giving values for (Elution volume/Void volume) of 1·0 were used in order to eliminate error due to possible adsorptive effects. Apparently, the nondialyzable, weakly acidic fraction (NWA) contributes the major band (mol. wt. $\geqslant$ 100,000) and the main component of the dialyzable weakly acidic fraction contributes the low molecular weight minor band observed in earlier studies on the undialyzed pigment.[1] The iron content of NWA represented a much higher percentage (about 50 per cent) of the ash compared[1] to the unfractionated pigment (about 10 per cent). The ash of NWA also contained a significant amount of silicon.

Acidic (HCl) hydrolysis of NWA under conditions similar to those used for the leaf pigments yielded trace amounts of quinic and small quantities of amino acids. The bulk of NWA appeared to be resistant to such hydrolysis as indicated by the large amount of visually unchanged pigment remaining, and later work (*vide infra*) confirmed this observation. Twenty-three components which reacted with ninhydrin were separated, including the eighteen amino acids previously described.[1] The major amino acids were glycine, leucine, alanine and valine; cystine was absent and the level of methionine corresponded to $<0{\cdot}1$ per cent of the sulfur content of NWA. Quinic acid was found by the thiobarbituric acid test in a barely detectable amount. No attempt was made to find caffeic acid by alkaline hydrolysis since considerable difficulty was experienced in detecting this acid even in hydrolysates of leaf pigment, which contains larger amounts of hydrolyzable chlorogenic acid, judging by the quinic acid yields. These data indicated that hydrolyzable chlorogenic acid (or, more exactly, quinic acid) and amino acids comprise only a minor part of the NWA molecule. Since only a small amount of molecular fragmentation of NWA occurred under these hydrolytic conditions, it was necessary to employ more drastic procedures.

By high temperature and prolonged alkaline fusion of NWA, more extensive rupturing was obtained. Fusion with KOH was conducted at 230–240° for 30 min and the products were extracted into ether. Passage of dry HCl into the ether yielded hydrochlorides which were hydrolyzed and the free bases readily separated by gas chromatography. Under these conditions, nicotine, nornicotine, cotinine and 3-ethylpyridine were identified as the sole products by cochromatography using conventional thin-layer and gas chromatographic procedures. The yield (about 0.3 per cent of NWA) of these bases in the fusion was much higher than the yields of quinic acid in the hydrolyses. Fusion at 260–270° for 45 min with concurrent distillation and workup of products as above gave more extensive fragmentation. Table 2 lists seventeen bases for which evidence of identity was obtained. Nicotine, nornicotine, metanicotine and cotinine were identified by the above criteria, but evidence for the lower boiling components was limited to cochromatography on Carbowax 20 M. The occurrence of nicotine or other bases as moieties in high molecular weight substances in either tobacco leaf or smoke has not been previously reported. Since many of the lower boiling compounds in Table 2 are pyrolytic products of nicotine and well-known smoke constituents, it is not known whether these are derived from nicotine (or a related alkaloidal moiety) in NWA or are linked independently in the pigment. The probable occurrence of 3,5-; 2-; and 2,6-substituted pyridine linkages is of considerable interest and, in the case of the 2- and 2,6-compounds, would indicate that these pyridine derivatives are not derived from nicotine or a related alkaloid in the pigment. The presence of 3-vinylpyridine does not obviate the possibility that all products are derived from the 3-pyridyl alkaloids since oxidation and reduction may occur concurrently during alkaline fusion.[15] The presence of pipiderine may

[15] M. PIATELLI, E. FATTORUSSO, S. MAGNO and R. A. NICOLAUS, *Tetrahedron* **19**, 2061 (1963).

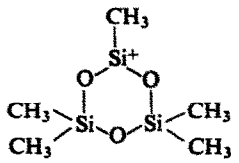
show the occurrence of anabasine in the pigment, but the latter has not been isolated from the fusion products.

TABLE 2. BASES IN KOH FUSION PRODUCTS OF NONDIALYZABLE, WEAKLY ACIDIC FRACTION OF THE PIGMENT

	Relative amounts*		Relative amounts*
N-Methylpyrrolidine	4	3,5-Lutidine	4
Piperidine	2	3-Vinylpyridine	2
Pyrrolidine	3	3-Ethylpyridine	1
Pyrroline	1	Pyrrole	2
N-Methylpyrrole	3	Cotinine	3
Pyridine	3	Metanicotine	1
2-Picoline	2	Nicotine	3
2,6-Lutidine	1	Nornicotine	1
3-Picoline	3		

* 4 = largest amount. See text for limitations on identities.

After removal of the hydrochlorides from the ether extracts of the fusion products obtained at 250–260°, evaporation of the ether extract yielded a residue, a small part of which was insoluble in methanol. The methanol-insoluble material was a yellowish solid which gave a distinctive i.r. spectrum identical with that of a silicone previously isolated from cigar smoke condensate in this laboratory.[16] In this earlier work, it was concluded that the silicone was a polymeric methylsiloxane, probably in the range of $-[(CH_3)_2SiO]-_{10-50}$. In the present work, the silicone is actually linked in the pigment and does not accompany the pigment as a contaminant since it is ether-soluble and should have been removed in the extraction prior to dialysis. If such was not the case, subsequent obstacles in the isolation (solubility in pH 10·0 buffer and dialysis) make the possibility of contamination very remote. The mass spectrum of the silicone from NWA showed a major peak at 207, which corresponds to the following ion known to result from the fragmentation of certain silicones[17]:



Such an ion would be expected in the fragmentation of the cyclic structure postulated previously for the silicone from cigar smoke. At lower fusion temperatures, e.g. 190°, the silicone was obtained in higher yield (about 5 mg/2·0 g pigment). Assuming the above methylsiloxane structure, this yield would account for essentially all of the silicon found in the elemental analysis.

The isolation of a silicone from a natural product always raises the question of possible contamination from an extraneous source. In the work reported here, no stopcock grease or other sources of silicones were used at any point in the study. Several reports of the occurrence

[16] A. I. SCHEPARTZ, *Tobacco Sci.* 4, 12 (1960).
[17] K. BIEMANN, *Mass Spectrometry, Organic Chemical Applications*, p. 171. McGraw-Hill, New York (1962).

of organic silicon compounds in natural products have appeared and these findings were reviewed recently.[18] In most cases, these reports concern the isolation of silicon complexed with polysaccharides, including cellulose and pectin which are present in tobacco leaf and may contribute significantly to certain products in tobacco smoke.[19,20]

In spite of the drastic conditions of fusion, a large amount of NWA could be recovered visually unchanged after the reaction by precipitation in the usual manner. Gel filtration of such precipitates gave major bands in the 30,000–100,000 molecular weight range and a minor component of $\leqslant 1000$, confirming that a very high degree of resistance to bond breaking exists in the pigment.

As previously noted,[1] the occurrence of this high molecular weight pigment in cigarette smoke may be explained by a mechanism other than high temperature distillation, sublima- the cigarette coal, leaf cells in the proximity of the coal might erupt and expel their cellular tion and related processes based on volatility. As a result of the sharp thermal gradient behind contents, including the leaf pigment, into the smoke stream. Expelled cellular particles could then serve as nuclei for further aerosol formation or be adsorbed on other particles. Such transitions might be accompanied by molecular degradations and/or polymerizations with low molecular weight smoke constituents, such as the picolines and lutidines. If the presence of alkaloids in the leaf pigment could be established, the validity of this mechanism might be fortified. Recently, evidence pertinent to this point has been obtained by other workers[21] in this laboratory. In preliminary experiments nicotine, nornicotine, 3-ethylpyridine and probably other substituted pyridines have been isolated from the pyrolytic products from Turkish tobacco leaf pigment.

The presence of nicotine in the smoke pigment raises a further question of the possible physiological activity of the substance. At present, biological tests to determine this are being initiated.

EXPERIMENTAL

Isolation

Commerical U.S. cigarettes were smoked on an automatic smoking machine under standard conditions and the smoke condensate was partitioned between ether and aqueous 1 N NaOH as previously described.[22] The alkaline extract was adjusted to pH 6·1 with concentrated aqueous HCl, resulting in a black tarry precipitate (*A*) which was filtered off. The filtrate was adjusted to pH 1·0, which yielded another precipitate (*B*).

After reprecipitation from aqueous alkali, *A* was extracted continuously with ether for 48 hr and the ether-insoluble material was dialyzed against phosphate buffer (pH 10·0) for 6 days with daily changes of buffer (tot. vol. 216 l./48 g). The nondialyzable and dialyzable fractions were precipitated independently at pH 3·0.

Analyses

For gel filtration, pigment fractions were dissolved in water at pH 12·0 and the pH adjusted slowly to 10·0 with aqueous HCl. Phosphate buffer (pH 10·0) was added to give a final phosphate concentration of 0·01 M. Polyacrylamide (25 g) was swelled in 0·01 M buffer and solutions containing 10–20 mg of the fractions were separated on columns prepared in the usual manner, using buffer as the eluting solvent. Void volumes were obtained using "Blue Dextran 2000" (Pharmacia).*

* Mention of a specific commercial product does not constitute an endorsement by the U.S. Department of Agriculture over similar items not mentioned.

[18] W. HEINEN, In *Moderne Methoden der Pflanzenanalyse* (Edited by H. F. LINSKENS and M. V. TRACEY), Vol. 6, p. 4. Springer Verlag, Berlin (1963).

[19] E. W. ROBB, W. R. JOHNSON, J. J. WESTBROOK and R. B. SELIGMAN, *Bull. Inform. CORESTA* No. 3, 81 (1966).

[20] W. S. SCHLOTZHAUER, I. SCHMELTZ and L. C. DONIO, *Abstr. 20th Tobacco Chemists Res. Conf.*, Winston-Salem, N.C., 1966.

[21] I. SCHMELTZ and W. S. SCHLOTZHAUER, Personal communication.

[22] I. SCHMELTZ, C. J. DOOLEY, R. L. STEDMAN and W. J. CHAMBERLAIN, *Phytochem.* **6**, 33 (1967).

Iron and silicon were determined by the α,α'-bipyridyl[23] and ammonium molybdate[24] methods, respectively.

Hydrolyses

For amino acids, a sample (400 mg) of pigment fraction was refluxed for 20 hr in 200 ml aqueous 6 N HCl. After filtration, the filtrate was evaporated to dryness and the residue was dissolved in water (4·0 ml). The pH of the solution was adjusted (aqueous HCl) to 2·0 and analyzed on an automatic amino acid analyzer. Separation of amino acids from hydrolysis mixtures were also performed using the paper chromatographic methods of Wright *et al.*[3,4]

For quinic acid, a similar hydrolysis was performed, and the residue from the filtered hydrolysate was diluted to 4·0 ml with water. This solution was analyzed by the thiobarbituric acid method.[6]

Alkaline Fusion

Eight g of pigment fraction were mixed with 80 g KOH, 4 g $K_2S_2O_4$ and 4 ml water in a system permitting collection of distillate. The temperature of the mixture was raised to the desired temperature (230–270°) in a period of 20–30 min and the desired temperature maintained for 30–45 min. The reaction mixture was cooled to about 70° and 400 ml water were added. The solution was extracted continuously with ether (500 ml) for 16 hr. The ether extract was combined with the distillate from the fusion and the combined ether solution (*D*) was dried over Na_2SO_4. For isolation of the bases, *D* was saturated with dry HCl. The precipitated hydrochlorides were separated by decantation and dissolved in water (25 ml). This solution was alkalinized to pH 11·0 at about 5°, and extracted with several portions (25 ml each) of ether. The dried ether extract was concentrated to 2–5 ml in a distillation column at a reflux ratio of 1:1. This ether concentrate was studied by gas and thin-layer chromatography.

For isolation of the silicone, the above ether solution (*D*) was concentrated to a volume of 2–5 ml after removal of the hydrochlorides. Addition of methanol (4–10 ml) gave a precipitate which was filtered and washed with methanol. This washed precipitate was used in the i.r. and mass spectrometric studies.

Chromatography

The bases were separated by gas chromatography on Carbowax 20 M and by two-dimensional thin-layer chromatography using the procedures previously detailed.[25,26] Identities were established by cochromatography with authentic compounds in all instances as reported earlier.

Acknowledgements—The authors thank W. J. Chamberlain, E. D. Talley and J. C. Dooley for technical assistance and E. Glock, American Tobacco Co., for providing a sample of cotinine.

[23] *Official Methods of Analysis*, 10th Ed., p. 192. Association of Official Agricultural Chemists, Wash., D.C. (1965).
[24] *Official Methods of Analysis*, 10th Ed., p. 8. Association of Official Agricultural Chemists, Wash., D.C. (1965).
[25] I. Schmeltz, R. L. Stedman, W. J. Chamberlain and D. Burdick, *J. Sci. Food Agr.* **15**, 774 (1964).
[26] E. Hodgson, E. Smith and F. E. Guthrie, *J. Chromatog.* **20**, 176 (1965).