Qualitative Differences in the Alkaloid Fraction of Cured Tobaccos

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A study was made of nicotine, nornicotine, anabasine, myosmine, 2,3′-bipyridine, oxynicotine, 3-pyridyl methyl ketone, nicotinic acid, and other at present unidentified but probably related compounds in samples of cured leaf of some types of tobacco grown most extensively in the United States. In certain samples in which nornicotine was the principal alkaloid, rather than nicotine, much more myosmine was found than in the predominantly nicotine-containing samples. No evidence was found of any consistent difference in the proportion of these pyridine compounds related to tobacco type as ordinarily recognized in the trade or to the manufactured product for which the tobacco would be likely to be used.

Tobacco is grown commercially in widely scattered areas of the United States and other countries, and in each area under different climatic and soil conditions. Varieties, cultural practices, and harvesting and curing methods have developed in each area which differ from those used in the other areas.

The purposes for which the tobacco leaf produced under these various conditions is used are closely related to the conditions of growth and curing. For example, it is generally accepted in the tobacco trade that a cigarette to be sold in this country should be composed of a majority of tobacco leaf grown in certain areas from Virginia to Georgia on relatively light soils but with well defined fertilizer applications. This tobacco should be picked leaf by leaf and cured by a process known as flue curing. The next most prominent component of the cigarette blend should be grown in an area located generally west of the Appalachian mountains on a heavier soil with very different fertilizer treatment. This burley tobacco should be stalk-cut and air-cured by an entirely different procedure. Small proportions of other domestic and oriental types also go into this blend. Cigar tobacco is grown in other districts located either north or south of the areas where cigarette tobacco is grown and is handled in several different ways related to its use as wrapper, binder, or filler. Pipe tobacco is made principally from grades of burley leaves normally produced at different stalk positions than those used for cigarettes.

Thus, variety, climate, soil, various field practices, leaf position on the plant, and methods of harvesting and curing are among the variables which produce leaf of different chemical composition, which in turn influences the use for which the leaf is deemed acceptable. Manufacturers are unanimous in their agreement as to the main features of this selection of specific tobaccos for specific purposes, though they may differ as to the exact proportions of different tobaccos in their blends. However, the chemical methods now used by most laboratories are inadequate to distinguish, for example, certain samples of burley tobacco which would be used in cigarettes from certain samples of cigar tobacco.

In view of the current interest in possible effects on the consumer of tobacco products, the importance of all chemical constituents of tobacco is now being actively investigated by numerous agencies. As nicotine-type alkaloids are characteristic of all tobaccos, a detailed study of this group of compounds has particular significance at this time.

A previous paper from this laboratory (*8*) described methods of separating more different kinds of alkaloids and related compounds from tobacco samples than had been done heretofore. The present paper reports the use of these methods on a few representative samples of different tobacco types grown in the United States. It seemed possible that qualitative differences in the alkaloidal constituents of these different types might exist which were related to the characteristic farm practices by which they were produced, and to the use to which they were put in manufacture. The estimates given of the quantities of some of the substances separated, especially those of unknown structure, leave much to be desired. However, as it has been demonstrated (*5*) that conventional methods for "nicotine" give values more than ten times the true nicotine content in some tobacco samples, a start should be made toward estimating some of the other substances present. Most samples of present commercial tobaccos contain predominantly nicotine and give correct results by conventional methods, but those methods do not indicate which samples give erroneous results.

Materials and Methods

Samples representative of cigarette grades of flue-cured (U. S. Types 11b and 13), burley (U. S. Type 31), and Maryland (U. S. Type 32) grown in their normal areas under conventional practices have been analyzed to compare these types. In addition, samples of Maryland tobacco of grades corresponding to different heights on the same plants and other samples grown in the presence and absence of tobacco mosaic disease have been analyzed to study the effect of these variables. A sample of fermented or resweated Pennsylvania cigar filler tobacco (U. S. Type 41) provided by W. G. Frankenburg was analyzed, rather than unfermented leaf, as this was more nearly analogous to the condition in which this type is used in manufacture. A more complete description of the samples, including varieties and specific practices and locations where they were grown and cured, together with the detailed results obtained, is available as a mimeographed report (*6*).

The chemical methods were similar to those described previously (*8*), except for the following modifications.

The previous method for extraction of isolated substances from chromatographed paper strips with 0.25*N* hydrochloric acid for spectrophotometric determination is inadequate if the piece of paper contains a large quantity of an alkaloid. Therefore it is necessary to use successive extractions or an elution method such as described by Wyatt (*9*). The same extraction method must be applied to the blank paper cut at the same R_f for spectrophotometric comparison.

The paper, after chromatography and color development, was examined in ultraviolet light as well as in daylight. Also, if spectrophotometric readings were not anticipated and color was to be developed in the whole sheet, the *p*-

aminobenzoic acid (PABA) was added at 0.25% w./v. to the organic phase of the chromatographic solvent. This eliminated the need for spraying the paper and so minimized the production of toxic fumes and resulted in more uniform treatment and greater sensitivity. Thus 0.1 microgram of nicotine, nornicotine, or anabasine could be detected on the paper.

Results

The principal results of the analyses of these samples are shown in Table I. First the quantities of each identified compound found in the sum of all solvent fractions are indicated, followed by a figure for the sum of all isolated but not identified substances. The amount shown as "not isolated" in line C is the difference between total determined by ultraviolet spectrophotometry before chromatography (line D) and the sum of all the identified and unidentified isolated substances. This value probably results from (1) the alkaloidal material which was lost in the course of the extensive manipulation, (2) substances, probably not 3-pyridines, which absorb in the region of 260 mμ but do not react to cyanogen bromide to a visible degree, and so were located in the paper at R_f values at which no color appeared, and (3) the incorrectness of some of the assumptions with respect to the relationship between ultraviolet absorption and quantity. The exact contribution of each source cannot be evaluated at present, but it seems probable that the latter sources of error are more serious than the first one. The above classification of substances present is neither complete nor accurate. However, it is an improvement over the current practice, wherein all the substances that react to a given test are reported as nicotine or part as nicotine and the rest as nornicotine.

Alkaloid Composition

1. No evidence was found of any consistent difference in the proportion of the individual nicotine-type alkaloids related to tobacco type. The number of samples analyzed was not great enough to make this result altogether conclusive, however.

2. The principal alkaloidal constituent of two flue-cured samples—Gold Dollar and Red Free strain of 401—of the burley (Kentucky 16), and of the resweated Pennsylvania cigar tobacco was nicotine.

3. The principal alkaloid of the Cherry Red strain of 401 flue-cured and of the strain of Robinson Medium Broadleaf Maryland tobacco used was nornicotine.

4. Among the Robinson Medium Broadleaf samples of different farm grades, corresponding approximately to different leaf levels or degrees of maturity on plants from the same plot, there was no pronounced difference in proportion of the different identified constituents. The total quantity of "alkaloidal" material differed between the grades in a manner similar to that found by conventional analytical methods.

5. Some differences in alkaloidal composition between plants heavily infected with tobacco mosaic virus and those not so infected appear in these data, but the number of samples is not great enough to attempt an interpretation at the present time. The total amount of alkaloidal material found was less in the infected plants.

6. In samples in which nicotine is the principal alkaloid, anabasine is sometimes the next highest in concentration—more prominent than nornicotine. Though many samples exist in which nornicotine is second to nicotine in concentration, the prevailing opinion that this is always so seems to be based on the extensive use of the method of Bowen and Barthel (*1*). However, these authors stated that other steam-volatile amines besides nornicotine would be included in the value which they designated as nornicotine, but they did not consider this important on the basis of the information then available. Results of tests conducted in this laboratory with pure anabasine and myosmine show that each of these compounds is reported almost quantitatively as nornicotine by the Bowen and Barthel procedure.

7. Myosmine, or compounds with which it may be in equilibrium (*2*, *4*) and so indistinguishable by the methods used, was found in considerable quantity only in those samples containing a large proportion of nornicotine and may have been derived from the latter.

8. Oxynicotine has been found in all samples of cured tobacco analyzed, though the concentration was small. A concentration about ten times the average of these cured tobaccos and so of the same order of magnitude as that reported by Frankenburg and Gottscho (*3*) was found in one sample of resweated Pennsylvania Broadleaf which was analyzed by the present method. The concentration found in predominantly nicotine samples of cured tobacco is not signifi-

Table I. Alkaloids and Related Substances in Samples of Cured Tobacco Leaf

(Based on air-dry weight of sample)

	Flue-Cured, %			Burley Ky. 16, %	Maryland, Robinson Medium Broadleaf, %					Pennsylvania Broadleaf Resweated, %
		401				Dull				
	Gold Dollar	Red Free	Cherry Red		Bright	bright	Dull	Mosaic free	Mosaic infected	
A. Identified substances										
Nicotine	0.843	1.588	0.072	2.238	0.547	0.470	0.402	0.337	0.275	1.143
Nornicotine	0.005	0.028	1.160	0.039	1.853	2.445	2.478	0.554	0.569	0.013
Anabasine	0.020	0.060	0.128	0.042	0.108	0.267	0.111	0.037	0.059	0.014
Myosmine	0.000	0.000	0.020	0.007	0.096	0.140	0.139	0.132	0.030	0.018
2,3′-Bipyridine	0.000	0.001	0.001	0.002	0.003	0.006	trace	0.001	0.002	0.007
Oxynicotine	0.002	0.016	0.006	0.035	0.009	0.051	0.014	trace	0.003	0.141
3-Pyridyl methyl ketone	0.000	0.000	0.001	0.000	0.000	0.003	0.000	0.001	0.001	0.001
Nicotinic acid	0.005	0.011	0.026	0.045	0.024	0.080	0.021	0.074	0.018	0.074
Total	0.875	1.704	1.414	2.408	2.640	3.462	3.165	1.136	0.957	1.411
B. Isolated but not identified[a]	0.214	0.183	0.442	0.162	0.316	0.390	0.258	0.333	0.283	0.275
C. Not isolated[b]	0.098	0.631	0.553	0.258	0.373	0.447	0.664	0.466	0.203	0.191
D. Total[c]	1.187	2.518	2.409	2.828	3.329	4.299	4.087	1.935	1.443	1.877
Estimated number of substances isolated showing color with CNBr and PABA	16	23	27	24	22	24	23	18	24	26

[a] Sum of estimated quantities of substances giving spots colored after treatment with CNBr and PABA but distinguishable from all available model compounds.

[b] Difference between D and A + B.

[c] Sum of estimated quantities of substances showing ultraviolet absorption similar to that of pyridines found in all solvent fractions before chromatography.

cantly different from that in predominantly nornicotine samples.

9. Nicotinic acid was found in all samples. The quantity found in the better grades of flue-cured tobacco seems to be much lower than in air-cured samples, which is in line with the much shorter exposure of these samples to conditions under which oxidative enzymes would be expected to be active.

10. 3-Pyridyl methyl ketone, called 3-acetyl pyridine in the previous paper (*8*), was detected in half the samples reported here but only at the lower limit of the sensitivity of the methods used. In the previous paper the R_f of this compound was reported as 0.61. Later the principal color development was found at R_f 0.88, though some color remained at 0.61. The characteristic ultraviolet absorption spectrum is now found at 0.88 and not at 0.61. New solutions of the same sample of alkaloid and another independently synthesized sample obtained from P. C. Teague (*7*) also gave the principal color and the ultraviolet absorption at R_f 0.88.

11. Though the estimated number of substances isolated which show color with cyanogen bromide and *p*-aminobenzoic acid, as given at the bottom of Table I, is large in the case of all samples, most of these substances are present in very small amounts. The numbers given are based on best judgment with respect to the identity or nonidentity of substances derived from different solvent extracts of the tobacco samples. Judgment is based on similarity of the R_f value, the characteristics of the ultraviolet absorption curve, and the colors of the spots, both in visible and ultraviolet light, after treatment with color reagents. As all these criteria and especially the absorption curve can be influenced by admixture with other substances, the number of substances present which react with cyanogen bromide and *p*-aminobenzoic acid cannot be stated categorically. Additional tables showing solvent fractions from which these substances were obtained, R_f values, ultraviolet absorption curve characteristics, and calculated quantities in each sample are contained in the mimeographed report (*6*). A careful study of these tables shows that certain of these unidentified substances appear to be identical as obtained from a number of different tobacco samples.

12. It is evident from the method of calculation that the various errors involved, especially in the assumptions used, would tend to pile up in the "Not Isolated" position of Table I. Eight to 25% of the total "alkaloidal" material falls in this class in different samples, but there is no obvious relationship between the size of this quantity and the character of the sample. Much of this material may not be 3-pyridines.

13. More detailed data and results are available as a mimeographed report (*6*).

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GRAIN STORAGE

Effects on Corn of Storage in Airtight Bins

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ONE OF THE PROBLEMS associated with modern mechanized methods of grain harvesting is the increased amount of grain harvested at moisture contents above the safe storage level. Mechanical drying methods suitable for removing the excess moisture have been widely explored and considerable practical development work has been accomplished. As an alternative to drying, hermetic or sealed storage bins have been suggested for holding grain of high moisture, principally feed grains, on the farm. Spoilage of high moisture grain is primarily due to microorganisms. Exclusion of oxygen from the storage promises to reduce this cause of grain spoilage.

The principal development of hermetic storage for damp grain has been in France. Vayssière (*7*), reporting to the Food and Agriculture Organization of the United Nations in 1947, described a hermetic storage as one where the product is protected from any exchange of gases or liquids from the outside environment. In the storage of high moisture grain, mold and overheating were forestalled without preventing development of acidity caused by anaerobic fermentation. Other advantages claimed by French investigators were the suffocation of insects and other pests present at the time of storage and the positive prevention of the movement of insects and moisture into the storage.

Oxley (*6*), British grain storage investigator, states that the principle on which airtight storage is based is the inhibition of aerobic organisms (which include the majority of fungi) by the depletion of oxygen and production of carbon dioxide in the interseed spaces. Oxley also reports that according to British observation of work in France by Blanc and others, airtight storage was satisfactory for preserving the condition of wheat at 16.3% moisture for 18 months. Other lots stored at 18.9% appeared satisfactory, but these observations were not conclusive.

Underground storages for grain have been used in some countries for many centuries. The exchange of air in underground storages may be sufficiently limited to approach hermetic conditions. Gattoni (*5*) reported that large underground "silos" used for storing dry grain in South America were effective in controlling insects and preserving the quality of the grain.

Bottomley, Christensen, and Geddes (*3, 4*) investigated the influences of oxygen concentration on mold growth and biochemical changes in stored yellow corn and concluded that nearly anaerobic conditions greatly reduce but do not entirely prevent mold development, and thus spoilage of high moisture