Brown Pigment in Tobacco Leaf During Air-Curing

Roger A. Andersen,* Thomas H. Vaughn, and Richie H. Lowe

Brown pigment isolated by gel filtration was estimated quantitatively in field-grown tobacco during air-curing. Leaves of a high-phenolic variety had about two times the estimated amount of pigment as leaves of a low-phenolic variety. There was about a one-fold increase in the amount of pigment from start to finish of the curing cycles in leaves from both of these varieties. Carbohydrate material was present in the pigment, but this detectable carbohydrate was removed by high voltage electrophoresis at pH 5.2. Electrophoretically purified pigment was ninhydrin negative prior to hydrolysis, but 19 amino acids were present in hydrolysates. Quantitative amino acid analyses of hydrolysates showed proline was the principal amino acid, while aspartic acid, gluatmic acid, glycine, alanine, valine, serine, leucine, and threonine followed in order of descending amounts.

Soluble nondialyzable, polymeric brown pigments have been extracted from tobacco leaf at a single stage of cure or autolysis, and subsequently purified by Chortyk (1967), Chortyk *et al.* (1966), Jacobson (1961), and Wright *et al.* (1960). Wright *et al.* (1960) showed that these pigment fractions contained protein, chlorogenic acid, rutin, and iron as part of their compositions, and suggested that the dark-brown coloration of air-cured tobacco leaf resulted in part from these high molecular weight brown pigments. The compositional findings were confirmed (Chortyk, 1967; Chortyk *et al.*, 1966; Jacobson, 1961). Extractions of these pigments were carried out either with water (Jacobson, 1961; Wright *et al.*, 1960) or aqueous alkali at pH 10 (Chortyk, 1967; Chortyk *et al.*, 1966).

The purpose of our investigation was to employ a suitable modification of the available extraction and purification procedures for brown pigments and develop a quantitative estimation of the main pigment fraction. This modified method was used to determine the variation of brown pigment during air-curing of a high-phenolic tobacco variety and a low-phenolic tobacco variety. Some composition studies were undertaken to further clarify the nature of the isolated pigment fractions.

MATERIALS AND METHODS

Plant Growth, Curing, and Sampling Conditions. *N. tabacum*, Ky. Iso 6 F.C. 402 ("F.C. 402"), a variety high in phenols, and Ky. Iso 1 Ky. 16 ("Ky. 16"), a low-phenolic variety, were used (Sheen and Calvert, 1969; Stokes, 1963). Seeds were germinated and the seedlings grown to transplant size on vermiculite contained in 50-ml propylene tubes with a small opening at the bottom. The plants were watered by subirrigation with Hoagland's nutrient solution No. 1 (Hoagland and Arnon, 1950). After 5 weeks the plants, about 10 cm in height, were transplanted to field plots (June 28, 1968). The soil fertilizer application was 157 kg of N, 78.4 kg of P, and 224 kg of K per hectare. The plots consisted of 10–11 rows of each variety of tobacco, with border plants around the periphery. Leaf samples were taken at random on a given day from middle stalk positions of one plant in each of the rows. Border plants were not sampled. Samples were taken every 2 weeks from the time of setting to the final sampling date 11 weeks after transplanting. The tobacco was topped 7 weeks after transplanting, and 4 weeks later it was harvested for curing. Border plants were not cured.

The tobacco was air-cured in a conventional Burley tobacco curing barn. Samples of two or three leaves were taken from each of three plants of both varieties at 2-day intervals during a 6-week period. After all samplings, midribs were removed and the leaf tissue was immediately freeze-dried and stored *in vacuo* (Keller and Kasperbauer, 1969). Samples were packed in dry ice during transfer to the laboratory.

Extraction and Purification of Main Brown Pigment Fraction. One gram of freeze-dried tobacco ground to pass through a 40-mesh screen was extracted with 10 ml of 0.1*M* NH_4OH, pH 10, and 5 ml of *n*-hexane for 20 hr at room temperature with occasional stirring. The mixture was then filtered on a Buchner funnel which contained Celite on Whatman No. 1 paper. The filtrate was transferred to a separatory funnel; 15 ml of chloroform was added, and the mixture was shaken. The chloroform layer was separated and discarded. The aqueous layer was collected and the top layer was discarded. A $^1/_2$-ml aliquot of the middle layer was applied to the top of a Sephadex fine-grade G-25 gel (Pharmacia Fine Chemicals, Inc.) column 1.2 cm × 19 cm which had been preconditioned with 0.1*M* NH_4OH. The eluant was 0.1*M* NH_4OH. A proportional pump was used to add the ammoniacal solution continuously, and the column pressure was increased to provide a flow rate of 0.5 ml per min. The effluent was monitored with a Buchler Continuous Flow Ultraviolet Monitor with a read-out range of 220–300 mμ. Molecular weights of the pigment bands were estimated according to the R_f values (void volume/elution volume) on the same gel column previously described; the molecular weight exclusion limit of 5000 was provided for this gel by the manufacturer. R_f values of the pigment bands were referred to a chart prepared with plots of molecular weights *vs.* R_f values. Blue dextran 2000 (Pharmacia Fine Chemicals, Inc.), egg albumin (Nutritional Biochemicals Corp.), casein (Nutritional Biochemicals Corp.), bovine pancreas ribonuclease (Worthington Biochemical Corp.), chlorogenic acid (Nutritional Biochemicals Corp.), and rutin (Nutritional Biochemicals Corp.) were used in this calibration. Bands were dried *in vacuo* at ambient temperature. Water was added to the dried residue to obtain a solution of sufficient concentration for spectrophotometric or other measurement. Preliminary wavelength-absorbancy scans were performed on a Beckman DB recording spectrophotometer, and the only characteristic absorbance peak for the first and second eluted bands was obtained at 270 to 280 mμ in the 260 to 700 mμ scanning range

Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture, and Department of Agronomy, University of Kentucky, Lexington, Ky. 40506

* To whom correspondence should be addressed.

Table I. Amino Acids in Hydrolysates of Brown Pigment Main Band after High Voltage Electrophoresis

(Micromoles Amino Acid Per Gram Pigment)

	"F.C. 402" Tobacco Leaf		
	At Harvest	Cured 13 Days	Cured 39 Days
Aspartic Acid	424	263	158
Threonine	185	116	43
Serine	202	134	53
Glutamic Acid	364	198	108
Proline	2636	1166	753
Glycine	329	216	77
Alanine	353	170	58
Valine	244	135	51
Cystine	None detected	72	None detected
Methionine	33	19	5
Isoleucine	125	68	23
Leucine	194	119	32
Tyrosine	96	70	19
Phenylalanine	113	77	23
Ornithine	5	3	1
Lysine	136	78	27
Histidine	47	36	13
Aginine	111	81	24
Tryptophan	None detected	4	4

employed. The first eluted band of each sample was routinely measured in 10.0 ml of H_2O at 280 mμ in a Beckman DU spectrophotometer.

For studies of pigment composition, the first and second bands that were treated or tested were obtained from samples of "F.C. 402" tobacco at harvest, and after 13, 29, and 39 days of conventional air-curing. The selection of these samples was made to provide a random range of curing periods. Electrophoretic purification was performed with a Brinkmann Model FF-1 continuous flow separator with the following conditions:

Sample flow (through inlet tubing): 0.16 ml/min
Solvent flow over plate: 2.30 ml/min
Voltage: 2000 V
Current: 0.09 amps
Buffer used in electrode chamber: 0.10*M* sodium acetate buffer, pH 5.2
Buffer used in curtain: 0.02*M* sodium acetate buffer, pH 5.2.

After electrophoresis, samples of pigment used for quantitative analysis were dialyzed against water for 4 days at 4° C to remove buffer salts.

Tests were made for the presence of free α-amino nitrogen in the bands with ninhydrin reagent, according to the method of Meyer (1957). Bands were hydrolyzed for 24 hr in 6*N* HCl for analysis of all amino acids except tryptophan, and in 14% $Ba(OH)_2$ for analysis of tryptophan. The hydrolysates were analyzed quantitatively for amino acid composition with the aid of a Technicon automated amino acid analyzer system. Tests for the presence of carbohydrate material were made upon pigment bands hydrolyzed in 6*N* HCl for 30 min according to methods described for paper chromatographic separation of simple sugars and disaccharides and their subsequent detection by means of spraying the dried chromatograms with an aqueous acetone solution of silver nitrate (Block *et al.*, 1955). Elementary analysis for carbon, hydrogen, nitrogen, and iron were performed by the Galbraith Laboratories, Inc., Knoxville, Tenn. Qualitative tests for caffeic acid, quinic acid, and quercetin moieties were made upon pigment bands as previously described (Wright *et al.*, 1960).

RESULTS AND DISCUSSION

Number of Pigment Bands Obtained on the Gel Column. Two brown pigment bands separated on the Sephadex G-25 gel column in all the samples. The front-moving band (main pigment band) contained the highest concentration of brown material; it was narrower and darker than the slower-moving band (secondary pigment band) which tailed considerably. The R_f (void volume/elution volume) of the main band was 1.0 and approximated the R_f values of proteins of molecular weights exceeding 5000, *i.e.*, Dextran 2000, egg albumin, and casein. The secondary band had an R_f of 0.79.

Composition Studies of the Pigment Bands. Positive tests for carbohydrate material in main band hydrolyzates were obtained for all samples tested, *i.e.*, in "F.C. 402" leaves conventionally air-cured for 13, 29, and 39 days. After electrophoretic purification of these same main bands, negative tests were obtained for carbohydrate material after hydrolysis. Therefore, carbohydrate material was a contaminant of the gel-purified pigment bands prior to high voltage electrophoresis.

The amino acid compositions of pigment hydrolyzates were determined for electrophoretically purified and dialyzed main pigment bands of "F.C. 402" leaves at harvest and after 13 and 39 days of conventional air-curing (Table I). In each sample, proline was the principal amino acid, while aspartic acid, glutamic acid, glycine, alanine, valine, serine, leucine, and threonine followed in order of descending amounts. Other amino acids were present in lesser amounts. Among these three samples the total amounts of amino acids per unit weight of main pigment band decreased with curing time. Previous investigators (Wright *et al.*, 1960) employed paper chromatography to detect 18 amino acids qualitatively in nondialyzable leaf pigment, and these corresponded to those found in our experiments. While these workers speculated that aspartic and glutamic acids were the preponderant amino acids, our quantitative results showed that proline was present in greatest amount.

Seventeen amino acids were detected in hydrolysates of secondary band pigment from "F.C. 402" tobacco conventionally air-cured for 29 days. Because high voltage electrophoretic purification was not performed in this analysis, the results were not considered as significant as those given above concerning main band hydrolyzates.

The main pigment band extracted from leaves of "F.C. 402" tobacco air-cured for 29 days was ninhydrin negative and the secondary pigment band of this tobacco was ninhydrin positive. These results provided evidence for the absence of a free α-amino nitrogen functional group on the main pigment band, and conversely, the presence of this functional group on the secondary pigment band. Presumably, the *N*-terminal amino acid of the protein material in the main pigment band was substituted, while in the secondary pigment band it was free.

Elementary analysis was performed on the main pigment band obtained from "F.C. 402" leaves conventionally air-cured for 29 days; the pigment was electrophoretically purified and then dialyzed. Results were: C = 43.9%, H = 5.7%, N = 4.9%, and Fe = 0.6%. Compared with the amounts of these elements in pigments described by Chortyk (1967) and Wright *et al.* (1960), our values for C, H, and N were similar. Because pure proteins contain 50 to 55% C, 6 to 7% H, and 12 to 19% N, it is apparent that the main pig-

ment band in this sample may contain a higher content of oxygen than the 20 to 23% content present in pure proteins. Based upon the N determination and the quantities of amino acids in hydrolysates of the main band pigments, the estimated protein content of pigments was 15 to 60% in the samples tested.

Caffeic acid and quinic acid moieties were present in main pigment band hydrolysates of a "F.C. 402" tobacco sample air-cured for 29 days. Quercetin moieties were not detected in this sample. Quercetin moieties were present, however, in secondary pigment band hydrolysates of this sample, while caffeic acid and quinic acid moieties were not detected.

Quantitative Estimation of the Main Pigment Band. A linear relationship existed between absorbance measurements of aqueous solutions at 280 mμ and concentration for a given sample of main pigment band derived from air-cured "F.C. 402" or "Ky. 16" tobacco. The characteristic 270 to 280 mμ absorbance peak for main pigment band indicated that unsaturated linkages were present in the pigment. Certain amino acids such as phenylalanine, tyrosine, and tryptophan have absorption maxima in this region. However, if pigment absorbance was caused entirely by amino acid moieties, the increased absorbance noted with increased cure time would be associated with increased levels of these aromatic amino acids in hydrolysates of pigment from samples with increased cure time. As already shown, however, the amino acid content of pigment decreased as a function of cure time. Aglycone moieties of plant phenols such as chlorogenic acid, rutin, tannins, and other unidentified plant phenols would contribute to absorbance increases in this region. Other unidentified aromatic or unsaturated moieties in the pigment may also have contributed to the observed absorbance maxima. The quantitative estimation of brown pigment which we used, *i.e.*, the absorbance at 280 mμ of a given aliquot of main pigment band extracted from 1 g of tobacco, has to be interpreted as an approximate estimate at best. This is true because nonuniformities of the amino acid composition of pigment at different curing times were found and other differences existed. It is probable that the main pigment band which moved with the solvent front on Sephadex G-25 gel consists of several pigments which could be further resolved on gel columns which have a higher molecular weight exclusion limit.

Major Pigment Band During Air-Curing. There was about a one-fold increase in the estimated amount of main pigment band from the start to finish of the curing cycle for the "F.C. 402" and "Ky. 16" leaves (Figure 1). The increases of pigment with curing time in the present experiment occurred when soluble chlorogenic acids and soluble rutin contents lowered and total soluble phenolic levels remained constant in these samples (*cf.* previous experiments, Andersen *et al.*, 1969). At comparable times of air-curing, the pigment in high-phenolic, "F.C. 402" leaves was higher than in "Ky. 16" leaves. The higher phenolic content probably accounts for the higher pigment values in "F.C. 402" leaves compared to "Ky. 16" leaves because caffeic acid and quinic acid moieties were present in main pigment band, and chlorogenic acid was found to be associated with brown pigment fractions (Chortyk, 1967). Our present results and previous findings (Andersen *et al.*, 1969) allow us to suggest that pigment formation in leaf is directly related to endogenous levels of specific plant phenols such as chlorogenic acid. Because pigment levels increased with cure time and, conversely, chlorogenic acid and rutin levels steadily decreased with cure time, it is probable that pigment formation is an irreversible process during conventional air-curing of tobacco leaf. Our results corroborate

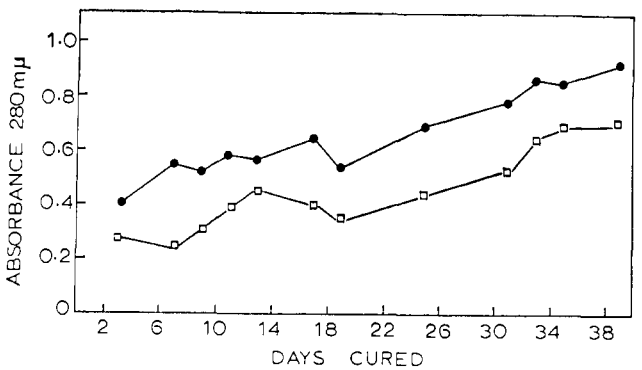

Figure 1. Relative absorbance of major pigment fraction in leaves of conventionally air-cured "F.C. 402" (●—), and 'Ky. 16' (□—). Absorbance value for 10.0 ml aqueous solution of pigment isolated from aliquot containing extract of 50 mg tobacco. Standard deviation (σ) = 0.05

the view that pigment formation involves, in part, a chlorogenic acid–rutin–protein–iron complex formation (Chortyk, 1967; Wright *et al.*, 1960).

Two bands of pigment were present after extraction of "F.C. 402" leaves sampled during growth from 3 to 11 weeks after transplant. The main pigment band was measured quantitatively, and the amino acid composition was determined for one sample at harvest (Table I). The range of absorbance values at 280 mμ for samples was 0.30 to 0.34 (σ = ±0.05). The relative proportions of amino acids in the sample at harvest were very similar to those found in main pigment band from samples of cured "F.C. 402" tobacco leaf. These data suggest two possibilities, namely: (1) artifact pigment was formed during the 20-hr extraction of leaves; or (2) brown pigment was present in leaves during growth. The alkaline solution (pH 10) used in the analysis probably prevented polyphenoloxidase-mediated oxidation of *o*-dihydroxyphenols, a presumed intermediate in pigment formation (Clayton, 1959). However, there is a possibility that some autooxidation of *o*-dihydroxyphenols occurs at pH 10. In several tests, however, addition of the antioxidants ascorbic acid and cysteine to the extraction and filtration media did not change the values for brown pigment.

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LITERATURE CITED

Andersen, R. A., Lowe, R., Vaughn, T., *Phytochemistry* **8,** 2139 (1969).
Block, R. J., Durrum, E. L., Zweig, G., "Paper Chromatography–Paper Electrophoresis," pp. 128–132, Academic Press, Inc., New York (1955).
Chortyk, O. T., *Tobacco Sci. XI,* 137 (1967).
Chortyk, O. T., Schlotzhauer, W. L., Stedman, R. L., *Beitr. Tabakforsch.* **3,** 422 (1966).
Clayton, R. A., *Arch. Biochem. Biophys.* **81,** 404 (1959).
Hoagland, D. R., Arnon, D. I., California Agr. Exp. Sta. Circ. 347 (1950).
Jacobson, J. L., *Arch. Biochem. Biophys.* **93,** 580 (1961).
Keller, C. J., Kasperbauer, M. J., J. AGR. FOOD CHEM. **17,** 327 (1969).
Meyer, H., *Biochem. J.* **67,** 333 (1957).
Sheen, S. J., Calvert, J., *Tobacco Sci. XIII,* 10 (1969).
Stokes, G. W., *Science* **141,** 1185 (1963).
Wright H. E., Burton, W. W., Berry, R. C., *Arch. Biochem. Biophys.* **86,** 94 (1960).

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