Amino Acid Composition in Soluble Tobacco Fractions Containing Brown Pigments

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Brown pigments in air-cured and flue-cured tobacco and blended cigarettes were isolated into six fractions by means of solubility in acidic and alkaline solution and in ethanol. Amino acid composition of the pigment fractions analyzed by gas–liquid chromatography revealed that aspartic and glutamic acids occurred in great quantities, followed by glycine, alanine, valine, and serine in all fractions, irrespective of tobacco type and curing methods. The composition was comparable between the same fraction of different tobacco types but differed among the fractions. Proline content was extremely high in the nonprecipitated, alcohol-soluble brown pigment fraction. The major brown pigment in cured tobacco is alkali-soluble, acid-precipitable, and alcohol-insoluble. This pigment consisted of more than 50% as proteins whose amino acid composition is similar to that of fraction I protein. Brown pigments of the Kentucky 1R1 reference cigarettes differed from smoke pigments in weight distribution of pigment fractions and in amino acid composition. The present results suggest that the distribution of proteins and amino acids in brown pigment fractions is not affected by tobacco types but can be modified by cultural practices and curing methods.

Brown pigments in tobacco leaves are formed by enzymatic oxidation of polyphenols during leaf ripening and curing. They vary in molecular weight from <3000 to >100000 and consist of water-soluble and water-insoluble fractions. The former may be further separated into dialyzable and nondialyzable fractions (Chortyk, 1967; Wright et al., 1960, 1964). Their chemical properties may be related to the protein moiety in the pigment complex. Pyrolytic analyses of tobacco brown pigments have revealed that they contribute to formation of polynuclear aromatic hydrocarbons, bases and phenols, some of which exhibit carcinogenic or cocarcinogenic activity in experimental animals (Chortyk et al., 1966). These same groups of compounds are also present in the pyrolysate of proteins and amino acids (Patterson et al., 1969). Data obtained from pyrolysis of amino acid mixtures can be useful in predicting the composition of protein pyrolysates (Smith et al., 1974). Since the aromatic ring in amino acids facilitated the formation of a much larger fraction of aromatic hydrocarbons than was obtained from aliphatic amino acids, it has been suggested that amino acid structure can affect the gross composition of the pyrolysate and consequently the composition of tobacco "tar" (Patterson et al., 1969). A recent study substantiated the fact that the amount of total free amino acids in cured leaves is positively correlated with the level of polynuclear aromatic hydrocarbons in cigarette smoke (Tso and Chaplin, 1977).

In comparing the data obtained from the alkali-soluble and acid-precipitated brown pigments of flue-cured, burley, Maryland, and Turkish tobaccos, Chortyk (1967) reported that the pigments of different tobacco types are essentially similar in elemental composition and molecular weight distribution; and these characteristics are apparently not affected by different curing methods. Elemental analyses revealed little information on the variation of amino acids among tobacco types. Furthermore, brown pigments other than alkali-soluble and acid-precipitated ones were not compared. In view of a correlation between the brown pigments in the leaf and the biological activity of pigment pyrolysate (Chortyk et al., 1966), a quantitative and qualitative comparison of all soluble fractions containing brown pigments from cured leaves of different tobacco types should be investigated. The present paper reports the amino acid composition of six soluble fractions in air-cured leaves of four tobacco types. Amino acid composition of these fractions from flue-cured and blended tobaccos and cigarette smoke condensate was also compared.

EXPERIMENTAL SECTION

Air-cured leaves of six Kentucky isogenic tobacco lines representing burley (KyIso 1 Ky 16, KyIso 3 Burley 37), flue-cured (KyIso 4 Hicks, KyIso 6 F.C. 402), fire-cured (KyIso 2 Ky 151), and Turkish (KyIso 7 Turkish) types were obtained from a field experiment which was conducted according to conventional burley cultural practices on the Agricultural Experiment Farm, University of Kentucky, Lexington, Ky. Flue-cured leaves of NC 95 produced at the Oxford Tobacco Research Station of USDA, Oxford, N.C., and the Kentucky 1R1 reference cigarettes and their smoke condensate provided by the Tobacco and Health Research Institute, University of Kentucky, were included for comparison.

Alkali-soluble and acid-precipitated brown pigments of the cured leaves (10 g of leaf powder, <40 mesh, preconditioned in a freeze-dryer overnight) were isolated into alcohol-soluble and alcohol-insoluble fractions according to the method of Chortyk et al. (1966) with certain modifications. An NH_4OH solution (pH 10) containing NaCN (5×10^{-3} M) was used in place of NaOH for extraction and dilute HCl was used to acidify the extract to pH 1 for precipitation of brown pigments. The use of NaCN prevents possible pigment formation due to reactions catalyzed by polyphenoloxidase and peroxidase in the extract. To recover the nonacid precipitable pigments, the remaining aqueous solution was freeze-dried and extracted with ethanol by vigorous shaking to yield the alcohol-soluble and alcohol-insoluble fractions. Similarly, the tobacco residue was further extracted with dilute HCl (pH 3) so as to solubilize all brown substances from the insoluble matter. This extract was then freeze-dried and fractionated into alcohol-soluble and -insoluble portions in the same manner as the other pigment fractions. Detailed fractionation procedures are schematically illustrated in Figure 1. There are six fractions containing

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Table I. Quantity (mg/g of Dry Leaf Weight) of Brown Pigment Fractions in Cured Tobacco Leaf

Tobacco	Brown pigment fraction A	B	C	D	E	F	Total quantity
Air-cured							
KyIso 1 Ky 16	15.15	44.49	5.06	2.04	133.24	458.58	658.56
KyIso 3 Burley 37	12.02	37.45	6.12	1.47	153.53	329.91	540.50
KyIso 2 Ky 151	7.01	18.36	7.55	Tr	173.82	477.17	683.91
KyIso 4 Hicks	9.21	33.43	6.92	1.48	172.13	531.44	754.61
KyIso 6 F.C. 402	10.38	36.42	9.90	3.91	139.54	444.60	644.75
KyIso 7 Turkish	12.55	28.68	8.26	1.31	170.37	531.42	752.59
Flue-cured							
NC 95	11.43	3.33	16.73	10.29	148.69	383.62	574.09

Table II. Amino Acid Composition (μmol/g of Pigment) in the Acid-Precipitated, Alcohol-Insoluble Brown Pigments (Fraction C) of Cured Tobacco Leaf

Amino acid	Air-cured KyIso 1 Ky 16	KyIso 3 Burley 37	KyIso 2 Ky 151	KyIso 4 Hicks	KyIso 6 F.C. 402	KyIso 7 Turkish	Flue-cured NC 95
Ala	507	413	278	296	263	296	368
Val	446	360	244	251	225	254	324
Gly	501	440	290	315	296	341	360
Ile	253	213	153	171	151	156	247
Leu	381	317	221	223	206	226	358
Pro	281	242	193	191	200	210	238
Thr	400	297	207	237	205	227	250
Ser	442	333	228	255	242	259	299
Met	78	68	32	40	31	44	83
Phe	203	173	128	132	128	134	167
Asp	751	619	418	449	458	470	421
Glu	518	439	307	314	367	343	409
Tyr	220	192	114	112	103	123	123
Orn	12	12	8	6	6	9	8
Lys	201	151	119	115	118	108	176
Arg	242	210	138	167	111	116	133
Cys	70	74	45	53	61	64	44
Total	5506	4562	3123	3325	3170	3380	4008

brown pigments and designated as brown pigment fractions A, B, C, D, E, and F. Extraction of smoke pigment from smoke condensate (20 g) followed the same procedure. All samples were processed in duplicate, and results reported herein are the average of the duplicated samples.

All brown pigment fractions were analyzed for amino acid composition. Pigment fractions (25 mg) were hydrolyzed with 25 mL of 6 N HCl in a stoppered glass tube in a nitrogen atmosphere for 20 h at 110–115 °C. The hydrolysates were flash-evaporated in vacuo to dryness at 40 °C, resuspended in 20 mL of 0.1 N HCl and filtered through millipore (0.2 μm) filter. Procedures of cation-exchange cleanup, amino acid derivatization, and quantification of gas–liquid chromatography were the same as reported by Kaiser et al. (1974).

RESULTS

The six brown pigment fractions isolated according to solubility characteristics varied in coloration from dark brown for fraction C to grayish yellow for fraction B. Air-cured leaves of different tobacco types showed a similar pattern of distribution for the quantity of the six pigment fractions (Table I). Fraction D contained the least amount, whereas fraction F showed the most. It should be pointed out that the large amounts of nonprecipitated pigment fractions (fractions E and F) are attributed to low molecular weight compounds such as carbohydrates, alkaloids, and organic salts. The proportion of six pigment fractions in the flue-cured leaf sample differed from those of air-cured KyIso lines by having a greater amount of fraction C but less of fraction B.

The data in Table II present the amino acid composition in the alkali-soluble and acid-precipitated pigment

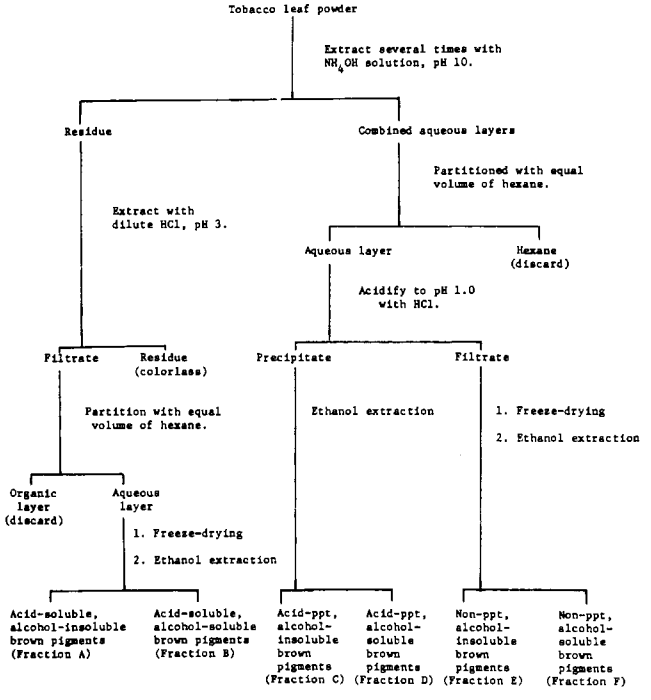

Figure 1. A scheme for fractionation of brown pigments in tobacco leaf.

(fraction C) which is the brown pigment extensively studied by others (Chortyk, 1967; Wright et al., 1960). Aspartic and glutamic acids are generally the major amino acids in this pigment. This also holds true in the remaining pigment fractions irrespective of tobacco types and curing

Table III. Quantity of Aromatic and Heterocyclic Amino Acids (μmol/g of Pigment Fraction) in Brown Pigment Fractions of Cured Tobacco Leaf

Tobacco[a]	Brown pigment fraction A	B	C	D	E	F
			Phenylalanine			
KyIso 1 Ky 16	6.05	5.38	203.40	13.92	6.86	27.89
KyIso 3 Burley 37	12.11	8.99	173.13	23.19	12.47	34.08
KyIso 2 Ky 151	4.04	3.26	128.34	*b*	2.91	26.12
KyIso 4 Hicks	3.63	2.75	131.97	9.69	3.63	12.52
KyIso 6 F.C. 402	2.42	2.40	128.34	10.44	2.47	18.40
KyIso 7 Turkish	6.26	3.54	134.39	6.12	3.03	17.53
NC 95	7.09	1.39	167.08	34.25	3.03	8.58
			Tyrosine			
KyIso 1 Ky 16	1.84	0.65	219.66	3.86	2.21	Tr
KyIso 3 Burley 37	6.35	4.10	192.06	7.68	11.65	4.91
KyIso 2 Ky 151	1.49	Tr	113.69	*b*	3.00	Tr
KyIso 4 Hicks	3.68	Tr	111.49	8.83	3.04	3.17
KyIso 6 F.C. 402	2.48	3.65	102.65	5.23	5.91	5.74
KyIso 7 Turkish	3.81	1.43	122.52	1.86	3.31	7.26
NC 95	4.31	Tr	122.52	22.85	Tr	2.06
			Proline			
KyIso 1 Ky 16	17.37	19.01	281.42	57.33	20.85	226.73
KyIso 3 Burley 37	40.82	25.37	241.47	56.36	33.61	211.03
KyIso 2 Ky 151	13.90	10.69	192.83	*b*	10.42	173.72
KyIso 4 Hicks	14.48	14.31	191.09	59.06	15.20	130.78
KyIso 6 F.C. 402	13.46	12.66	199.77	61.40	10.63	207.77
KyIso 7 Turkish	33.34	9.59	210.20	73.20	13.03	195.43
NC 95	25.79	3.00	237.99	86.86	26.93	200.30
			Methionine			
KyIso 1 Ky 16	1.12	0.79	77.74	2.01	1.34	Tr
KyIso 3 Burley 37	3.35	3.32	68.36	2.86	4.72	Tr
KyIso 2 Ky 151	0.89	Tr	32.17	*b*	2.14	Tr
KyIso 4 Hicks	3.13	Tr	40.21	9.38	3.69	1.54
KyIso 6 F.C. 402	3.35	1.33	30.83	1.74	4.10	Tr
KyIso 7 Turkish	1.85	Tr	44.23	1.51	2.68	Tr
NC 95	4.71	1.15	83.10	9.48	3.35	Tr

[a] All leaves were air-cured except NC95 which was flue-cured. [b] Not determined.

methods. Glycine, alanine, valine, and serine contents are the next highest in all cases. Amino acid composition of the other five brown pigment fractions in the air-cured leaves of different tobacco cultivars had a similar pattern among the same fraction but varied between the fractions. Among the aromatic and heterocyclic amino acids, proline content is highest in all six pigment fractions, especially in fraction F (Table III). This is in agreement with the increase of free proline during leaf curing reported by others (Hamilton, 1974; Weybrew et al., 1966). Phenylalanine and tyrosine were in higher concentrations in burleys than in other tobacco types. Within the flue-cured tobacco type these aromatic amino acids showed a higher concentration in the flue-cured leaves than the air-cured leaves. The quantitative difference of proline and methionine among the pigment fractions of air-cured tobaccos and between air-cured and flue-cured leaves showed a great contrast. Histidine was detected only in trace amounts in all cases, whereas tryptophan quantification was not possible by the present procedure because it is unstable under acid hydrolysis.

The fraction C pigment contains the greatest quantity of amino acids in all pigment fractions (Table IV). Among the tobacco types, two burleys have high amino acid content in alkali-soluble pigment fractions. The average quantity of amino acids in the fraction C pigment of two burleys was 5034 μmol/g of pigment. This is equivalent to 0.67 mg of amino acids/milligram of pigment, if one assumes that the average molecular weight of amino acids is 133. In other words, 67% of the weight of the pigment is composed of proteins, which is in close agreement with other findings (Andersen et al., 1970; Chortyk, 1967). The

Table IV. Amino Acid Quantity (μmol/g of Pigment Fraction) in the Brown Pigment Fractions of Cured Tobacco Leaf

Tobacco	Brown pigment fraction A	B	C	D	E	F
Air-cured						
KyIso 1 Ky 16	172	137	5506	435	242	960
KyIso 3 Burley 37	391	242	4562	672	525	1257
KyIso 2 Ky 151	129	79	3123	*a*	168	886
KyIso 4 Hicks	251	154	3326	448	168	538
KyIso 6 F.C. 402	129	118	3170	355	158	893
KyIso 7 Turkish	230	79	3380	261	137	690
Flue-cured						
NC 95	299	52	4008	877	240	463

[a] Not determined.

four nonburley cultivars, on the average, have 43% protein in the same pigment. Since all six cultivars were cultured according to burley practices in a field experiment and air-cured under the same conditions, the difference of protein content in fraction C pigment can be attributed to the characteristics of burley and nonburley tobacco types. Flue-cured leaves showed 53% protein content in this pigment, which is 10% higher than the air-cured dark tobaccos. The protein and/or amino acid in other pigment fractions of air-cured leaves ranges from 11.6% in fraction F to 1.7% in fraction B.

On the basis of amino acid content in the pigment fraction and pigment quantity per gram weight of dry leaf, one can calculate the quantitative distribution of amino acids in six pigment fractions in gram weight of cured tobacco leaf (Table V). Owing to the large pool size of

Table V. Distribution of Amino Acid Quantity (μmol/g of Dry Leaf Weight) in the Brown Pigment Fractions of Cured Tobacco Leaf

Tobacco	Brown pigment fraction						Total amino acids
	A	B	C	D	E	F	
Air-cured							
KyIso 1 Ky 16	2.61	6.11	27.86	0.89	32.28	440.20	509.95
KyIso 3 Burley 37	4.70	9.05	27.92	0.99	80.61	414.56	537.83
KyIso 2 Ky 151	0.90	1.44	23.58	a	29.21	422.98	478.11
KyIso 4 Hicks	2.31	5.61	23.01	0.66	28.88	285.79	345.81
KyIso 6 F.C 402	1.34	4.29	31.38	1.39	22.09	396.94	457.43
KyIso 7 Turkish	2.88	2.26	27.92	0.34	23.41	366.58	423.39
Flue-cured							
NC 95	3.42	0.17	67.06	9.02	35.64	177.49	292.80

[a] Not determined.

Table VI. Quantitative Comparison of Kentucky 1R1 Reference Cigarette and Its Smoke Condensate for Pigment Fractions and Total Amino Acids

Pigment fraction	Quantity, mg/g of dry wt		Total amino acids, μmol/g of pigment fraction	
	Cigarette	Smoke condensate	Cigarette	Smoke condensate
A	12.4	0.6	188	179
B	2.4	8.8	105	145
C	14.3	0.7	2274	Tr
D	6.7	7.7	132	109
E	286.1	112.9	136	9
F	331.8	245.8	572	595

free amino acids and peptides, the fraction F contains 84 and 61% of total amino acids and/or proteins in air-cured and flue-cured leaves, respectively. The level of protein in fraction C on leaf weight basis is comparable in air-cured leaves of six cultivars. However, the quantity is doubled in the flue-cured leaf. About 23% of total proteins or amino acids in the flue-cured leaf was in the form of acid-precipitated pigment (fraction C), whereas only 6% appeared in the air-cured tissue. The distribution of individual amino acids in pigment fractions did not follow the same proportion as amino acid pool. For example, proline in fraction F amounted to 94% of total proline in the air-cured leaf and 89% in the flue-cured sample, whereas its content in fraction C accounted for only 2 and 5% of total proline in the respective cured tobaccos.

The quantity of six pigment fractions from the Kentucky 1R1 reference cigarette followed a pattern closely similar to that of flue-cured leaves (Table VI). Its decreased amount of acid-precipitated pigment (fraction C) coincided with the increase of nonprecipitated ones (fraction F), suggesting that a continuous degradation of large molecular weight pigment proteins into low molecular weight proteins, peptides, and/or amino acids occurred during fermentation and storage. This is substantiated by the decrease in content of total amino acids in the acid hydrolysate of this pigment. The estimated protein content in the fraction C pigment of Kentucky 1R1 reference cigarette is about 30%. The fraction C pigment was degraded during pyrolysis, as evidenced by the disappearance of this pigment in smoke condensate. The amino acid composition in the soluble fractions of reference cigarettes differed from that of smoke condensate (Table VII). The latter contains high levels of alanine, glycine, serine, and glutamic acid, all of which are low molecular weight amino acids and may be derived from pyrolytic degradation of other amino acids and/or related leaf constituents. Almost 98% of amino acids in smoke condensate can be fractionated into the alkali-soluble, nonprecipitated pigment fraction (fraction F).

Table VII. Comparison of Amino Acid Composition in Soluble Fractions of Kentucky 1R1 Reference Cigarette and Smoke Condensate

Amino acid	Kentucky 1R1 reference cigarette, μmol/g of dry wt	Smoke condensate μmol/g of dry wt
Ala	16.28	70.56
Val	9.34	5.70
Gly	15.76	17.13
Ile	4.24	1.29
Leu	5.21	3.71
Pro	37.36	3.61
Thr	6.11	0.04
Ser	14.66	20.52
Met	2.02	0.05
Phe	6.76	1.45
Asp	70.54	3.57
Glu	50.64	14.56
Tyr	3.33	0.03
Orn	3.12	Tr
Lys	9.97	2.09
Arg	4.00	0.55
Cys	5.33	4.79
Total	264.67	149.65

DISCUSSION AND CONCLUSION

Brown pigments in cured tobacco leaves are highly heterogeneous in chemical and physical properties. The separation of the pigments into six fractions by solubility demonstrated differences in chemical characteristics. Since the amino acid composition of a given pigment fraction appears to be similar among tobacco types and between the same tobacco type differently cultured, the proteinaceous substances must exert a determinant role in solubility in the present fractionation procedure. The present procedure would recover all free amino acids and most of proteins. This permits quantitative and qualitative evaluation of these nitrogenous compounds in different tobacco types and of their alteration by cultural practices and curing methods with respect to brown pigment fractions. Carugno et al. (1974) reported that different tobacco varieties showed certain quantitative and qualitative differences in amino acid composition and only small quantitative variations in the composition of protein-bound amino acids. The amino acid composition of acid-precipitated (protein-bound) and nonprecipitated (protein-bound and free amino acids) fractions in the present study substantiates this generalization.

Wright et al. (1960) isolated a dark-brown pigment from a soluble, nondialyzable fraction of aged burley tobacco by precipitation with acetic acid. This pigment is a chlorogenic acid–rutin–protein–iron complex which also contains alkaloids and related bases and a silicone and

Table VIII. Comparison of Amino Acid Composition in Fraction I Protein and Acid-Precipitated, or Nonprecipitated, Alcohol-Insoluble Brown Pigments in Tobacco Leaf

Amino acid	Fraction I protein[a]	Acid-ppt, alcohol-insoluble pigment (fraction C)		Non-ppt, alcohol-insoluble pigment (fraction E)	
		Air-cured[b] leaf	Flue-cured leaf	Air-cured leaf	Flue-cured leaf
Phe	1.00	1.00	1.00	1.00	1.00
Ala	1.93	2.29	2.20	3.22	6.48
Val	1.43	1.98	1.94	1.88	2.68
Gly	2.39	2.43	2.16	5.15	6.82
Ile	0.80	1.22	1.48	1.16	1.76
Leu	2.13	1.75	2.14	1.58	2.01
Pro	1.39	1.47	1.43	3.31	8.89
Thr	1.53	1.75	1.50	3.12	3.74
Ser	1.19	1.96	1.79	4.49	8.17
Met	0.39	0.33	0.50	0.59	1.11
Asp	2.58	3.52	2.52	7.82	9.42
Glu	3.33	2.55	2.45	5.55	15.37
Tyr	1.39	0.96	0.74	0.93	0
Lys	1.42	0.90	1.05	1.22	2.71
Arg	1.20	1.10	0.80	1.78	4.64
t value		1.25	1.14	4.21[c]	9.48[c]

[a] The data are from Kawashima and Wildman (1970) as averages of large and small subunits of fraction I protein. All numbers are calculated as relative molar ratios to phenylalanine. [b] Average values of six KyIso Lines. [c] Significant difference at the 1% level of probability.

consists of glutamic and aspartic acids as the predominant amino acids in the acid hydrolysate (Dymicky et al., 1967; Wright et al., 1960). The fraction C pigment in the present study should be identical with that pigment on the basis of the purification method. It also has a high concentration of glutamic and aspartic acids. Andersen et al. (1970) purified a major brown pigment from a flue-cured variety by high-voltage electrophoresis and reported that the amino acid composition of acid hydrolysate of the pigment showed proline in predominance, followed by aspartic acid, glutamic acid, glycine, and alanine in order of descending amounts. Since their method of pigment purification differed from the present procedure, a meaningful comparison is difficult.

The formation of brown pigments during curing is usually preceded by a loss of soluble proteins. Fraction I protein in chloroplasts constitutes about 50% of soluble proteins in green tobacco leaves (Kawashima and Wildman, 1970). It has been suggested that the loss of 50% soluble proteins in cured leaf is attributable to a total degradation of fraction I protein, whereas there is no decrease in simple cytoplasmic soluble proteins (Gains and Miles, 1975). However, one may point out that the degradation of fraction I protein occurs soon after chloroplast breakdown, and its degradation products become simple soluble proteins in cytoplasm. Fraction I protein in tobacco leaf is usually extracted with slightly basic solutions and becomes less soluble in acidic solutions. This solubility property resembles the acid-precipitated, alcohol-insoluble brown pigment (fraction C). When one compares the relative amino acid composition of this pigment with that of fraction I protein, a good agreement is obtained (Table VIII). This holds true for both air-cured and flue-cured leaves and suggests that fraction I protein and its degradation products become the protein moiety in the alkali-soluble and acid-precipitated brown pigment. Polyphenoloxidase and peroxidase have been implicated as the possible contributors of protein moiety in tobacco brown pigment (Sheen, 1974; Weybrew and Long, 1970). These soluble enzyme proteins are only in small quantities as compared with fraction I protein. Hence, their presence in this brown pigment, if any, will not be expected to alter the amino acid composition appreciably. Comparisons of amino acid composition between fraction I protein and nonprecipitated, alcohol-insoluble brown pigments (fraction E) showed significant difference at the 1% level of probability. This indicates that degradation products from proteins other than fraction I protein are in substantial quantities in this fraction. The same conclusion can be applied to other pigment fractions.

Flue-cured leaves of NC 95 contain less protein than the same tobacco type grown according to burley practices. This is explainable because of the low nitrogen fertilizer rate in the flue-cured tobacco region. However, flue-cured leaves yielded two to three times more fraction C pigment than air-cured leaves. This suggests that flue-curing results in a great incorporation of fraction I protein and its degradation products into this pigment. In contrast, air-curing prolongs the enzymatic degradation of proteins and other leaf constituents, possibly including brown pigments. As a result, it decreases the amount of fraction C pigment and accumulates fraction F in air-cured leaves.

Pyrolysis of proteins and amino acids yields polynuclear aromatic hydrocarbons in addition to carbon monoxide, hydrogen cyanide, phenol, and other gases (Smith et al., 1974). Aromatic and heterocyclic amino acids are better precursors of polynuclear aromatic hydrocarbons than aliphatic amino acids (Patterson et al., 1969). The greater the proportion of protein in pigment fractions, the more one would expect an increase in hazardous compounds in cigarette smoke. However, if one uses both protein quantity and amino acid composition in tobacco leaf as criteria for smoking hazards, the nonprecipitated brown pigment fractions are at least equal to or probably surpass the acid-precipitated brown pigments in hazardous potential. The present results revealed that tobacco genotypes have minor effect on amino acid composition of brown pigment fractions, and consequently any qualitative modification of brown pigments in terms of altering amino acid composition through intraspecific hybridization may not be fruitful. On the other hand, cultural practices leading toward less concentration of nitrogenous compounds and facilitating the degradation or removal of these compounds before, during, and after curing could provide alternative avenues for modification of the protein and amino acid content in the brown pigment fractions of tobacco leaf.

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Effects of Application Rates on Maleic Hydrazide Residues in Burley Tobacco

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The fate and stability of maleic hydrazide (MH) applied to Burley Tobacco and soil was examined. Four dosages of MH, from one-tenth to twice the recommended amount, were applied to tobacco at various stages of maturity. Residual quantities of MH in green and cured plants, as well as in soil, were determined by our derivatization–gas chromatography method. The effects of different application rates to tobacco are discussed.

Maleic hydrazide (MH, 1,2-dihydro-3,6-pyridazinedione), a systemic plant growth regulator in worldwide use as a tobacco sucker inhibitor, has generated recent interest because of its almost ubiquitous presence in tobacco and tobacco products. These concerns have been stimulated to a great extent by possible European Economic Community import restrictions on tobacco with high MH residues and by the possible health-related effects of MH in test animals (Epstein et al., 1967; Epstein and Mantel, 1968).

The fate of MH in tobacco has been examined by a number of workers over a period of about 20 years (Anglin and Mahon, 1958; Lane, 1965; Davis et al., 1974; Cheng and Steffens, 1976). All of these used the analytical method of Wood (1953) with modifications by Anglin and Mahon (1958), Lane et al. (1958), and Hoffman (1961). This analysis is based on the hydrolytic reduction of MH to hydrazine by zinc in aqueous sodium hydroxide solution. The hydrazine is subsequently steam distilled into an acidic solution of ρ-dimethylaminobenzaldehyde to form an azine which has an absorption maximum at 455 nm. This method, when applied to tobacco, suffers from interferences caused by pyrrole, resorcinol, tryptophan, and possibly other leaf constituents. Because of these interferences and the ambiguous nature of photometric determinations, we felt that a more comprehensive study of residual MH in the tobacco plant and soil was necessary using the gas chromatographic method developed at our laboratory (Haeberer et al., 1974; Haeberer and Chortyk, 1974). In the study of the stability and fate of MH applied to tobacco and soil, the following questions require elucidation: How much MH remains in harvested tobacco leaves, stalks, and roots from a standard application? Will a twofold application be reflected in a twofold increase in MH residue? Does the tobacco plant absorb MH from the soil? How much persists in the soil after 100 days? Will the next crop absorb soil MH? How much MH remains in cured leaves? To answer these questions, several experiments were conducted. Various quantities of MH were applied to soil and to growing Burley tobacco at various stages of maturity. Subsequently, residual MH was determined and the significance of these findings are discussed.

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EXPERIMENTAL SECTION

Reagents. The agricultural formulation of maleic hydrazide as the diethanolamine salt, MH-30 (UniRoyal), was obtained commercially. It was applied without further refinement after dilution with water (35 L of water/liter of MH-30). Maleic hydrazide for standards was obtained from Eastman Kodak Co. as the practical grade. It was recrystallized twice from distilled water. *N,O*-Bis(trimethylsilyl)acetamide (BSA) was obtained from Analabs, Inc. as the pure reagent and used without further refinement. Methyl alcohol (Burdick and Jackson Laboratories, Inc.) was the "distilled-in-glass" grade. Ethyl