be involved, however. Flue-cured samples from two locations and three cultivars were relatively uniform. However, the Va. 509 sample differed from other burley samples, primarily in neutral lipid content. Thus, cultivars, location of production, or local curing conditions may be involved in the differences among burley samples, and location and cultivar differences may be involved in the differences in lipid composition between burley and flue-cured tobacco.

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Protein Composition and Classification of Tobacco

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Protein composition and classification of flue-cured tobacco were studied during maturation and flue curing, and flue-cured leaf was contrasted with that of air-cured Burley and Maryland types. Most of the N in immature leaves was proteinaceous (77% PN:23% NPN) and composition was 23% simple and 77% residual proteins. Upon maturity PN had declined (53% PN:47% NPN) and protein composition for mature leaf was 36% simple:64% residual proteins. After flue curing PN had further declined (38% PN:62% NPN) as simple proteins increased (49% simple:51% residual protein). Simple proteins made up about half the total protein for all three types of cured tobacco, although Maryland had a higher PN ratio (51% PN:49% NPN) than flue cured or Burley (38% PN:62% NPN). More of the simple proteins for all three types of cured tobacco were glutelins. The enzyme proteins (albumin and globulin) increased slightly during flue curing. The data suggest that fraction I protein was located in the residual protein fraction and was rapidly broken down during flue curing.

Few significant studies have appeared in the recent literature on the protein composition of tobacco leaf (Stedman, 1968). Yet the degradation of leaf proteins is an extremely important aim of tobacco processing. It is believed that these compounds are associated with poor smoking quality (Pogel et al., 1957; Johnstone and Plimmer, 1959). Shmuk (1953) demonstrated that tobacco quality is positively correlated with soluble sugars and negatively correlated with proteins. Increased alkalinity of cigarette smoke caused by a preponderance of ammonia and other alkaline products of partial combustion of proteins causes a harsher and more disagreeable smoke and increases irritation of mucous membranes. Abdallah (1970) stated that a decrease in protein content always increased taste quality but some amount of protein is needed to enhance taste sensation.

Studies on green tobacco leaf have shown the presence of a soluble cytoplasmic protein termed "fraction I" and a minor heterogenous protein component termed "fraction II" (Wildman et al., 1949). Recently fraction I protein, once thought to be ribulose diphosphate carboxylase until Anderson et al. (1968) showed it was significantly different in molecular weight in some microorganisms, has been designated by Kawashima and Wildman (1970) as a particular high molecular weight protein found wherever chlorophyll a is present. Fraction I protein is rapidly broken down during curing; however, appreciable amounts of fraction II protein remain after curing (Pogel et al., 1957). Kawashima et al. (1967) reported a marked increase of smaller molecular weight proteins during curing. Extensive studies reveal that many of the proteins possessing much enzymatic activity remain relatively stable during curing (Johnstone and Plimmer, 1959). Rapid proteolysis occurs during curing with liberation of amino acids from hydrolyzed proteins.

Proteins from fresh leaves are usually grouped into the three categories of chloroplastic, cytoplasmic, and nuclear proteins (Miller, 1957). The classical Osborne method of classifying proteins according to solubility has traditionally been used on storage proteins, namely seeds. Commercial tobacco, although a leaf, is bought and sold in a dried state (not fresh leaf). Chemical analysis of commercial tobacco is normally preceded by drying the leaf to a moisture-free state and mill grinding the leaf into a fine dry powder.

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Table I. Chemical Profile of Major Nitrogenous Constituents in Three Types of Tobacco Samples

Sample	% total alkaloids[a]	% total N[a]	% PN[a]	% NPN[a]	% composition of total N	
					PN	NPN
Flue cured						
Green leaf (immature)	0.90	4.38	3.38	1.00	77	23
Green leaf (mature)	2.46	1.73	0.92	0.81	53	47
Cured leaf	2.74	1.92	0.73	1.19	38	62
Burley (cured leaf)	4.24	3.62	1.36	2.26	38	62
Maryland (cured leaf)	2.67	2.20	1.12	1.08	51	49

[a] Values can also be expressed as milligrams since they are based on a 100-mg sample.

Since dried tobacco powder is not in a fresh leaf state with sap intact the normal concept of classifying leaf proteins as chloroplastic, cytoplasmic, and nuclear is not as applicable as classifying them by solubility such as the Osborne method. Another advantage of the Osborne method for classifying dried tobacco tissue is that the simple proteins, proteins which yield only amino acids upon hydrolysis, are separated by solubility and amino acid composition of these fractions can later be studied and compared.

The purpose of this work was to study the composition and classify the tobacco proteins by the Osborne solubility scheme during the maturation of flue-cured tobacco, to observe the effect of flue curing on the protein composition and classification of mature green leaf, and to compare the protein composition and classification of flue-cured leaf with that of air-cured Burley and Maryland type tobaccos.

MATERIALS AND METHODS

Three types of tobacco were grown in the field during 1973 under recommended cultural practices. Flue-cured Hicks was produced at Tifton, Ga., Burley 21 at Greeneville, Tenn., and Maryland Catterton at Marlboro, Md. Green leaf samples, referred to in this study as immature, were harvested approximately 4 weeks after transplanting when about 38 cm (15 in.) tall. Green leaf mature samples were replicated four times in the field and harvested as they ripened with four harvests required to strip the stalk. Upon harvest, leaves were divided into two lots. The first lot was dried in a microwave oven (Amana Radar Range Model IRR-2, 1600 W) as described by Stephenson et al. (1971) to immediately halt enzymatic activity. The second was flue cured in a conventional manner. Analyses were run on each rep and the average appears in the tables for green leaf mature and cured leaf. Burley and Maryland type tobacco were air cured in their respective conventional manner. Samples were composited over stalk positions, midribs were removed, and dried laminae were ground in a Wiley mill to pass a 2-mm screen. Chemical analyses (Table I) were conducted by methods described by Gaines (1971).

Extraction of Proteins. Proteins were extracted based on solubility differences by the classical Osborne method (1907) as described by Lund and Sandstrom (1943).

(A) One gram of the ground tissue was weighed into a 100-ml beaker and stirred for 1 hr in 20 ml of distilled water (pH 5.88). The slurry was transferred to a 40-ml centrifuge tube and centrifuged 5 min at 2500 rpm. The supernatant containing the peptizates was decanted in a beaker and second and third extractions were made by stirring the residue for 20 min in 20 ml of distilled water with a glass rod. The three extractions were combined and diluted to a 70-ml volume. To a 10-ml aliquot 5 ml of 10% trichloroacetic acid (Cl_3CCOOH) was added and sample was placed in an 80° bath for 20 min to precipitate the water-soluble protein. After centrifugation 8 ml of concentrated sulfuric acid was added to the precipitate and the dissolved fraction was transferred to a 100-ml Kjeldahl flask. The percent N of the digested protein was determined by the Kjeldahl method (Gaines, 1971) and reported as albumin N.

(B) The residue in the 40-ml centrifuge tube, after the water-soluble protein had been removed, was next extracted with 5% potassium chloride (pH 5.65). Three extractions were made according to the procedure described in paragraph A. The saline-soluble N is reported as the globulin fraction and percent N was determined on a 10-ml aliquot.

(C) The residue in the 40-ml centrifuge tube from paragraph B was extracted with 70% ethyl alcohol at 70° according to the procedure described in paragraph A. This fraction represents the prolamin fraction and a 10-ml aliquot was used for N determinations.

(D) The residue after fractions A, B, and C had been removed was next extracted with 0.2% potassium hydroxide (pH 12.20). Three extractions were made according to paragraph A. This fraction represents the glutelin fraction and percent N was determined on a 10-ml aliquot.

(E) The residue from paragraph D was extracted in 20 ml of 5% Cl_3CCOOH for 20 min at 80° to remove any remaining nonprotein N. The residue was recovered after centrifugation and is listed as residual protein N.

RESULTS

Major Nitrogenous Constituents in Three Types of Tobacco. Chemical analysis of the three types of tobacco is shown in Table I to present a profile of the major nitrogenous constituents of the samples used in this study. As flue-cured tobacco matured total N decreased. Most of the N in early growth was proteinaceous (77%), but as the leaves matured and became fully expanded, a definite increase in nonprotein N (NPN) was noted. After curing, an even higher amount of NPN (62%) was observed. Our PN/NPN ratio for the cured leaf sample agrees well with the ratio of 39%/61% reported by Darkis and Hackney (1952) for better grades of flue-cured tobacco. A similar ratio (38%/62%) was found in Burley tobacco (Table I), but a slightly higher ratio was found in Maryland tobacco (51%/49%). Darkis and Hackney (1952) reported the same trend for better grades of Burley and Maryland tobaccos.

The total N/total alkaloids ratio for all three types of cured leaf ranged from 0.70 to 0.85 indicating a good balance between the nitrogen and nicotine levels (Bacot, 1960) in the samples selected for the protein classification study.

Classifying Protein Fractions. The classification of proteins based on solubility differences is still essentially that arrived at by Osborne in 1907. As with all solubility classification schemes, there is overlap between fractions. However, if fairly well-defined procedures are followed, i.e., conditions of pH and extraction, the four groups may be obtained relatively free from one another and still with their own distinctive properties. An attempt to relate the various schemes for classifying protein fractions has been

Table II. Various Schemes for Classifying Protein Fractions

Basis of classification	Protein fractions			
Protein species (Osborne)	Albumin	Globulin	Prolamin	Glutelin
Solubility	Aq soln	Salt soln	70% aq EtOH soln	Alkali soln
Starch gel electrophoretic mobility	Fast moving	Fastest moving	Slow moving	Immobile
Location in cell	Cytoplasm (attached to mem)	Cytoplasm (mem + endoplasmic reticulum)	Granules (protein bodies)	
Function in cell	Metabolic (enzymic)	Structural	Storage	
Amino acid composition: basic amino acids	More		Less	
Molecular weights	Lower		Higher	

Table III. Protein Classification (Based on Solubility) of Three Types of Tobacco; Percent Actually Found in Various Nitrogen Fractions[a]

Sample	% albumin N	% globulin N	% prolamin N	% glutelin N	% residual protein N	% total protein N
Flue cured						
Green leaf (immature)	0.15	0.16	0.15	0.33	2.59	3.38
Green leaf (mature)	0.07	0.08	0.07	0.10	0.60	0.92
Cured leaf	0.09	0.10	0.06	0.11	0.37	0.73
Burley (cured leaf)	0.16	0.15	0.16	0.23	0.65	1.36
Maryland (cured leaf)	0.09	0.12	0.12	0.22	0.57	1.12

[a] Values can also be expressed as milligrams since they are based on a 100-mg sample.

Table IV. Protein Classification (Based on Solubility) of Three Types of Tobacco; Percent of Total Protein N

Sample	% albumin	% globulin	% prolamin	% glutelin	% simple proteins	% residual proteins
Flue cured						
Green leaf (immature)	4	5	4	10	23	77
Green leaf (mature)	8	9	8	11	36	64
Cured leaf	12	14	8	15	49	51
Burley (cured leaf)	12	11	12	17	52	48
Maryland (cured leaf)	8	11	11	19	49	51

described (Kent, 1970) and a modification of this scheme is shown in Table II.

Proteins of the albumin and globulin classes include the enzyme proteins (Redman, 1971) which are of particular importance during tobacco curing. These are cytoplasmic proteins in which their function in the cell is believed to be enzymic and structural in the case of globulins. Prolamins and glutelins are granule protein bodies located in the cell and their chief function is for storage. The prolamins derive their name from the large quantities of proline and ammonia they liberate on hydrolysis. Only small amounts of basic amino acids are present. The glutelins generally make up a large percentage of the proteins in plants as large quantities are found in the storage organs. The glutelins and prolamins are termed gluten proteins in wheat and comprise up to 80–90% of the total proteins (Redman, 1971).

Prolamin and glutelin are often referred to as insoluble protein by cereal chemists (Kent, 1970), because of their insolubility in water and salt solution. They are often linked together because of similar properties, composition, and source of origin (Table II). Consequently, soluble protein refers to albumin and globulin, and has been shown to have lower molecular weights and faster electrophoretic mobility on starch gel than the other fractions (Table II). The amino acid composition of soluble and insoluble proteins of wheat (Redman, 1971) reveals they have similar amino acid sequences and quantitative values.

The residual protein refers to proteins other than simple proteins that are not soluble and are bound by a nonprotein group. These probably are conjugated and derived proteins and include some of the nucleoproteins, phosphoproteins, chromoproteins, glucoproteins, lipoproteins, etc. These proteins are separated from NPN by their insolubility in Cl_3CCOOH.

Classification of Tobacco Proteins. The classification of tobacco proteins is shown in Tables III and IV. The data in Table III show the actual amounts of N found on a dry

Table V. Effect of Stirring vs. Homogenizing on the Soluble Protein Fractions[a]

Sample	% albumin N		% globulin N		% prolamin N		% glutelin N		% total	
	Stird	Homogd	Stird	Homogd	Stird	Homogd	Stird	Homogd	Stird	Homogd
Flue cured										
Green leaf (mat.)	0.07	0.06	0.08	0.09	0.07	0.06	0.10	0.11	0.32	0.32
Cured leaf	0.09	0.08	0.10	0.10	0.06	0.08	0.11	0.11	0.36	0.37

[a] Values can also be expressed as milligrams as they are based on a 100-mg sample.

Table VI. Effect of Cl_3CCOOH Precipitation on 10-ml Aliquots of the Soluble Protein Fractions[a]

Sample	% globulin N		% prolamin N		% glutelin N		Total	
	10-ml aliq.	10-ml aliq., Cl_3CCOOH ppt.	10-ml aliq.	10-ml aliq., Cl_3CCOOH ppt.	10-ml aliq.	10-ml aliq., Cl_3CCOOH ppt.	10-ml aliq.	10-ml aliq., Cl_3CCOOH ppt.
Flue cured								
Green leaf (mat.)	0.08	0.07	0.07	0.06	0.10	0.10	0.25	0.23
Cured leaf	0.10	0.09	0.06	0.06	0.11	0.10	0.27	0.25

[a] Values can also be expressed as milligrams as they are based on a 100-mg sample.

weight basis in the various protein fractions and Table III shows the percentage these amounts comprise of the total protein N.

Most of the PN was residual (Tables III and IV). Only 23% of the total protein N made up simple proteins in the leaves of young growing plants, and the glutelin fraction comprised the largest amount of simple proteins (10% of the total protein N, Tables III and IV). After the leaves matured and ripened, much of the N in the leaves had declined. Yet, despite a reduction in the actual amounts found (Table III), an increase in the percent of simple proteins was observed (36%, Table IV). Still most of the PN was in the residual form (64%). We did not determine if this pattern was typical during the growth of Burley and Maryland tobacco, but for flue cured, as the plants matured, the ratio of simple proteins to the total amount of PN increased, and more of these simple proteins were glutelins.

Residual protein N decreased during flue curing from 0.60 to 0.37% (Table III). This breakdown of bound protein during curing is not reflected in an increase in simple proteins (Table III) as most of the fractions remained the same. This reduction of residual protein N, however, does lower the total protein N level from 0.92 to 0.73% (Table III) which accounts for the apparent gain in simple proteins (Table IV). It was obvious from Table III that flue curing had little effect on the simple proteins; in fact a slight increase in the enzymic proteins (albumin and globulin) was observed.

Almost equal amounts of simple and residual proteins were found in the three types of tobacco (excluding green leaf) although Burley had slightly more simple proteins (52%, Table IV). The data in Table IV indicate that more of the simple proteins of all three types of tobacco were glutelins. Flue cured was relatively high in enzymic proteins (albumins and globulins) and Maryland relatively low (Table IV) based on these observations.

Extracting Soluble Protein Fractions. It was reported by Pirie (1955) that histological studies show some cells have not been torn open after usual grinding procedures and the extent to which chloroplasts are destroyed varies with species and physiological state. To see if some of the cells had not been torn open and entrapped protein remained intact, we homogenized the tissue in sequence with the respective solvents in a Virtis 45 homogenizer at top speed for 5 min and compared the N content of these precipitated proteins (precipitated with 5% Cl_3CCOOH as described) from this treatment with the N content of the precipitated proteins extracted by the mild stirring of the Osborne treatment. The results in Table V show about the same N values for these two treatments which indicate that homogenizing offers no advantage over stirring. Apparently no protein was left bound in the cells after stirring.

Is the N of the Soluble Protein Fractions Proteinaceous? In the solubility scheme used in reporting the data in Tables III and IV, only the aliquot from the albumin fraction was precipitated with 10% Cl_3CCOOH. This is because much nonprotein N is soluble in water. To determine whether the other soluble protein fractions (globulin, prolamin, and glutelin) were in fact proteinaceous, 5 ml of 10% Cl_3CCOOH was added to a 10-ml aliquot from each of the fractions and the samples were placed in an 80° bath for 20 min to see if a precipitate formed. A precipitate formed in each of the fractions. Nitrogen determinations were made on the precipitates from each fraction and compared with the N content of the 10-ml aliquots. The results shown in Table VI indicate the N content of the precipitates is about the same as the N content of the 10-ml aliquots for each fraction. These data show that the N of the 10-ml aliquots from each fraction is for the most part proteinaceous, and can be almost totally precipitated with Cl_3CCOOH.

In another test to see if the Cl_3CCOOH precipitate was in fact protein we used the xanthoproteic test for proteins. The test is specific for proteins containing amino acids which was well suited for our means because simple proteins yield amino acids upon hydrolysis. A yellow color produced when concentrated nitric acid is added to a protein whether solid or liquid confirms the presence of protein. We got an intense yellow color for each of the precipitates from the four fractions and this could be repeated continuously.

To determine if the water-soluble fraction (albumin) did contain enzyme, we tested for one that was sure to be present in tobacco, amylase. The other fractions were also tested for amylase. The test was to add a portion of the fraction to a starch solution treated with iodine to produce a blue color. If amylase was present it would cause the blue color to disappear by hydrolyzing starch and interfering with the starch–iodine complex. Of the four fractions tested, only the water-soluble fraction caused the blue color to

disappear. This test indicated that amylase was present only in the water-soluble fraction. This test was repeated using various temperatures, volumes, and concentrations of starch solution, iodine, and aliquots from the four fractions and the water-soluble fraction was the only fraction that caused the disappearance of the blue color and the color disappeared in a manner of minutes.

DISCUSSION

It has been reported (Redman, 1971) that the albumin and globulin classes include the enzyme proteins. Table IV shows an increase in the percent of total protein N for these two protein classes in flue-cured tobacco during curing. Albumin increased from 8% before curing to 12% after curing and globulin increased from 9% before curing to 14% after curing. Although there is a slight increase in the N actually found in these two fractions (Table III) what made this apparent gain was the big loss in residual protein N during curing from 0.60 to 0.37%, a loss of 38% residual protein during curing. Simple proteins had a net increase during flue curing of 0.04% (Table III), which is only 7% of the residual protein N. Therefore, a 31% loss in residual protein N is unaccounted for, since it was not picked up by the simple proteins. It is suggested that fraction I protein was located in the residual protein N fraction of the green mature leaf, and during curing was rapidly broken down. It is well known that fraction I is rapidly broken down during curing (Pogel et al., 1957; Johnstone and Plimmer, 1959; Kawashima et al., 1967), and although it is referred to as a soluble protein, it is usually extracted by a phosphate buffer ranging in pH from 7 to 8. Probably only a portion of this protein would have been soluble in extractions employed in this study. Water-soluble albumins were extracted in distilled water (pH 5.88), globulin in 5% KCl (pH 5.65), and glutelins in 0.2% KOH (pH 12.20). Therefore, probably most of fraction I protein was left intact after simple protein extraction in green mature leaf, but was broken down during flue curing.

Perhaps it was not surprising to find the enzyme proteins (albumin and globulin) remained relatively stable during flue curing as Axelrod and Jagendorf (1951) found phosphatase, invertase, and peroxidase activities remained constant in autolyzed leaf and Garner (1951) stated there is evidence for the presence even after flue curing of protease, lipase, emulsin, amylase, invertase, phosphatase, glycolase, pectase, ketone aldehyde mutase, oxidase, peroxidase, catalase, and reductase activities, although specific amounts were not cited. Pogel et al. (1957) found pectin methylesterase remained relatively unchanged during curing. Fraction II proteins possess much enzymatic activity and are relatively stable during curing (Johnstone and Plimmer, 1959; Kawashima et al., 1967). Zelitch and Zucker (1958) reported activity for some oxidative enzymes disappeared during curing but acknowledged activities for nonoxidative enzymes persisted even beyond 12 days (Frankenburg, 1946; Axelrod and Jagendorf, 1951; Pogel et al., 1957; Barrett, 1957).

The enzyme proteins, albumin and globulin, are reported to be cytoplasmic proteins (Kent, 1970; Redman, 1971). However, Sheen and Townes (1970) in their zymogram studies of soluble leaf proteins found a decrease in the number of bands during simulated air curing and concluded that cytoplasmic and structural proteins in membrane and cell wall disintegrated during curing, except for three glycoprotein bands. The results of our study suggest the cytoplasmic proteins Sheen and Townes found to disintegrate during curing were not albumin or globulin, or any of the simple proteins.

Also the data reported here suggest the familiar phrase "half of the soluble leaf protein is lost during curing" is misleading and applies mainly to fraction I protein, as simple proteins, technically the soluble proteins, remained relatively stable during curing.

Perhaps future studies on how increased fertilizer rates affect these levels of simple proteins and studies on the amino acid composition of simple protein fractions will tell us more about how various kinds and quantities of proteins affect the smoking quality of tobacco.

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