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STEROL DISTRIBUTION WITHIN GREEN AND AIR CURED TOBACCO

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Abstract—Stigmasterol and β-sitosterol varied greatly within the tobacco plant. Stigmasterol increased significantly in the shaded leaves. It was the predominant sterol at the plant base and in the basal leaf sections. β-Sitosterol predominated at the plant apex and in the leaf tip where light intensities were the highest. The other two major sterols in tobacco, campesterol and cholesterol, increased slightly from the base to plant apex. These same sterols increased slightly from basal to tip leaf sections. In the green plant, they decreased from the base to the plant apex. Light does not appear to have a large effect on the distribution of campesterol and cholesterol. There was a general decrease in total sterol during the final maturation and curing phases.

INTRODUCTION

RECENTLY, Heftmann[1] emphasized an earlier hypothesis[2] that plant sterols may function in a manner similar to animal sterols. In animals, sterols may serve as membrane stabilizers, precursors of other steroids, or as hormones. In the non-hormonal function the principal animal sterol, cholesterol, has been suggested to act as a stabilizer of the phospholipid molecule in red blood cell membranes.[3]

There have been similar attempts to examine plant cells to localize sterol presence and determine possible functions. Cholesterol was reported to constitute approximately 80% of the total sterol fraction isolated from chloroplast of *Phaseolus vulgaris* leaves.[4] Kemp and Mercer,[5] working with *Zea mays*, found the major portion of sterols in the mitochondrial and microsomal fractions rather than in the nuclear and chloroplast fractions. Recently, Knight[6] reported that sterols in the chloroplasts from several plant sources could be divided into two fractions.

In studies to determine the influence of sterols in plant membranes, Grunwald[7] presented evidence that certain exogenously applied sterols acted as membrane stabilizers, whereas other sterols had the reverse action. Cholesterol, β-sitosterol and stigmasterol decreased the methanol-initiated betacyanin leakage from red beet tissue. Campesterol, the other major tobacco sterol, was not included in these leakage experiments. In a further effort to determine if sterols were associated with the membrane containing fractions, Grunwald[8] examined their distribution in the organelles of tobacco leaf tissue. Free sterols, sterol glycosides and sterol esters were isolated from all cellular fractions.

The distribution of total sterol within the tobacco plant has been reported.[9] The total

[1] E. HEFTMANN, *J. Am. Oil Chem. Soc.* **47**, 90A (1970).
[2] E. HEFTMANN, *Ann. Rev. Plant Physiol.* **14**, 225 (1963).
[3] K. C. WINKLER and H. G. BUNGENBERG DEJONG, *Arch. Necrl. Physiol.* **25**, 431 (1941).
[4] E. T. MERCER and K. L. TREHARNE, In *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. 1, Academic Press, New York (1966).
[5] R. J. KEMP and E. T. MERCER, *Biochem. J.* **110**, 119 (1968).
[6] B. A. KNIGHTS, *J. Am. Oil Chem. Soc.* **42**, 90A (1970).
[7] C. GRUNWALD, *Plant Physiol.* **43**, 484 (1968).
[8] C. GRUNWALD, *Plant Physiol.* **45**, 663 (1970).
[9] D. L. DAVIS, P. D. LEGG and G. B. COLLINS, *Crop Sci.* **10**, 545 (1970).

sterol pattern within the plant was characterized by a linear increase from the plant base to the apex. Within a leaf, the sterol distribution was also linear from the basal section of the leaf to the tip. In an earlier study, Stedman and Rusaniwskyj[10] reported that the central region of the tobacco plant appeared to have slightly more sterols. Thus, the sterol quantities appear to be associated with some physiological phenomenon, such as cellular differentiation or light response.[11]

Based on studies of cellular[5,8] and total sterol distribution within the tobacco plant,[9] information is needed on component sterol distribution within the plant to assess the response of sterols to environmental factors. Also, these studies should yield indirect information on translocation and interconversion of sterols. The specific objectives of the present study were, (1) to determine which component sterols were responsible for differences in total sterol in the plant, (2) to examine the influence of the final maturation and curing phases, and (3) relate shifts in component sterols to light regimes which have been shown to influence plant sterols.

RESULTS AND DISCUSSION

Component Sterols

The four major component sterols in tobacco are cholesterol, campesterol, stigmasterol, and sitosterol.[8,12,13]

TABLE 1. QUANTITY OF COMPONENT STEROLS WITHIN AIR-CURED PLANTS OF *Nicotiana tabacum* L.

Stalk position leaf section	Cholesterol		Campesterol		Stigmasterol		Sitosterol		Total
	μg/g	wt. %	μg/g	wt. %	μg/g	wt. %	μg/g	wt. %	mg/g
Top 1/4									
Tip 1/3	247 b*		379 b		506 a		921 b		2·05 b
Middle 1/3	216 ab		328 ab		487 a		787 b		1·82 ab
Basal 1/3	191 a		309 a		492 a		618 a		1·61 a
Average	218 c	12·0	339 b	18·6	495 a	27·0	775 b	42·4	1·83 b
Third 1/4									
Tip 1/3	241 a		339 a		468 a		823 b		1·87 a
Middle 1/3	217 a		331 a		527 a		710 ab		1·79 a
Basal 1/3	216 a		318 a		647 a		623 a		1·80 a
Average	225 c	12·4	329 b	18·1	547 ab	30·1	719 b	39·4	1·82 b
Second 1/4									
Tip 1/3	202 a		345 a		543 a		711 c		1·80 a
Middle 1/3	193 a		282 a		585 a		505 b		1·57 a
Basal 1/3	193 a		264 a		689 a		342 a		1·49 a
Average	196 b	12·1	297 a	18·3	606 b c	37·5	519 a	32·1	1·62 a
Lower 1/4									
Tip 1/3	168 a		304 a		532 a		608 b		1·61a
Middle 1/3	182 a		316 a		638 a		587 b		1·72 a
Basal 1/3	182 a		278 a		780 b		435 a		1·68 a
Average	177 a	10·6	299 a	17·9	650 c	39·0	543 a	32·5	1·67 ab

* Values not followed by the same letter are significantly different at $p \leq 0{\cdot}05$. Comparisons are within vertical categories.

[10] R. L. STEDMAN and W. RUSANIWSKYJ, *Tob. Sci.* **4,** 17 (1960).
[11] P. B. BUSH, C. GRUNWALD and D. L. DAVIS, *Plant Physiol.* **47,** 745 (1971).
[12] R. L. STEDMAN, *Chem. Revs.* **68,** 153 (1968).
[13] C. GRUNWALD, C. J. KELLER and L. P. BUSH, *J. Agric. Food Chem.* **19,** 216 (1971).

TABLE 2. STEROL COMPOSITION IN THREE LEAF SECTIONS OF AIR-CURED *Nicotiana tabacum* L.

Leaf section	Cholesterol		Campesterol		Stigmasterol		Sitosterol		Total
	μg/g	wt. %	μg/g	wt. %	μg/g	wt. %	μg/g	wt. %	mg/g
Tip 1/3	214 b*	11·7	342 b	18·7	512 a	27·9	766 c	41·7	1·83 b
Middle 1/3	202 a	11·7	314 a	18·2	559 a	32·5	647 b	37·6	1·72 ab
Basal 1/3	195 a	11·9	293 a	17·8	652 b	39·5	504 a	30·8	1·64 a
Average	204	11·8	316	18·2	574	33·3	639	36·7	1·73

* Values not followed by the same letter are significantly different at $p \leqq 0{\cdot}05$. Comparisons are within vertical categories.

Cholesterol. Cholesterol accounted for approximately 11 to 14% of the total sterol in green and air-cured tobacco lamina (Tables 1 and 3). This sterol ranged from 168 to 247 μg/g of air-cured tissue and from 219 to 262 μg/g dry wt. in green tissue. Cholesterol decreased slightly in the lower leaves during the 6 weeks' maturation period following removal of the influorescence and 12 weeks' curing. The upper leaves of the green and air-cured plants contained similar amounts of cholesterol.

Campesterol. This sterol appears to be the most stable phytosterol in tobacco leaf tissue. It usually accounts for about 18% of the total sterol in tobacco and averages about 325 μg/g air-cured leaf (Table 1). The average was found to be slightly higher in the green tissue (Table 3). The pattern of distribution within the plant was similar to that for cholesterol. In the air-cured material there was a gradual increase from the lower part of the plant to the apex. This same pattern was observed within the sections of the leaf, with the tip section having the highest level of campesterol. In the green material, this sterol was highest at the base of the plant. It appears that during periods of maturation, senescence or curing, campesterol and cholesterol decrease slightly in the lower portions of the plant without a corresponding increase in the upper portions. This indicates degradation or a conversion of these two sterols to other steroids or related compounds in the more mature tissues.

Translocation upward into other leaves did not appear to occur since there was no increase in these two sterols in upper leaves. This does not exclude translocation into the vascular tissue of the leaf mid-vein and stalk. Davis *et al.*[9] reported a distribution of total sterol in transport tissue similar to leaf material, but in much lower quantities. Studies on other compounds have shown that losses in organic compounds during air-curing are

TABLE 3. QUANTITY AND RELATIVE PROPORTION (wt. %) OF COMPONENT STEROLS WITHIN GREEN, FLOWERING PLANTS OF *Nicotiana tabacum* L.

Stalk position	Cholesterol		Campesterol		Stigmasterol		Sitosterol		Total
	μg/g	wt. %	μg/g	wt. %	μg/g	wt. %	μg/g	wt. %	mg/g
Top 1/3	219 a*	14·0	313 a	20·1	478 a	30·7	548 a	35·2	1·56 a
Middle 1/3	238 ab	13·2	341 a	18·9	660 b	36·7	564 a	31·2	1·80 b
Lower 1/3	262 b	11·7	402 b	18·0	1010 c	45·2	562 a	25·1	2·24 c
Average	240	13·0	352	19·0	716	37·5	558	30·5	1·87

* Values not followed by the same letter are significantly different at $p \leqq 0{\cdot}05$. Comparisons are within vertical categories.

greater in plants with leaves attached to the stalk than in primed leaves.[14] Hamilton[15] found an increase in insoluble nitrogen in leaf mid-vein and stalks during curing. However, the gain in these plant parts did not account for the total losses from the leaf lamina. Tso[16] stated that respiratory losses account for a large decrease in dry matter during slow stalk curing. Thus, it appears that organic compounds, such as sterols, may be translocated into the stalk during air-curing; however, catabolic reactions may account for the predominant changes.

Stigmasterol—sitosterol. These two sterols will be discussed together since variation in levels appear to be related. These are both C_{29} sterols which differ only in one double bond at the C_{22} position in the C_{17} side chain of the molecule. Stigmasterol possesses the unsaturated side chain. The variation between these two sterols is far greater than for cholesterol and campesterol. Stigmasterol levels are greater in the lower part of the plant (Table 1) and basal section of the leaf (Table 2), while the opposite is the case for sitosterol. The higher proportion of the latter was always in the upper plant parts and tip sections of the leaf. In the air-cured leaf, stigmasterol accounted for 39·0% of the total sterol in the lower one-fourth of the plant and only 27·0% in the upper quarter, while the comparable values for sitosterol were 32·5 and 42·4% (Table 1). Stigmasterol decreased in proportion from 45·2% in the lower one-third of the green plant to 30·7% in the upper one-third, while sitosterol increased from 25·1 to 35·2% in these same two positions (Table 3). For the most part, the pattern of quantities of the two sterols was comparable to the above-mentioned percentages. Stigmasterol decreased from 650 to 495 μg/g from the lower to top quarter of the air-cured plant, while sitosterol increased from 543 to 775 μg/g (Table 1). The higher levels of stigmasterol were more pronounced in lower leaves of the green plant. There were no differences in sitosterol levels within the green plant. On a relative proportion (wt. %) basis, sitosterol did account for an increased proportion in the upper green leaves. The pattern among the leaf sections was the same as that observed for the stalk positions. Stigmasterol was usually higher in the basal sections, while sitosterol was the predominant sterol in the tip sections (Table 1). A point worth noting is that stigmasterol varies about the same within the leaf at each stalk position. The major difference in stigmasterol appears to be a higher level in the basal section of the leaf, while sitosterol has a linear distribution within each position. There was always a lower level of the latter sterol in the basal leaf section and a higher level in the tip section. There was very little difference in stigmasterol levels in the three sections in the upper plant parts.

Grunwald *et al.*[3] reported that stigmasterol and sitosterol in tobacco fluctuated more during the growing season than did cholesterol and campesterol. This is in agreement with the present report on changes within the plant. Variations in these two sterols are not unique in tobacco. Bush *et al.*[11] found that etiolated barley shoots had twice as much sitosterol as stigmasterol. Further it was observed that once the etiolated barley seedlings were exposed to light, there was a shift in the ratio of free stigmasterol to sitosterol to favor stigmasterol.

Based on the information in the literature that light influences phytosterols and that all membranes possess sterols,[8] some explanations may be given for the distribution of these two sterols within the tobacco plant. First, there may be a qualitative and/or quantitative influence of light on leaf sterols. Under conditions where the leaves were shaded (by upper leaves) in the latter part of the growing season, stigmasterol levels increased. Further, when

[14] C. O. JENSEN, *Ind. Engng Chem.* **44**, 306 (1952).
[15] J. L. HAMILTON, Personal communication.
[16] T. C. TSO, *Encyclopedia Chem. Tech.* **20**, 503 (1969).

the entire leaf is exposed to light, such as the upper leaves, there appears to be a uniform distribution within the leaf. It is unlikely that light has a direct effect on sitosterol since there was still a linear distribution within the upper leaves on the plant. The higher levels of sitosterol appear to be associated with tissues which have smaller cells per unit of leaf area; therefore, more membrane per unit area. The lower sitosterol levels are associated with areas where the tissue has larger cells such as the basal portion of the leaf or lower parts of the plant where cell membranes had begun to break down as a result of natural senescence.

The changes in relative amounts of stigmasterol and sitosterol may represent an interconversion. In an early report, Bennett *et al.*[17] suggested that sitosterol is a precursor to stigmasterol. This was later supported by Rowe.[18] Waters and Johnson[19] reported that the two sterols were not directly related biosynthetically by a hydrogenase–dehydrogenase system. However, Bennett and Heftmann[20] have demonstrated the conversion of sitosterol into stigmasterol in *Digitalis lanata*. Relatively low yields were obtained in these experiments. Our results indicate a close association of these two sterols in tobacco; however, there were instances in different plant parts where an increase in one sterol was not accompanied by a quantitative decrease in the other sterol. It does appear that as the tobacco plant develops sitosterol may be converted into stigmasterol under natural environmental conditions.

Translocation does not appear to be an explanation for the differences in stigmasterol and sitosterol levels within the plant, since there is the general increase–decrease relationship.

The ratios of component sterols were similar for the two tobacco varieties, with the only differences being that the variety with the highest sterol level contained a slightly higher level of sitosterol.

Total Sterols

The distribution of total sterols within the Burley tobacco plant was comparable to those reported previously.[9] There was a linear increase from the base of the plant to the apex and from the basal to tip section of the leaf (Tables 1 and 2).

There was a general decrease in total sterols during the final maturation and curing phases. This is in agreement with the results of Grunwald *et al.*[13]

EXPERIMENTAL

Plant material. Ky. 10 and Ky. 12, two varieties of tobacco (*Nicotiana tabacum* L.), were field grown and analyzed for component sterol distribution for 2 years at Lexington, Kentucky. Three field replications were used for this study in each of the years. These two varieties differed slightly in maturity, with Ky. 12 being the later maturing variety. The plot at Lexington had been in a bluegrass sod for several years. Available nitrogen levels were raised to 112 kg/ha and phosphorous and potassium levels to 336 kg/ha.

The tobacco flowers were removed (normal topping) when 50–75% of the flowers of the influorescence were open and at a level two leaves below the first floral branch. The green samples were obtained by removal of a leaf from three stalk positions of 20 plants in each of the three replications at the time of topping and the entire leaf freeze-dried. Lamina from the three stalk positions were processed separately, but the material from the 20 plants were combined in each replication. The entire plants were harvested 6 weeks after topping and air-cured in a conventional barn for about 12 weeks prior to removal of leaves from the stalks. Air-cured leaves were separated into four approximately equal stalk position group to examine sterols from the base to the plant apex. Within each of these groups, a sample of leaves were sectioned at right angles to the mid-vein into three approximately equal sections (basal, middle, and tip).

[17] R. D. Bennett, E. Heftmann, W. H. Preston and J. R. Hann, *Arch. Biochem. Biophys.* **103,** 74 (1963).
[18] J. W. Rowe, *Phytochem.* **4,** 1 (1965).
[19] J. A. Waters and D. F. Johnson, *Arch. Biochem. Biophys.* **112,** 387 (1965).
[20] R. D. Bennett and E. Heftman, *Steroids* **14,** 403 (1969).

The mid-veins were removed from the green freeze-dried and air-cured leaves. The resulting laminae were ground in a mill equipped with a 40-mesh screen. Constant moisture was achieved and samples (5 g) were weighed directly in a Soxhlet for the sterol determination.

Total sterols analysis. Total sterols were determined by a gravimetric method developed by Stedman and Rusaniwskyj.[21] The extraction allowed hydrolysis of glycosides and esters, yielding total values. Laboratory replication and standards were used as internal checks on the extraction procedures.

Individual sterol analysis. The digitonide obtained by the above-mentioned procedure was broken with an overnight treatment with 2 ml of pyridine containing 50 μg/ml of cholestane as an internal standard. Digitonin was precipitated with 10 ml ether and removed by centrifugation (12,000 **g**) for 30 min.[21] The filtered ether–pyridine mixture was evaporated by a slow air stream under a safety hood. The dried free sterols were dissolved in ethyl acetate (1 ml) prior to injection into a gas chromatograph.

Gas chromatography. Approximately 1–2 μl of sample solution were injected into each column of Barber–Colman Model 5000 equipped with dual coiled glass columns (1·8 m × 4 mm) and hydrogen flame ionization detectors. The dual injection method allowed a check on the analysis since each column system functioned as an independent unit. The glass columns were packed with 5% OVIOI on Anakrom ABS (80–90 mesh).[22]

Operating temperatures of 255, 275, and 300° were selected for the column oven, injection blocks and ionization detector oven, respectively. Helium was used as the carrier gas at a flow rate of 100 ml/min. It was found that even with close adjustment, the two detectors did not respond identically to a given sterol quantity; standards were therefore included in the analysis. A combined authentic standard of cholesterol, campesterol, stigmasterol and β-sitosterol was analyzed twice daily and used to correct for the relative responses of the detectors.

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[21] R. L. STEDMAN and W. RUSANIWSKYJ, *Tob. Sci.* **3**, 44 (1959).
[22] C. GRUNWALD, *Anal. Biochem.* **34**, 16 (1970).

Key Word Index—Nicotianum tabacum; Solanaceae; tobacco; Sterol changes in growth.