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Sterol Distribution in Intracellular Organelles Isolated from Tobacco Leaves[1]

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ABSTRACT

All membrane-containing fractions isolated from tobacco leaves contained free sterols, sterol glycosides, and sterol esters. The three sterol forms increased, on a dry weight basis, with a decrease in particle size. The supernatant fraction contained only trace amounts of sterol. The major sterols in all cellular fractions, in the order of decreasing amounts, were: stigmasterol, β-sitosterol, campesterol, and cholesterol. The 500*g* pellet contained the largest percentage of free sterol, while the 46,000*g* pellet contained the largest percentage of esterified sterol. The individual sterol composition of the free sterol and sterol glycoside fraction was very similar; however, the composition of the sterol ester fraction varied widely among intracellular fraction. The intracellular distribution pattern of cholesterol-^{14}C added to the isolation medium provided evidence that the intracellular sterol distribution pattern is not an artifact. These results support the suggestion that sterols in plant cells may have a physiological function associated with membranes.

Although the biosynthesis and identification of sterols in plants have received much attention in recent years, their function in higher plants is unknown. In 1930, Schönheimer *et al.* (12) suggested that sterols in plants were merely waste products. In 1963, Heftmann (5) challenged this hypothesis because high concentrations of steroids had been reported in reproductive organs and meristems of plants. It has since been shown that sterols in plants may stabilize membranes in the same way steroids function in animal cell membranes (2). It has been suggested that sterols might be involved in controlling the permeability of membrane (2).

It has been reported that chloroplasts isolated nonaqueously from *Phaseolus vulgaris* leaves contained a sterol component which was identified by gas chromatography as cholesterol, and this component constituted about 80% of the total sterol fraction (11). Similarly, chloroplasts isolated from *Spinacia oleracea* leaves contained cholesterol, β-spinasterol, and stigmast-7-enol (1). More recently Kemp and Mercer (7) reported that in *Zea mays* the nuclear fraction contained the greatest proportion of cholesterol. Apparently, the cholesterol content in corn shoot chloroplasts (7) is markedly different from that found in bean chloroplasts (11).

If, as suggested (2), the sterols in plant cells are associated with membranes, one should find relatively large quantities in the membrane-containing cell fractions. The objective of this study was to examine the sterol distribution and composition of intracellular organelles isolated from mature *Nicotiana tabacum* leaves.

MATERIALS AND METHODS

Tobacco plants (*N. tabacum* L. var. Ky-12) were grown in the greenhouse for 90 days. The plants were in flower at this time. Five hundred grams of fresh tobacco leaves were homogenized in a 4-liter Waring blender at 3 C in 1 liter of 0.1 M phosphate buffer at pH 7.5 containing 0.5 M sucrose, 0.01 M NaCl, and 0.04 M disodium EDTA. A total of 10 kg of tobacco was used in this experiment. The homogenate was filtered through four layers of cheesecloth to remove most of the cell debris. Fractionation was by centrifugation with four Sorvall RC-2 automatic superspeed refrigerated centrifuges with SS-34 rotors. The crude cell-free extract was centrifuged at 500*g* for 10 min, 2,500*g* for 30 min, 16,000*g* for 30 min, and 46,000*g* for 90 min. All pellets thus obtained were dried at 80 C and ground in a Sorvall Omni-Mixer.

The total, free, glycosidic and esterified sterols were isolated by a modified method of Stedman and Rusaniswkyi (14). Samples of 6 g dry weight were extracted with 250 ml of acetone in a Soxhlet apparatus for 24 hr, cooled, and divided into two equal aliquots. One aliquot was evaporated to dryness under vacuum, and 25 ml of 95% ethanol containing 0.13 ml H_2SO_4 were added and refluxed for 12 hr to cleave the sterol glycosides. Fifteen milliliters of 10% KOH in 95% ethanol were added and refluxed for 30 min to hydrolyze the esterified sterols. This sample gave total sterols since both the glycosides and esters have been hydrolyzed. The second aliquot was also taken to dryness under vacuum and 30 ml of 95% ethanol were added, heated for a few minutes and cooled. This sample gave free sterols. The sterols were extracted from the above mixtures three times with 30 ml of *n*-hexane and enough water to obtain two layers. The *n*-hexane fractions of each sample were combined, back extracted twice with 90% methanol, and taken to dryness under vacuum. The ethanol and methanol fractions of the second aliquot (sample from which only the free sterols were extracted) were combined and evaporated to dryness under vacuum, dissolved in 25 ml of 95% ethanol containing 0.13 ml H_2SO_4. After refluxing for 12 hr the sterol glycosides were extracted as free sterols as described above. Thus, values for free, glycosidic and total sterols were obtained directly and values for esterified sterols were calculated.

The resultant *n*-hexane-extracted residues (free, glycosidic, and total sterols) were dissolved in 20 ml of boiling absolute

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ethanol, and added to this mixture were 10 ml of hot 2% digitonin in 80% (w/v) ethanol and 5 ml of hot water. The samples were allowed to cool and remain at room temperature overnight. The precipitate was washed three times with 80% ethanol and three times with diethyl ether. The white sterol-digitonide precipitate was dried overnight at room temperature.

The qualitative and quantitative sterol analysis was performed by gas chromatography with a F & M model 402 equipped with a flame ionization detector (6). The column was 1.80 m U-shaped glass with a 6 mm internal diameter and packed with Anakrom ABS 80/90 mesh coated with 5% OV-101 (3). The column temperature was 250 C and the flash heater temperature was kept at least 25 C above that of the column. Helium was the carrier gas at a flow rate of 100 ml/min. The sterol-digitonide precipitate was broken with 2.0 ml of pyridine containing a known amount of internal standard (cholestane), heated at 70 C for 2 hr and subsequently left at room temperature for 12 hr. The digitonin was removed by precipitation with 40 ml of diethyl ether. The ether layer was recovered, taken to dryness, and taken up in ethyl acetate for injection into the gas chromatograph. The quantitative analysis of individual sterols was carried out by measuring the peak areas of the sterols and the internal standard, making corrections for the differences in relative weight response (4). A more complete discussion of the analytical procedure to determine individual sterols by gas chromatography has been reported elsewhere (3, 4).

A Packard model 4322 Tri-Carb scintillation spectrometer was used for the determination of cholesterol-^{14}C. Corrections were made for the quenching effect of chlorophyll by adding known quantities of cholesterol-^{14}C. The chlorophyll determination was made spectrophotometrically by the method of Kirk (8). Each fraction was exhaustively extracted with 80% aqueous acetone at room temperature in the dark. The absorbance of the acetone extract was measured at 663 and 645 nm with a Beckman DU spectrophotometer.

RESULTS AND DISCUSSION

All intracellular organelle fractions isolated in aqueous medium contained sterol (Table I). The amount of total sterol per gram dry weight of tissue increased with a decrease in particle size. The cell residue fraction when compared to the tobacco leaf contained only a small amount of sterol, 0.23 mg of sterol per g dry weight, suggesting that the cell wall contains very little, if any, sterol. No special effort was made to remove the membranous particles from this fraction; the 500*g* pellet which was made up of cell debris, nuclei, and some chloroplasts was also low in sterol content, 0.33 mg of sterol per gram dry weight. The 2,500*g* fraction which contained the largest amount of chlorophyll (Table VI) was somewhat higher in sterol content, 0.76 mg of sterol per g dry weight. The 16,000*g* fraction which was mainly mitochondria contained 1.44 mg of sterol per gram dry weight. The 46,000*g* fraction or microsomal pellet contained the largest amount of sterol, 8.34 mg/g dry weight. The supernatant contained only trace amounts of sterol, less than 0.01 mg/g dry weight, and it is reasonable to assume that this is due to the incomplete removal of microsomes. Kemp and Mercer (7) found that in corn the mitochondrial and microsomal fractions also contained the bulk of the sterols; however, they did not analyze the supernatants.

The 500*g*, 2,500*g*, 16,000*g*, and 46,000*g* pellets were analyzed for free sterols and sterol glycosides. The esterified sterol values were calculated from the above data (Table I). The free sterols and sterol glycosides increased with a decrease in particle size, however, the percentage of free sterols and sterol glycosides decreased. In the 500*g* pellet, 70% was free sterol, while in the 46,000*g* pellet only 11% was in this form. The percentage of sterol glycoside also decreased with particle size; however, the decrease was not so pronounced—a decrease from 12% in the 500*g* pellet to 3% in the 46,000*g* pellet. Conversely, the esterified sterols increased in absolute and percentage values, with a decrease in particle size. The 500*g* pellet contained mainly free sterols, while the 46,000*g* pellet contained mainly sterol esters. At the present, any physiological implication can only be indirect.

Table I. *Sterol Distribution of Intracellular Organelle Fractions Isolated from Tobacco Leaves.*

Isolation medium: 0.1 M potassium phosphate buffer, pH 7.5, containing 0.5 M sucrose, 0.01 M NaCl, and 0.04 M EDTA.

Fraction	Sterols						
	Total	Free		Glycoside		Esters	
	mg/g dry wt	*mg/g dry wt*	%	*mg/g dry wt*	%	*mg/g dry wt*	%
Tobacco leaf	1.79	0.27	15	0.04	2	1.48	83
Residue in cheesecloth	0.26	...	...	...	...	...	...
500*g* for 10 min pellet	0.33	0.23	70	0.04	12	0.06	18
2,500*g* for 30 min pellet	0.76	0.35	46	0.07	9	0.34	45
16,000*g* for 30 min pellet	1.44	0.44	31	0.10	7	0.90	62
46,000*g* for 90 min pellet	8.34	0.95	11	0.26	3	7.13	86
Supernatant	<0.01	...	...	...	...	...	...

Table II. *Composition of Total Sterol Fraction of Intracellular Organelles Isolated from Tobacco Leaves*

Fraction	Total Sterol	Stigmasterol	β-Sitosterol	Campesterol	Cholesterol
	mg/g dry wt	%	%	%	%
Tobacco leaf	1.79	41	36	14	9
500*g* pellet	0.33	41	37	14	8
2,500*g* pellet	0.76	40	37	15	8
16,000*g* pellet	1.44	40	36	15	9
46,000*g* pellet	8.34	40	38	14	8

The four major tobacco sterols (13, 6)-cholesterol (cholest-5-en-3β-ol), campesterol (24α-methylcholest-5-en-3β-ol), stigmasterol (24β-ethylcholest-5,22-dien-3β-ol), and β-sitosterol (24β-ethylcholest-5-en-3β-ol)—were found in all membrane-containing fractions (Table II). The most abundant sterol in all cellular fractions was stigmasterol, which accounted for roughly 40% of the total sterols. The next highest sterol was β-sitosterol at 37%, followed by campesterol and cholesterol at 14 and 8%, respectively. Only very minor variations in sterol composition were detected in the total sterol fraction among intracellular pellets, and part of these variations, about 3%, may be due to the method employed (4).

The composition of the free sterols is given in Table III. Stigmasterol, in all cellular fractions, accounted for about 50% of the free sterols, which is 10% higher than its content in the total sterol fraction. β-Sitosterol was the next highest component in all cellular fractions at 25%. However, this is 10% lower than the β-sitosterol content in the total sterol fraction. The percentage of campesterol and cholesterol in all cellular pellets was essentially the same as in the total sterol fraction. These results do not agree with those reported by Kemp and Mercer (11) for 21-day-old corn shoots. They found that the nuclear fraction had the highest free cholesterol content, 20 to 22%, while the mitochondrial and microsomal fraction contained only 1%. Conversely, it has been reported that cholesterol in *Phaseolus vulgaris* leaf chloroplast constituted 80% of the total sterol fraction (11); however, the samples were not analyzed for free sterols. Kemp and Mercer (7) found in their free sterol fractions

Table III. *Composition of Free Sterol Fraction of Intracellular Organelles Isolated from Tobacco Leaves*

Fraction	Free Sterols	Stigmasterol	β-Sitosterol	Campesterol	Cholesterol
	mg/g dry wt	%	%	%	%
Tobacco leaf	0.27	48	26	16	10
500*g* pellet	0.23	49	25	15	11
2,500*g* pellet	0.35	48	24	16	12
16,000*g* pellet	0.44	52	25	14	9
46,000*g* pellet	0.95	52	23	15	10

Table IV. *Composition of Glycoside Sterol Fraction of Intracellular Organelles Isolated from Tobacco Leaves*

Fraction	Sterol Glycosides	Stigmasterol	β-Sitosterol	Campesterol	Cholesterol
	mg/g dry wt	%	%	%	%
Tobacco leaf	0.04	48	26	16	10
500*g* pellet	0.04	50	26	13	11
2,500*g* pellet	0.07	46	27	16	11
16,000*g* pellet	0.10	46	26	16	12
46,000*g* pellet	0.26	46	27	16	11

at least twice as much β-sitosterol as stigmasterol, while in tobacco stigmasterol was generally 20% higher (Table III).

The composition of sterol glycosides follows the same pattern as that of the free sterols (Table IV). No information is available in the literature as to the intracellular sterol glycoside distribution in any other plant tissue, and any comparison will have to await further studies with other plant species.

The largest variation in sterol composition was found in the esterified sterol fraction (Table V). The sterol ester fraction of the 500g pellet contained 82% β-sitosterol and only 3% stigmasterol. Cholesterol accounted for less than 1% of this fraction. The 2,500*g* pellet contained 52% esterified β-sitosterol and 13% esterified stigmasterol, and the 46,000*g* pellet contained about equal quantities of β-sitosterol and stigmasterol. The cholesterol content increased from less than 1% in the 500*g* pellet to 8% in the 16,000*g* and 46,000*g* pellets. Campesterol was constant at 14% in all cellular fractions. These sterol composition values are unlike those of Kemp and Mercer (7) obtained from corn shoots. They found cholesterol to be the dominant sterol ester in the nuclear and chloroplastidic fraction, and β-sitosterol to be the dominant sterol ester in the mitochondrial and microsomal fraction. They reported that stigmasterol never accounted for more than 9% of the esterified sterol fraction in any intracellular organelles. These differences in sterol composition are probably due to species difference.

It may be argued that the sterol distribution pattern in this analysis and those of other researchers (1, 7, 11) may have been due to a partitioning effect of the sterols between the aqueous phase of the supernatant and the lipid phase of the cytoplasmic organelles. In order to test this possibility, free labeled cholesterol-^{14}C (6 μc/liter) was added to the aqueous isolation medium before the leaf tissue was homogenized. The cholesterol-^{14}C distribution pattern is presented in Table VI. About 18% of the labeled cholesterol, based on a per gram dry weight basis, was recovered from the cell residue fraction, and 82% was found in the crude homogenate. The 500*g* fraction contained 64% of the total radioactive cholesterol and, as the intracellular organelle size decreased, a decrease in radioactive cholesterol was also found. The supernatant contained only 0.2% of the ^{14}C label. The distribution of the added cholesterol-^{14}C was very different

Table V. *Composition of Esterified Sterol Fraction of Intracellular Organelles Isolated from Tobacco Leaves*

Fraction	Esterified Sterols	Stigmasterol	β-Sitosterol	Campesterol	Cholesterol
	mg/g dry wt	%	%	%	%
Tobacco leaf	1.48	39	38	14	9
500*g* pellet	0.06	3	82	15	<1
2,500*g* pellet	0.34	31	52	14	3
16,000*g* pellet	0.90	33	43	15	9
46,000*g* pellet	7.13	38	40	14	8

Table VI. *Intracellular Distribution of Adsorbed Cholesterol-^{14}C and Chlorophyll*

Cholesterol-^{14}C at 6 μc/liter was added to the aqueous isolation medium before the tobacco leaves were homogenized.

Fraction	Chlorophyll	Cholesterol-^{14}C	
	mg/g dry wt	*cpm/g dry wt*	%
Residue in cheesecloth	0.78	1,372	18.1
500*g* pellet	2.42	4,810	63.6
2,500*g* pellet	3.11	1,043	13.8
16,000*g* pellet	1.57	216	2.9
46,000*g* pellet	0.44	106	1.4
Supernatant	<0.01	12	0.2

from that of the free sterol distribution pattern. The largest amount of sterol on a weight basis was found in the 46,000*g* fraction (Table I); however, this fraction had only 1.4% of the ^{14}C label. The largest amount of radioactive cholesterol was recovered from the 500*g* fraction, which had a relatively low sterol content. From visual observations it appeared that the distribution of cholesterol-^{14}C and chlorophyll may have had some relationship; however, after determining the total chlorophyll content of each fraction it was clear that the 2,500*g* fraction, which contained the greatest amount of chlorophyll, contained only 14% of the ^{14}C label (Table VI). A relationship between chlorophyll and radioactive cholesterol could not be established.

These results suggest that the intracellular sterol distribution in tobacco leaves is not due to a partitioning effect of the sterols between the aqueous isolation medium and the lipid phase of the organelles, and apparently most of the sterols are associated with membrane-containing organelles. The data reported in this paper support the suggestion that phytosterols may be involved in the structure and function of membranes (2), much the same way as in animal cells (15), and microorganisms (9, 10). However, further work is needed to assign specific functions to the quantitative and qualitative sterol make-up of intracellular organelles.

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