Studies on the Sterols and Sterol Esters of the Intracellular Organelles of Maize Shoots

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1. The composition of the esterified and unesterified sterols of the nuclear, chloroplastidic, mitochondrial and microsomal fractions of 21-day-old maize shoots was examined. 2. The microsomal and mitochondrial fractions contain the bulk of the sterols of the tissue. 3. Only 1% of the sterol isolated from all the organelles is esterified. 4. The nuclear fraction has the greatest proportion of esterified sterol and the microsomal fraction the least. 5. 4-Demethyl sterols constitute the bulk of both esterified and unesterified sterols in all organelle fractions. 6. Cholesterol is the major esterified 4-demethyl sterol of the nuclear and chloroplastidic fractions, but only the nuclear fraction has an appreciable proportion of unesterified cholesterol. 7. Sterol esters of linolenic acid are more abundant in the mitochondrial and microsomal fractions than in the other two fractions.

Previous work in our Laboratory has shown that there is a greater proportion of esterified cholesterol in the shoots of maize (*Zea mays*) seedlings than in the roots or seed residues (Kemp & Mercer, 1968). Also, chloroplasts isolated non-aqueously from dwarf-bean (*Phaseolus vulgaris*) leaves contain a sterol, identified by gas-liquid chromatography as cholesterol, that constitutes about 80% of the total sterol fraction (Mercer & Treharne, 1966). Similarly, chloroplasts isolated from spinach (*Spinacia oleracea*) leaves contain cholesterol along with α -spinasterol and stigmast-7-enol, all of which may be esterified with palmitic acid (Eichenberger & Menke, 1966). The aim of the investigation reported here was to decide whether cholesterol resides predominantly in the chloroplast or is distributed throughout the plant cell, and to determine the composition of the sterol and sterol ester fractions of the intracellular organelles.

EXPERIMENTAL

Maize seeds [*Zea mays* var. South African White Horse Tooth, obtained from Gunsons (Seeds) Ltd., London, E.C. 3] were soaked in water for 24 hr. and then planted 0.5 in. deep in moist vermiculite contained in stainless-steel trays (200 seeds/14 in. $\times$ 12 in. $\times$ 2 in. tray). They were allowed to germinate for 21 days at 28° under continuous illumination. After 14 days the seedlings were watered each day with a proprietary nutrient solution (10.6% N, 4.4% phosphate, 1.7% K) diluted 1:1000 with water. At the end of the growth period the shoots (25 cm. long) were excised immediately above the first leaf and washed with deionized water. The other parts of the seedlings were discarded.

Isolation of intracellular organelles. Two batches of maize shoots (each 225 g. wet wt.) were cut up into small pieces with scissors, and each batch was then homogenized with 260 ml. of ice-cold grinding medium (0.5 M-sucrose-0.05 M-tris-1 mM-EDTA, pH 7.4) for three periods of 1 min. interspaced by 1 min. cooling periods in an MSE Ato-Mix blender operating at half-maximum speed. The homogenate was then strained through two layers of muslin and centrifuged for 15 min. at 2000g in a Sorvall RC-2B centrifuge (SS-34 rotor). The 2000g supernatant was decanted from the sedimented crude chloroplast fraction and centrifuged for 15 min. at 10000g in the same rotor to sediment mitochondria. The microsomal fraction was sedimented by centrifuging the 10000g supernatant for 2 hr. at 75000g in a Beckman Spinco model L ultracentrifuge (no. 30 rotor). The 75000g supernatant was discarded. The crude chloroplast pellet was resuspended in grinding medium, and 10 ml. samples were carefully layered on top of discontinuous sucrose density gradients in plastic centrifuge tubes. Each gradient consisted of three layers of grinding medium containing 35% (w/v) sucrose (top layer, 5 ml.), 45% (w/v) sucrose (middle layer, 7.5 ml.) and 60% (w/v) sucrose (bottom layer, 5 ml.) respectively (Kirk, 1963). The tubes were centrifuged for 15 min. at 2000g and then for a further 60 min. at 44000g in a Beckman Spinco model L ultracentrifuge (SW 25.1 swinging-bucket rotor). This procedure resulted in a distinct band of chloroplasts in the middle layer and a sediment of nuclei at the bottom of the tubes. The chloroplastidic and nuclear fractions were recovered by careful pipetting. All the procedures described in this section were carried out at 0–2°.

Lipid extraction and chlorophyll determination. The chloroplastidic, nuclear, mitochondrial and microsomal fractions were then exhaustively extracted with acetone. The acetone extracts were diluted with 4 vol. of water and extracted several times with peroxide-free diethyl

ether to remove the lipid. The ethereal extracts were washed with water, dried over anhydrous Na_2SO_4 and finally evaporated to dryness under N_2 . Samples containing one-thousandth of the lipid from each organelle were dissolved in aq. 80% (v/v) acetone and the content of total chlorophyll ($a+b$) was determined spectrophotometrically by the method of Kirk (1967).

Chromatography of the lipid. The remaining lipid from each organelle was chromatographed on columns of acid-washed Brockmann grade III alumina (Woelm anionotropic) with light petroleum (b.p. 40–60°), 2% E/P* and 40% E/P for development. The sterol esters were eluted in the 2%-E/P fraction and the unesterified sterols in the 40%-E/P fraction (Kemp, Goad & Mercer, 1967).

Other procedures. Saponification of sterol esters and the 40%-E/P fraction, thin-layer and gas-liquid chromatography of sterol esters, free sterols and fatty acids, and colorimetric determinations of sterols were carried out as described in the preceding paper (Kemp & Mercer, 1968). When amounts of sterol were too small for colorimetric estimation, known samples were analysed by gas-liquid chromatography, and the absolute quantities of the components were measured by comparison of their peak areas with those of known quantities of the same compounds chromatographed under identical conditions. This procedure had an error of less than 5%.

RESULTS

The chloroplastidic, nuclear, mitochondrial and microsomal fractions were isolated from 450g. wet wt. of 21-day-old maize shoots. The lipid was

* Abbreviation: E/P, solution of diethyl ether in light petroleum (b.p. 40–60°).

extracted from each fraction and its content of chlorophyll was determined. Table 1 shows that the bulk of the lipid was in the mitochondrial and microsomal fractions. It is also evident from Table 1 that the mitochondrial, microsomal and, to a smaller extent, nuclear fractions were contaminated with chloroplasts or chloroplast fragments.

The lipid from each fraction was then chromatographed on alumina. The crude sterol ester (2%-E/P) fraction from this chromatogram was purified by thin-layer chromatography and divided into two parts. One part (three-quarters of the total) was saponified and the resulting free sterols and fatty acids were isolated and analysed separately. The other part (one-quarter of the total) was analysed directly by a gas-liquid-chromatographic procedure capable of separating sterol esters according to the number of carbon atoms in their molecules. The free sterols obtained from the sterol esters on saponification were separated by thin-layer chromatography into 4,4-dimethyl, 4 α -methyl and 4-demethyl sterols. Each type of sterol was then determined colorimetrically, and its composition was determined by gas-liquid chromatography. The fatty acids obtained from the sterol esters were methylated with diazo-methane and, after purification by thin-layer chromatography, the methyl esters were analysed by gas-liquid chromatography. The crude unesterified sterol (40%-E/P) fraction from the alumina chromatogram was separated into 4,4-dimethyl, 4 α -methyl and 4-demethyl sterols by

Table 1. *Distribution of lipid, chlorophyll and esterified and unesterified sterols in the intracellular organelle fractions from maize shoots*

Organelle fraction	Wt. of lipid (mg.)	Wt. of chlorophyll (mg.)	Chlorophyll as % of lipid	Esterified sterol		Unesterified sterol		Total sterol as % of lipid
				Wt. ($\mu\text{g.}$)	$\mu\text{g./mg. of lipid}$	Wt. ($\mu\text{g.}$)	$\mu\text{g./mg. of lipid}$	
Nuclear	8.5	0.585	6.9	20.7	2.43	67.7	7.96	1.04
Chloroplastidic	138.4	49.6	36.0	38.4	0.28	976.8	7.05	0.73
Mitochondrial	339.9	87.4	26.0	103.4	0.30	8870	26.00	2.6
Microsomal	313.5	53.8	17.0	96.9	0.31	16932	54.10	5.4

Table 2. *Weights of esterified and unesterified 4,4-dimethyl, 4 α -methyl and 4-demethyl sterols of the intracellular organelle fractions from maize shoots*

Organelle fraction	4,4-Dimethyl sterols		4 α -Methyl sterols		4-Demethyl sterols	
	Unesterified ($\mu\text{g.}$)	Esterified ($\mu\text{g.}$)	Unesterified ($\mu\text{g.}$)	Esterified ($\mu\text{g.}$)	Unesterified (mg.)	Esterified ($\mu\text{g.}$)
Nuclear	1.5	1.5	1.7	1.1	0.065	18.1
Chloroplastidic	38.5	9.2	20.0	1.2	0.918	28.0
Mitochondrial	80.3	20.3	70.9	4.0	8.718	79.1
Microsomal	181.9	31.1	121.9	3.3	16.628	62.5
Total	302.2	62.1	214.5	9.6	26.329	187.7

Table 3. *Composition of the 4-demethyl sterols derived from the sterol esters of the intracellular organelles of maize shoots*

Organelle fraction	% of sterols determined after gas-liquid-chromatographic separation on 3% OV-1			
	Cholesterol	Campesterol	Stigmasterol	β -Sitosterol
Nuclear	76	1	1	22*
Chloroplastidic	52	10	3	35
Mitochondrial	32	11	9	48
Microsomal	32	9	8	51

Organelle fraction	% of sterols determined after gas-liquid-chromatographic separation on 1% QF-1		
	Cholesterol	Campesterol+stigmasterol	β -Sitosterol
Nuclear	90	2	8
Chloroplastidic	53	11	36
Mitochondrial	35	16	49
Microsomal	32	16	52

* A contaminant, probably a long-chain alcohol, co-chromatographed with β -sitosterol on the 3% OV-1 column. This was only found in the nucleus. The correct percentage of β -sitosterol in the esterified 4-demethyl sterol fraction is probably 8% as determined with the 1% QF-1 column, which separated the β -sitosterol from the contaminant.

Table 4. *Composition of the unesterified 4-demethyl sterols of the intracellular organelles of maize shoots*

Organelle fraction	% of sterols determined after gas-liquid-chromatographic separation on 3% OV-1			
	Cholesterol	Campesterol	Stigmasterol	β -Sitosterol
Nuclear	22	9	18	51
Chloroplastidic	2	13	26	59
Mitochondrial	1	12	26	61
Microsomal	1	12	27	60

Organelle fraction	% of sterols determined after gas-liquid-chromatographic separation on 1% QF-1		
	Cholesterol	Campesterol+stigmasterol	β -Sitosterol
Nuclear	21	28	51
Chloroplastidic	3	34	63
Mitochondrial	1	37	62
Microsomal	1	37	62

thin-layer chromatography and each sterol type was determined colorimetrically and analysed by gas-liquid chromatography.

Table 2 shows the amounts of esterified and unesterified 4,4-dimethyl, 4 α -methyl and 4-demethyl sterols isolated from the intracellular organelle fractions. The unesterified 4-demethyl sterols represented 97.2% of the total sterol isolated from all the organelle fractions and of this 61.4% and 32.2% was in the microsomal and mitochondrial fractions respectively. Only 1% of the total sterol isolated from all the organelles was esterified. The percentages of esterified sterol in the nuclear, chloroplastidic, mitochondrial and microsomal

fractions were 23.4, 3.4, 1.15 and 0.57% respectively. The percentages of esterified sterol in the total 4,4-dimethyl, 4 α -methyl and 4-demethyl sterols were 17, 4.3 and 0.7% respectively.

Table 3 shows the composition of the esterified 4-demethyl sterol fraction isolated from the intracellular organelles. The composition was much the same in the microsomal and mitochondrial fractions, with β -sitosterol and cholesterol comprising about 50% and 30% of the total respectively. The chloroplastidic and nuclear fractions had higher proportions of cholesterol (52 and over 75% respectively) and lower proportions of β -sitosterol (35 and 8% respectively). The proportions of

Table 5. *Composition of the 4 α -methyl sterols derived from the sterol esters of the intracellular organelles of maize shoots*

Organelle fraction	% of sterols determined by combining the results of gas-liquid-chromatographic separations on 3% OV-1 and 1% QF-1			
	Obtusifoliol	24-Methylenelophenol	Cycloeucalenol	24-Ethylidenelophenol
Nuclear	—*	—*	—*	—*
Chloroplastidic	7	64	Trace	29
Mitochondrial	9	74	Trace	17
Microsomal	8	68	Trace	24

* Not detected.

Table 6. *Composition of the unesterified 4 α -methyl sterols of the intracellular organelles of maize shoots*

Organelle fraction	% of sterols determined by combining the results of gas-liquid-chromatographic separations on 3% OV-1 and 1% QF-1			
	Obtusifoliol	24-Methylenelophenol	Cycloeucalenol	24-Ethylidenelophenol
Nuclear	—*	—*	—*	—*
Chloroplastidic	20	67	Trace	13
Mitochondrial	21	64	Trace	15
Microsomal	13	49	16	22

* Not detected.

Table 7. *Composition of the 4,4-dimethyl sterols derived from the sterol esters of the intracellular organelles of maize shoots*

Organelle fraction	% of sterols determined after gas-liquid-chromatographic separation on 3% OV-1			
	α -Amyrin	β -Amyrin	Cycloartenol	24-Methylenecycloartanol
Nuclear	—*	—*	—*	—*
Chloroplastidic	29	30	16	25
Mitochondrial	20	26	28	26
Microsomal	18	31	28	23

* Not detected.

campesterol and stigmasterol were low in all the organelle fractions and particularly so in the nuclear fraction.

Table 4 shows the composition of the unesterified 4-demethyl sterol fraction from the intracellular organelles and presents a different picture from that shown by Table 3. In all four organelle fractions β -sitosterol was the most abundant component (51–63%) with considerable proportions of campesterol and stigmasterol. Only the nuclear fraction had an appreciable proportion (22%) of cholesterol.

The esterified and unesterified 4 α -methyl sterols were analysed by a combination of the gas-liquid-chromatographic results from two columns, 3% OV-1 and 1% QF-1 (Kemp & Mercer, 1968). It was

found that the 4 α -methyl sterols from all the organelle fractions contained considerable amounts of a non-sterol component, which, unless removed, interfered with the gas-liquid-chromatographic separation of the 4 α -methyl sterols. This component was removed by crystallization from hot cyclohexane solution, when it was deposited as white flakes. Infrared spectroscopy of the crystals revealed the presence of $-\text{[CH}_2\text{]}_n-$ and $-\text{OH}$ groups, suggesting a long-chain alcohol. Tables 5 and 6 respectively show the composition of the esterified and unesterified 4 α -methyl sterols isolated from the organelle fractions. Neither esterified nor unesterified 4 α -methyl sterols could be detected in the nuclear fraction. Outside the nucleus 24-methylenelophenol was the main esterified and

Table 8. *Composition of the unesterified 4,4-dimethyl sterols of the intracellular organelles of maize shoots*

Organelle fraction	% of sterols determined after gas-liquid-chromatographic separation on 3% OV-1			
	α -Amyrin	β -Amyrin	Cycloartenol	24-Methylenecycloartanol
Nuclear	—*	—*	—*	—*
Chloroplastidic	31	10	21	38
Mitochondrial	20	25	21	34
Microsomal	34	7	32	27

* Not detected.

Table 9. *Composition of the fatty acid fraction derived from the sterol esters of the intracellular organelles of maize shoots*

Fatty acid*	% of fatty acids determined after gas-liquid-chromatographic separation on 25% DEGS			
	Nuclear fraction	Chloroplastidic fraction	Mitochondrial fraction	Microsomal fraction
12:0	1.8	16.6	11.6	12.2
14:0	8.3	10.4	10.2	10.2
14:1	3.6	4.5	3.8	4.2
15:0	6.3	3.6	2.7	3.0
15:1	2.4	3.0	1.8	2.5
16:0	22.0	13.6	11.1	15.8
16:1	15.5	7.1	7.0	6.9
17:0	2.6	1.8	1.6	1.1
17:1	2.4	1.6	0.9	0.9
18:0	6.5	4.9	3.4	6.2
18:1	9.0	6.7	3.5	5.5
18:2	1.4	1.8	3.7	2.8
18:3	0.5	4.1	14.8	10.0
19:0	1.9	0.7	0.9	0.7
20:0	3.9	1.8	3.7	1.5
21:0	1.1	0.4	0.5	0.5
22:0	3.9	3.5	7.8	2.6
> 22	6.9	13.9	11.0	13.4

* The number before the colon is the number of carbon atoms in the fatty acid; the number after the colon is the number of double bonds.

unesterified 4 α -methyl sterol. Cycloeucalenol was present in above trace amounts only in an unesterified form and only in the microsomal fraction. Obtusifoliol and 24-ethylidenelophenol were present in all the organelles in both esterified and unesterified form.

Tables 7 and 8 respectively show the composition of the esterified and unesterified 4,4-dimethyl sterols isolated from the organelle fractions. Neither esterified nor unesterified 4,4-dimethyl sterols could be detected in the nuclear fraction. Cycloartenol, 24-methylenecycloartanol and the pentacyclic triterpenes α - and β -amyrin were generally distributed in the esterified and unesterified 4,4-dimethyl sterol fractions isolated from the other organelles.

Table 9 shows the composition of the fatty acid

fraction derived from the sterol esters isolated from the organelles. The most abundant fatty acids in the nuclear fraction were the C₁₆ fatty acids, palmitic acid and palmitoleic acid, which represent 22% and 15.5% of the total respectively. The most abundant fatty acids of the chloroplastidic fraction were lauric acid (16.6%), myristic acid (10.4%) and palmitic acid (13.6%). The mitochondrial and microsomal fractions were particularly rich in lauric acid (11.6 and 12.2% respectively), myristic acid (10.2 and 10.2% respectively), palmitic acid (11.1 and 15.8% respectively) and linolenic acid (14.8 and 10.0% respectively).

Table 10 shows the results of the gas-liquid-chromatographic separation of the sterol esters on 3% SE-30, a system that resolves them according

Table 10. *Composition of the sterol ester fraction of the intracellular organelles of maize shoots*

The sterol ester fraction was separated into the sterol esters of saturated, monounsaturated, diunsaturated and triunsaturated fatty acids by chromatography on thin layers of AgNO₃-impregnated Kieselgel G with benzene-hexane (2:3, v/v) used for development. Each type of sterol ester was then separated by gas-liquid chromatography on 3% SE-30 into its components according to the number of carbon atoms in the molecule. A, B, C and D denote sterol esters of saturated fatty acids, monounsaturated fatty acids, diunsaturated fatty acids and triunsaturated fatty acids respectively. + and +++ denote the relative proportions of the most abundant sterol esters of that type in the organelle concerned. They are used when the sensitivity of the gas-liquid chromatographic system to overloading prevented the detection of minor components, which were almost certainly present. Blank spaces in the Table indicate that sterol esters of that type could not be detected.

Carbon no.	% of each sterol ester															
	Nuclear fraction				Chloroplastidic fraction				Mitochondrial fraction				Microsomal fraction			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
39	17.9				21.4	6.8			12.9	11.7			16.5			
40													0.9			
41	23.4				25.2				23.1				22.6			
42	6.8												0.9			
43	25.8				33.0	48.6			32.4	47.2			25.8			
44	2.5								2.3				3.2			
45	10.9				6.8	31.1	+		15.3	29.4	+		14.8		+	
46							+	+	2.3		+	+	3.2		+	+
47	12.7				13.6	13.5	++++	++++	9.4	11.7	++++	++++	8.9		++++	++++
48									2.3			+	3.2			
49																
50																
51																

to the number of carbon atoms in their molecules. As we reported in the preceding paper (Kemp & Mercer, 1968), this system is very sensitive to overloading and consequently only the major components were detected. With the nuclear fraction only the sterol esters of saturated fatty acids gave gas-liquid-chromatographic traces from which their composition could be determined by peak-area measurement. The most abundant components of this fraction had carbon numbers of 41 and 43. Since cholesterol was the most abundant esterified sterol and palmitic acid was the most abundant fatty acid in the nuclear fraction, the component having a carbon number of 43 was probably mainly composed of cholesteryl palmitate. Similarly that having a carbon number of 41 was probably cholesteryl myristate.

The most abundant sterol esters of saturated fatty acids in the other organelle fractions had carbon numbers of 39, 41, 43, 45 and 47. In these organelles the most abundant sterols were cholesterol and β -sitosterol. Consequently the component with a carbon number of 39 was probably cholesteryl laurate, that with 41 was probably a mixture of cholesteryl myristate and β -sitosteryl laurate, that with 43 was probably cholesteryl palmitate and β -sitosteryl myristate, that with 45 was probably cholesteryl stearate and β -sitosteryl

palmitate and that with 47 was probably cholesteryl arachidate and β -sitosteryl stearate.

The most abundant sterol esters of mono-unsaturated fatty acids in the chloroplastidic and mitochondrial fractions had carbon numbers of 43, 45 and 47, which probably corresponded to cholesteryl and β -sitosteryl esters of myristoleic acid, palmitoleic acid and oleic acid.

The most abundant sterol esters of di- and tri-unsaturated fatty acids in the extranuclear fractions had a carbon number of 47 and probably corresponded to β -sitosteryl linoleate and linolenate.

DISCUSSION

All experimental work involving the splitting of the cells of a particular tissue into intracellular components by homogenization and differential centrifugation is open to the criticism that the organelle preparations are impure. Table 2, which shows that the mitochondrial, microsomal and, to a smaller extent, nuclear fractions contained chlorophyll, indicates that these fractions were contaminated with chloroplast fragments. The chloroplast fraction, however, was prepared by a procedure claimed to lead to little contamination by other organelles (Kirk, 1963). The nuclear fraction was also considered to be more than 90% pure. In spite

of the impurity of the other two fractions, it is possible to make certain observations.

It is clear from Tables 3 and 4 that cholesterol was not located entirely or even mainly in the chloroplasts. The nuclear fraction contained a greater proportion of both esterified and unesterified cholesterol than the chloroplastidic and other organelle fractions. Cholesterol, however, did constitute over 50% of the esterified sterols of the chloroplastidic fraction. The cholesterol content of the maize shoot chloroplasts was markedly different from that found in chloroplasts of *Phaseolus vulgaris* (Mercer & Treharne, 1966). This may be due to a species difference or to the different way in which the chloroplast fractions were prepared. The *Phaseolus* chloroplasts were prepared by the non-aqueous technique developed by Stocking (1959), whereas the maize chloroplasts were prepared by using aqueous solutions. It is possible that the organic solvents used in the former case preferentially leached out, perhaps owing to a different location within the chloroplasts, the C₂₈ and C₂₉ sterols (e.g. campesterol, stigmaterol and β -sitosterol), leaving the chloroplast preparation with an abnormally high cholesterol content. It is known that the non-aqueously prepared chloroplasts lost the outer component of their double external membrane.

The nuclear and chloroplastidic fractions had much lower sterol contents than the mitochondrial and microsomal fractions both in absolute terms and when expressed as a percentage of the total lipid of the fraction (Table 1). There are several

possible interpretations of this. It could be taken as supporting the view that sterol synthesis in plants is bound up with the mitochondrial and microsomal fractions. Sterol synthesis was reported to be intense in mitochondria in lettuce leaves (Nicholas & Knapp, 1965), and work with *Wistaria sinensis* suggested that microsomes are involved in sterol synthesis and mitochondria in the metabolism of sterol precursors by other pathways (Breccia & Rampi, 1967). Alternatively, since sterols are known to be important components of membranes, it could be indicative of either the relative proportions of membranous structures in the four organelles, or of qualitative differences in these membranes.

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