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CHANGES IN THE LEVELS AND COMPOSITION OF THE ESTERIFIED AND UNESTERIFIED STEROLS OF MAIZE SEEDLINGS DURING GERMINATION

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Abstract—The levels and composition of the esterified and unesterified sterol fractions of the shoot, root, scutellum and endosperm of maize seedlings during the first 2 weeks of germination have been studied. During the germination period it was found that (i) the level of "fast-reacting" sterol esters remains constant in all four morphological units; (ii) the level of "slow-reacting" sterol esters increases in the scutellum but remains constant in the shoot, root and endosperm; (iii) the levels of both "fast-reacting" and "slow-reacting free sterols increase in the shoot and root but remain constant in the scutellum and endosperm; (iv) the composition of the shoot, scutellum and endosperm sterol mixtures does not change, β-sitosterol being the most abundant component; (v) the composition of the sterol mixture in root shows a decrease in β-sitosterol and an increase in stigmasterol such that stigmasterol becomes the most abundant component.

INTRODUCTION

IN RECENT years, studies on plant sterols have centred round their biosynthesis and their function has been somewhat neglected. By analogy with animal sterols, unesterified plant sterols may have a structural function in membranes, whilst plant sterol esters may be concerned in transport. The present work is concerned with the changes in esterified and unesterified sterol levels and composition in the various morphological regions of maize during germination. It was hoped that such a study would throw some light upon the function of sterols and sterol esters in plants as well as providing information about the germination process itself. A preliminary report of the work has already appeared.[1]

RESULTS

Levels of Esterified and Unesterified Sterols during Germination

Batches of 100 maize seedlings were germinated for 4, 6, 9, 11 and 13 days on moistened cotton wool at 30° under constant illumination. The seedlings of each batch were then dissected into four morphological units, the shoot, root, scutellum and endosperm, from which the total lipid was extracted. The lipid samples were fractionated by chromatography on columns of acid-washed, Brockmann grade III alumina using mixtures of light petroleum, b.p. 40–60°, and diethyl ether for development. Three fractions were taken. Fraction 1 was eluted with light petroleum, fraction 2 with 2% (v/v) ether in light petroleum and fraction 3 with 40% (v/v) ether in light petroleum. Fraction 1 contained waxes and hydrocarbons and was discarded, fraction 2 contained esterified sterols and fraction 3 contained free or unesterified sterols. Aliquots of fractions 2 and 3 were assayed for "fast-reacting" and

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[1] R. J. KEMP, L. J. GOAD and E. I. MERCER, *Biochem. J.* 103, 53P (1967).

"slow-reacting" sterols by the Moore and Baumann modification of Liebermann-Burchard colorimetric procedure.[2] The so-called "fast-reacting" sterols show maximum absorption at 620 nm 90 sec (at 25°) after addition of the colour reagent (5% conc. H_2SO_4 in acetic anhydride) whilst "slow-reacting" sterols show maximum absorption 30–33 min after addition of the reagent. The speed of development of absorption at 620 nm appears to be dependent upon the presence of a 7,8 double bond. Thus "fast-reacting" sterols are usually Δ^7 or $\Delta^{5,7}$-sterols whilst "slow-reacting" sterols are usually Δ^5-sterols.

The results of the Moore and Baumann assay are shown in Figs. 1–4.*

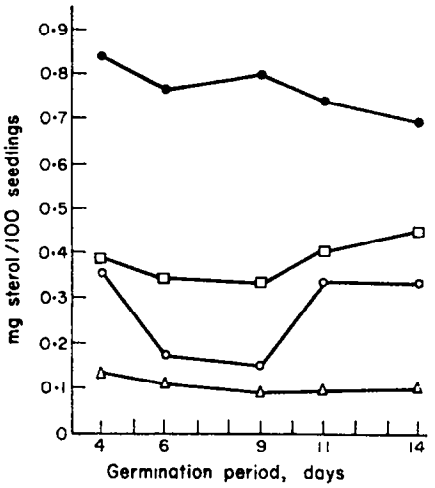

FIG. 1. FAST-REACTING ESTERIFIED STEROLS.
—△—, shoot; —△—, root; —□—, scutellum; —●—, endosperm.

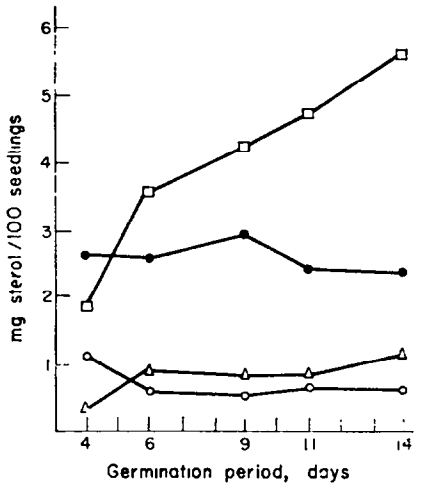

FIG. 2. SLOW-REACTING ESTERIFIED STEROLS.
—△—, shoot; —△—, root; —□—, scutellum; —●—, endosperm.

[2] P. R. MOORE and C. A. BAUMANN, *J. Biol. Chem.* **195**, 615 (1952).

* Revised versions of these figures will be published as an Erratum in the next issue.

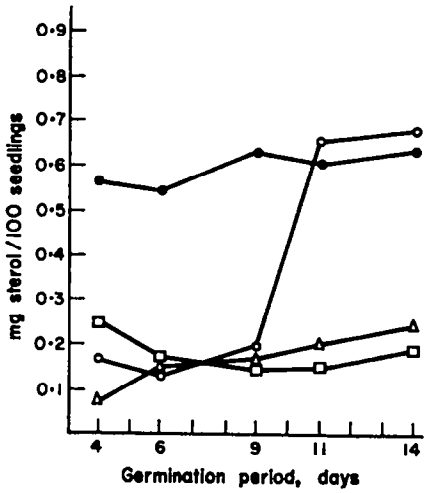

FIG. 3. FAST-REACTING UNESTERIFIED STEROLS.

—○—, shoot; —△—, root; —□—, scutellum; —●—, endosperm.

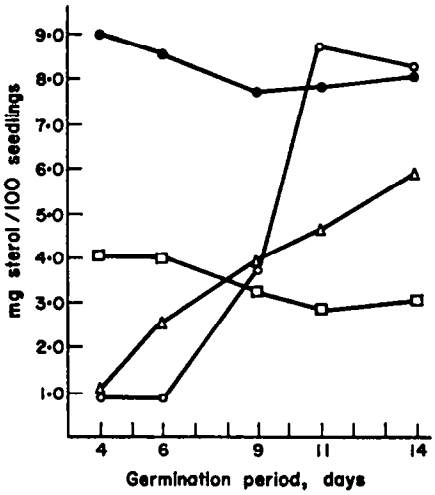

FIG. 4. SLOW-REACTING UNESTERIFIED STEROLS.

—○—, shoot; —△—, root; —□—, scutellum; —●—, endosperm.

The levels of "fast-reacting" esterified sterols (Fig. 1) do not change significantly in any of the four morphological units throughout the germination period. The levels of "slow-reacting" esterified sterols (Fig. 2) show little change in shoot, root and endosperm but increase three-fold in the scutellum. The levels of both "fast-reacting" and "slow-reacting" unesterified sterols (Figs. 3 and 4) show marked increases in shoot and root but remain constant in the scutellum and endosperm. Although the endosperm appears to undergo little change in sterol level during the germination period, it has a high content of fast- and slow-reacting esterified and unesterified sterols relative to the other parts of the seedling.

Composition of Esterified and Unesterified Sterols

The major part of fractions 2 and 3 from 4-, 9- and 13-day seedlings (see previous section) were analysed for their component sterols. Fraction 2 was saponified so as to hydrolyse the sterol esters and the sterol-containing unsaponifiable material isolated. This was then chromatographed on thin layers of silica gel G using chloroform for development. Fraction 3, because it contained only unesterified sterols, was chromatographed directly on the same thin-layer system. The 4-demethyl zone, from each thin-layer chromatogram, was scraped off and eluted with ether. The 4-demethyl sterols were then analysed by gas–liquid chromatography on 1% SE-30 and 1% QF-1 and found to contain three major components, campesterol, stigmasterol and β-sitosterol. These are 3β-hydroxyΔ^5-sterols which differ from one another only in the nature of their side chain (see Fig. 5); they are all "slow-reacting" sterols.

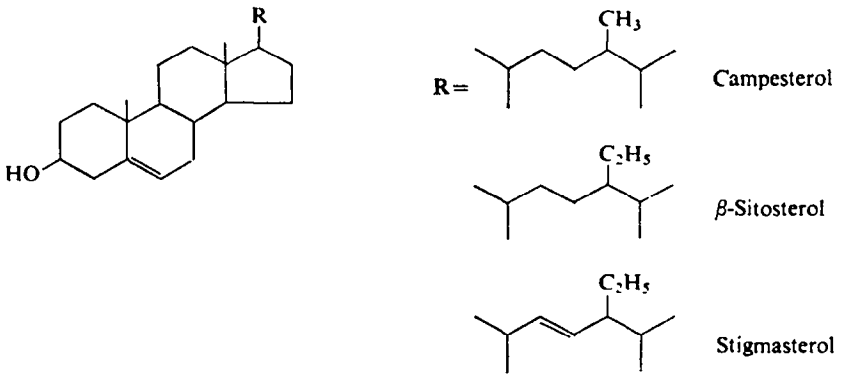

FIG. 5.

The amount of these three sterols in the 4-demethyl sterol mixture from each fraction was determined by measuring peak areas from the chromatographic trace. The 1% SE-30 column separated, in order of elution, campesterol, stigmasterol, and β-sitosterol, whereas the QF-1 column did not separate campesterol from stigmasterol but did separate saturated sterols (stanols) from their Δ^5-sterol counterparts. All fractions contained, as well as the three major sterols, a minor component which co-chromatographed with cholesterol on SE-30 and QF-1, but which was not positively identified. The endosperm 4-demethyl sterol mixture, whether formerly esterified or not, was shown by the QF-1 column to contain at least three stanols as well as the main sterol components; this is in agreement with earlier work.[3] The variation in level of the three major sterols in the esterified and unesterified 4-demethyl sterols of maize seedlings germinated for 4, 9 and 13 days is shown in Table 1.

In the shoot endosperm and scutellum there was virtually no change in the composition of the "ester" sterol and the "free" sterol mixtures during the germination period. The scutellum and endosperm sterol mixtures show a high proportion (57–77 per cent) of β-sitosterol, a characteristically low proportion (trace–17 per cent) of stigmasterol and fairly constant intermediate level (19–28 per cent) of campesterol. There is also a great similarity between the composition of the "ester" sterol mixture and the "free" sterol mixture of the scutellum and endosperm. The shoot sterol mixtures have a slightly lower proportion (50–69 per cent) of β-sitosterol and again a constant proportion (18–22 per cent) of

[3] R. J. ANDERSON, *J. Am. Chem. Soc.* **46**, 1450 (1924).

campesterol; however, the proportion of stigmasterol varies from "ester" sterol (13–17 per cent) to "free" sterol (29–30 per cent).

TABLE 1. COMPOSITION OF "FREE" STEROLS AND "ESTER" STEROLS OF GERMINATING MAIZE SEEDLINGS FROM GLC ANALYSIS

Germination period (days)	1% SE-30 columns (%)			1% QF-1 column (%)	
	β-Sitosterol	Stigmasterol	Campesterol	β-Sitosterol	Stigmasterol+ campesterol
			Shoot-sterol esters		
4	65	13	22	74	26
9	64	17	19	66	34
13	69	13	18	64	36
			Shoot-free sterols		
4	51	29	20		
9	50	29	21	55	45
13	50	30	20		
			Root-sterol esters		
4	67	17	16	66	34
9	57	26	17	58	42
13	49	32	19	54	46
			Root-free sterols		
4	37	37	26	37	63
9	21	49	30	24	76
13	20	51	29	23	77
			Scutellum-sterol esters		
4	70	10	20		
9	69	9	22	74	26
13	69	10	21		
			Scutellum-free sterols		
4	57	17	26		
9	59	14	27	62	38
13	57	15	28		
			Endosperm-sterol esters		
4	76	trace	24		
9	77	trace	23	70	30
13	77	trace	23		
			Endosperm-free sterols		
4	69	10	21		
9	69	11	20	71	29
13	71	10	19		

The lack of change in the composition of the 4-demethyl sterol mixtures during germination which appears characteristic of the shoot, scutellum and endosperm is not repeated in the root, however. Both the "ester" sterol and the "free" sterol mixtures of the root showed the same pattern of change, a marked fall in the proportion of β-sitosterol and a marked rise in the proportion of stigmasterol during the germination period. The "ester" sterol mixture showed a fall in the proportion of β-sitosterol of 18 per cent (67→49 per cent) and a rise in the proportion of stigmasterol of 15 per cent (17→32 per cent). The proportional changes in the "free" sterol mixture were almost identical to those of the "ester" sterol mixture but differed markedly in that the proportion of stigmasterol increased to such an extent that it was the major sterol at the end of the experimental period. The proportion of campesterol in the "ester" and "free" sterol mixtures was again remarkably constant being 16–19 per cent and 26–30 per cent respectively.

DISCUSSION

The function of sterols in plants is not clear. It is not, however, unlikely that they have a structural role, possibly as an integral part of the lipid layer of cell membranes in the same way that cholesterol has in animal tissues. If this were so, then actively growing plant tissues would be expected to accumulate sterol as a consequence of increased membrane production; similarly non-growing tissues would be expected to have constant sterol levels. These membrane sterols would be unesterified. Such a picture is in fact found in the germinating maize seedling. We have found that there is a considerable increase in the level of unesterified sterol in the rapidly developing shoot and root during the germination period whilst the endosperm and scutellum have a virtually constant level of unesterified sterol. We suggest, therefore, that unesterified sterol has a structural function in plant tissues.

The role of esterified sterols poses an even more difficult problem. By analogy with cholesterol esters in animal tissues, one might postulate that it is as esters that sterols are transported. However there is no evidence that sterols are transported from one tissue to another in plants. On the other hand there is evidence that sterols are transported within the plant cell from one organelle to another,[4] and it may well be that they are transported from their site of synthesis to other intracellular organelles as esters. The finding that the level of sterol esters does not change in the shoot and root during germination is in accord with an intracellular transport process. The three-fold increase in sterol ester level in the scutellum is, however, puzzling. It may be due to *de novo* synthesis of sterol ester in the scutellum or to hydrolysis of sterol glycosides and esterification of the free sterol produced. Preliminary experiments in which scutella have been incubated with [2-^{14}C] mevalonic acid show little, if any, labelling of slow reacting sterols, either free or esterified, and thus suggest that there is little *de novo* sterol synthesis in the scutellum.

The analyses of the 4-demethyl sterols from the four morphological units of maize seedlings at different stages of germination show that the C-28 sterol campesterol constitutes a remarkably constant proportion of the mixture. Of the other two main 4-demethyl sterols, β-sitosterol is quantitatively more important than stigmasterol in all tissues except the root. Here, there is a progressive decrease in the proportion of β-sitosterol and a progressive increase in the proportion of stigmasterol during the germination period. This is particularly marked in the unesterified sterol fraction where the proportion of β-sitosterol drops from 37 to 20 per cent whilst the proportion of stigmasterol rises from 37 to 51 per cent. The root

[4] W. E. DAVIES, Ph.D. Thesis, University of Wales (1963).

thus appears to have a greater requirement for stigmasterol than the other tissues. The increase in level of stigmasterol and decrease in that of β-sitosterol in the root is indicative of the biosynthetic relationship of these sterols suggested by other workers.[5]

EXPERIMENTAL

Seed Germination

Maize seeds of the South African White Horsetooth variety were soaked in water for 24 hr and then germinated on moist cotton wool at 30° for periods of 4, 6, 9, 11 and 13 days under continuous light. After each period of germination 100 healthy seedlings were harvested and dissected into shoot, root, scutellum and endosperm.

Extraction and Chromatography of Lipid

Tissues were homogenized in acetone with an Ultra-Turrax homogenizer. The acetone extract was filtered off and the residue extracted with two further volumes of acetone. The bulked acetone extract was diluted with several volumes of water and extracted four times with diethyl ether. The ethereal extract was washed with water, dried (Na_2SO_4) for 30 min and then evaporated to dryness under N_2.

The lipid was then dissolved in the minimal volume of light petroleum (b.p. 40–60°) and chromatographed on columns of acid-washed alumina weakened with water to Brockmann grade III. The columns were developed with light petroleum and increasing proportions of diethyl ether in light petroleum (E/P). Three fractions were collected, fraction 1 eluted with light petroleum, fraction 2 eluted with 2% (v/v) E/P and fraction 3 eluted with 40% (v/v) E/P.

Saponification and Extraction of Unsaponifiable Material

The lipid fraction was dissolved in the minimal volume of ethanol and 60% aqueous KOH added to the extent of 1 ml/10 ml ethanolic solution. The mixture was refluxed for 1 hr, then cooled, diluted with four volumes of water and extracted several times with diethyl ether. The bulked ethereal extract was washed free of alkali with water, dried (Na_2SO_4) for 30 min and then evaporated to dryness under N_2.

Thin-layer Chromatography

Fractions of lipid and unsaponifiable material were chromatographed on 0·25 mm layers of silica gel G into which Rhodamine 6G had been incorporated. The chromatograms were developed with $CHCl_3$ and the position of compounds located by examination under u.v. light. The various sterols appeared as pinkish zones on a pale background. This thin-layer system separates sterols according to methyl substitution at C-4 thus giving three sterol zones, the 4,4′-dimethyl sterols, the 4α-methyl sterols and the 4-demethyl sterols. The lipid and unsaponifiable material dissolved in cyclohexane were spotted on to the plates as narrow bands with β-sitosterol and lanosterol alongside as markers. After development the sterol zones were scraped off and eluted with diethyl/ether.

Gas–Liquid Chromatography

The 4-demethyl sterols were analysed with an Aerograph 1520 Gas Chromatograph using 2 m columns of two types. One was 1% SE-30, the other was QF-1. Both liquid phases were supported on 80–100 mesh, acid-washed, silanized Gas-Chrom Q. Column temperature was 220°, the nitrogen gas flow rate was 40 ml/min and a hydrogen flame detector was used. For identification purposes a mixture of cholestane, cholesterol, campesterol, stigmasterol and β-sitosterol was chromatographed immediately after the maize sterols. The quantity of each sterol component was determined by peak area measurement.

Estimation of "Fast-" and "Slow-reacting" Sterols

Aliquots of fractions 2 and 3 were each dissolved in 1 ml glacial acetic acid and 2 ml colour reagent (5% A.R. conc. H_2SO_4 in A.R. acetic anhydride) added. The mixtures were incubated in darkness at 25° and the absorbance at 620 nm was measured after 90 sec and 33 min using a Beckmann DB Spectrophotometer. The absorbance values were converted into mg "fast-" or "slow-reacting" sterol using previously prepared standard curves for which ergosterol and β-sitosterol were taken as representative "fast-" and "slow-reacting" sterols respectively.

[5] R. D. Bennett, E. Heftmann, W. H. Preston, Jr. and J. R. Haun, *Arch. Biochem. Biophys.* **103**, 74 (1963).

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