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STUDIES ON PHYTOSTEROL BIOSYNTHESIS: OBSERVATIONS ON THE ESTERIFIED STEROLS OF HIGHER PLANTS

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Abstract—In *Pisum sativum* leaves and *Zea mays* leaves and roots, [2-^{14}C] mevalonic acid is rapidly incorporated into both the free and the esterified 4,4-dimethyl, 4α-methyl and 4-desmethyl sterols. Gas-liquid chromatography has demonstrated the presence of cycloartenol, 24-methylene cycloartanol, cycloeucalenol, obtusifoliol, lophenol, 24-methylene lophenol, 24-ethylidene lophenol, cholesterol, campesterol, stigmasterol and β-sitosterol in maize leaves.

PHYTOSTEROL biosynthesis is currently under investigation in several laboratories[1–9] and a number of sterols have been implicated as possible precursors of β-sitosterol. These include the 4,4-dimethyl-sterols, cycloartenol and 24-methylene cycloartanol, and the 4α-methyl sterols, cycloeucalenol, 24-methylene-lophenol and 24-ethylidene lophenol. The incorporation of [1-^{14}C] acetate, [2-^{14}C] mevalonate and [methyl-^{14}C] methionine into these compounds has also been demonstrated.[2–7] A previous report from our laboratory indicated that both the free and the esterified sterols of maize (*Zea mays*) become rapidly labelled following the administration of [2-^{14}C] mevalonate.[10,11] During continuing studies on phytosterol biosynthesis we have made further observations on the esterified sterols which may be relevant to future considerations of phytosterol biosynthesis.

RESULTS AND DISCUSSION

Under the conditions described previously[4] DL-[2-^{14}C] mevalonate (2 μC) was incubated for 4 hr with chopped leaves (4·0 g) of seven day germinated peas (*Pisum sativum*) and the total lipid (37·0 mg) isolated. Chromatography on alumina gave fractions which contained hydrocarbons, including squalene (4·4 mg, 39,600 dpm); esterified sterols (2·0 mg, 33,000 dpm); 4,4-dimethyl sterols (3·1 mg, 147,600 dpm); 4α-methyl sterols (1·2 mg, 120,700 dpm)

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and 4-desmethyl sterols including β-sitosterol, stigmasterol and campesterol (2·0 mg, 333,700 dpm). The association of the bulk (70–90%) of the radioactivity of the above fractions with the sterol groups indicated was confirmed by silica gel TLC followed by elution and radioassay of the appropriate sterol bands. Following saponification of the esterified sterols with ethanolic potassium hydroxide, the sterols were separated on alumina to give fractions containing relatively non-polar material (7430 dpm); 4,4-dimethyl sterols (8080 dpm), 4α-methyl sterols (11,350 dpm) and 4-desmethyl sterols (2600 dpm) respectively. TLC followed by radioautography demonstrated that most of the radioactivity in each of these fractions co-chromatographed with the respective sterol groups indicated above. However it was noted that the 4-desmethyl sterol fraction gave two very close running spots but which were nevertheless well separated from the less polar 4α-methyl sterols. The incorporation of [methyl-^{14}C] methionine into chopped pea leaf sterols resulted in similar results, radioactivity was again incorporated into the three sterol groups in both the free and the esterified forms.

The incorporation of [2-^{14}C] mevalonic acid into the free and esterified sterols of maize (*Zea mays*) shoots and roots has also been examined. Maize was germinated for seven days in the light and the shoots and roots removed from 25 seedlings. The shoots were allowed to take up 4·0 ml of DL-[2-^{14}C] mevalonate (4·0 μc), water was added when all the mevalonate solution had been imbibed. The roots were washed, chopped into small portions and incubated with 7·5 ml of DL-[2-^{14}C] mevalonate (7·5 μc). After 21 hr at 25° the total lipid was extracted from the shoots and from the roots. This was chromatographed on alumina (Brockmann grade III) and fractions eluted with light petroleum (hydrocarbons); 2 per cent diethyl ether in light petroleum (esterified sterols) and 40 per cent diethyl ether in light petroleum (free sterols). The free sterols and the sterols obtained by saponification of the esterified sterols were separated by TLC into the three sterol structural groups. The results (Table 1) show that, as with pea leaves radioactivity was incorporated by both maize shoots and roots into the 4,4-dimethyl, 4α-methyl and 4-desmethyl sterols of the esterified and free sterols. The values in parentheses (Table 1) represent the percentage of the radioactivity associated with each sterol type which was precipitated with digitonin. With the 4,4-dimethyl sterols it is known that digitonin precipitation is incomplete.[4] The proportions indicated in Table 1 therefore probably represent an underestimate of the radioactivity associated with this type of sterol. However the results obtained do show that a very considerable proportion of the radioactivity in every case was present in 3β-hydroxyl sterols belonging to each of the sterol structural types.

In both peas and maize, whilst the 4-desmethyl sterols are heavily labelled in the free form they contain a markedly lower proportion of the radioactivity in the esterified form when compared with the corresponding 4,4-dimethyl and 4α-methyl sterols. As previously reported[12] the esterified 4-desmethyl sterols of maize shoots and roots have a quantitatively different composition from the free sterols, indicating that the esterified and free 4-desmethyl sterols are not in a state of simple equilibrium. Also variations occur in different tissues within the same plant.[12] Similar variations have been observed in the leaves, stems and roots of *Phaseolus vulgaris* plants.[13]

The 4,4-dimethyl and 4α-methyl sterols of pea leaves have previously been shown to contain cycloartenol, 24-methylene cycloartanol, 24-methylene lophenol and 24-ethylidene

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lophenol.[4] With similar experimental methods the sterols of maize shoots have now been investigated. The non-saponifiable lipid (1·45 g) was obtained from 2–3 week old maize shoots (890 g) and the sterols (207·6 mg) obtained by digitonin precipitation. Separation by alumina column and preparative silica gel TLC gave the 4,4-dimethyl (6·5 mg), 4α-methyl (7 mg) and 4-desmethyl sterols (167 mg). The 4-desmethyl sterols were shown by GLC (1 per cent QF-1 and 1 per cent SE-30) to contain β-sitosterol (~75 per cent), campesterol (~9 per cent), stigmasterol (~14 per cent) and cholesterol (~2 per cent). The 4,4-dimethyl sterols were acetylated and shown by silver nitrate-silica gel TLC and GLC to contain cycloartenol (~65 per cent), 24-methylene cycloartanol (~13 per cent) and two unidentified

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN THE ESTERIFIED AND FREE STEROLS OF MAIZE SHOOTS AND ROOTS FOLLOWING INCORPORATION OF [2-^{14}C] MEVALONIC ACID

Fraction	Shoots	Roots
[2-^{14}C] mevalonic acid administered (μC)	4	7·5
	dpm × 10^{-3}	
Total lipids	3,302	3,810
Hydrocarbon fraction (squalene)	129·7	23·4
Esterified sterol fraction		
Total radioactivity	724·8	1,305·3
4,4-dimethyl sterols	317·0 (24)†	750·0 (28)†
4α-methyl sterols	119·5 (69)	216·0 (50)
4-desmethyl sterols	104·0 (95)	99·7 (75)
Free sterol fraction		
Total radioactivity	2,046*	2,013*
4,4-dimethyl sterols	381 (31)	527 (42)
4α-methyl sterols	157 (70)	210 (70)
4-desmethyl sterols	583 (98)	420 (94)

* It is apparent that much of the radioactivity of this fraction was not recovered in the three sterol types. TLC followed by radioautography showed that radioactivity was associated with other unidentified compounds, and in particular with a very heavily labelled material running midway between the origin and the 4-desmethyl sterols.

† Figures in parentheses represent the percentage of the radioactivity of each fraction which was digitonin precipitable.

compounds (~22 per cent). The 4α-methyl sterols were also acetylated, and separated by silver nitrate-silica gel TLC into the four component bands.[5,14] GLC analysis of these provided evidence for the presence of lophenol (~5 per cent), cycloeucalenol (~18 per cent), obtusifoliol (~15 per cent)[15,16]; 24-methylene lophenol (~12 per cent), 24-ethylidene lophenol (~30 per cent) and a number of more minor unidentified compounds. These investigations did not differentiate between the free and esterified sterols, this aspect is currently being investigated. However it is relevant that in studies on the sterol composition of birchwood cycloartenol, 24-methylene cycloartanol and 24-ethylidene lophenol occur in

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the esterified form.[17,18] Also the 4,4-dimethyl and 4α-methyl sterols in grapefruit peel[14] and coffee beans[19] are esterified to an appreciable extent.

The work of Bloch[20] indicates that during cholesterol biosynthesis in animals, 3-oxo sterols are formed at the stage(s) in the biosynthetic sequence when the C4 methyl substituents are removed. Results have also been reported which provide strong evidence that 3-oxo sterols play an intermediate role in β-sitosterol biosynthesis in peas.[21] The explanation for the esterification of a portion of the 4,4-dimethyl and 4α-methyl phytosterols and for the rapid incorporation of [2-^{14}C] mevalonate into these esterified compounds is therefore obscure at the present time. Studies are now in progress in our laboratory to investigate this question in more detail.

EXPERIMENTAL

Techniques were generally as described previously.[4,5,14] Total lipid was extracted by homogenization of the plant material with acetone, dilution of the acetone extract with water and extraction of the lipid with diethyl ether in the usual manner. The lipid was separated on columns of alumina (Brockmann, grade III) eluted sequentially with light petroleum (hydrocarbons); 2 per cent diethyl ether in light petroleum (E/P) (esterified sterols); 6 per cent E/P (4,4-dimethyl-sterols); 9 per cent E/P (4α-methyl sterols); 15 per cent E/P (4-desmethyl sterols). Thin layer chromatography (TLC) was on Kieselgel G incorporating Rhodamine 6 G.[22] Radioactivity was determined on a Packard Tri-Carb Spectrometer.[4] For the analysis of the maize leaf sterols the methods of extraction, saponification, sterol digitonide precipitation and separation of sterol acetates on silver nitrate-silica gel thin-layers were as described before.[4,5,14] Gas-liquid chromatography (GLC) was performed on a Varian-Aerograph 1522B instrument using 6 ft × $\frac{1}{8}$ in. columns of 1 per cent SE-30 or 1 per cent QF-1; all operating procedures were as described previously.[5] Identification of sterols was by comparison of retention times with authentic sterols which were analyzed immediately before or after the unknown sterol.

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