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THE OCCURRENCE OF BOUND, WATER-SOLUBLE SQUALENE, 4,4-DIMETHYL STEROLS, 4α-METHYL STEROLS AND STEROLS IN LEAVES OF *KALANCHOE BLOSSFELDIANA*

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Abstract—A bound, non-dialysable, water soluble fraction from which squalene, 4,4-dimethyl sterols, 4α-methyl sterols and sterols are released by treatment with alkaline pyrogallol or DMSO has been shown to occur in leaves of the higher plant, *Kalanchoe blossfeldiana*. The sterol composition of this and a second bound, water-soluble sterol fraction, broken down by acid treatment, were different and both were significantly different from the composition of the free sterol fraction Possible metabolic roles for bound, water-soluble sterols and their biosynthetic precursors are considered

INTRODUCTION

A BOUND, water-soluble sterol fraction that is refractory to extraction with organic solvents and from which free sterols are released after treatment with alkaline pyrogallol has been reported to occur in yeast[1,2] and *Euglena gracilis*.[3] These bound, water-soluble forms of sterols from yeast are considered by Adams and Parks[2] to be non-covalent saccharide complexes. They were non-dialysable and could be broken down by solution in dimethyl sulphoxide (DMSO), chromatography on silica gel and digitonin precipitation. I now report the occurrence of similar bound, water-soluble sterols together with their similarly bound biosynthetic precursors, squalene, 4,4-dimethyl sterols and 4α-methyl sterols in leaves of the higher plant *Kalanchoe blossfeldiana*.

RESULTS AND DISCUSSION

Aqueous extracts from leaves of both vegetative and flowering *K. blossfeldiana*, after exhaustive extraction with organic solvents, were found to contain bound squalene, 4,4-dimethyl sterols, 4α-methyl sterols and sterols. These substances were released from their bound, non-dialysable form either by treatment with alkaline pyrogallol or by solution in DMSO. They were then separated by TLC and analysed qualitatively and quantitatively by GLC. Bands from TLC plates corresponding to squalene and the other three groups of substances were extracted and individual substances were identified by comparison of their GLC retention times with those of authentic samples on two or more different stationary phases. Free counterparts of the bound substances referred to above were isolated from the organic solvent extracts, separated by column chromatography and TLC then analysed by GLC.

Table 1 shows that the amounts of free and bound water-soluble sterols and their

[1] B. G ADAMS and L W PARKS, *Biochem Biophys Res Commun.* **28**, 490 (1967).
[2] B G ADAMS and L. W PARKS, *J Lipid Res.* **9**, 8 (1968)
[3] R D BRANDT, G OURISSON and R J PRYCE, *Biochem Biophys. Res. Commun.* **37**, 399 (1969)

biosynthetic precursors found in leaves of both vegetative and flowering *K. blossfeldiana* are similar. Amounts of these bound, water-soluble substances present are *ca.* 0·1 per cent of their free counterparts. The somewhat lower amounts of bound, water-soluble substances obtained after dialysis of an aqueous fraction could be due to loss during the dialysis. Prior to the alkaline pyrogallol treatment referred to in Table 1 the aqueous fraction had been subjected to prolonged treatment with mineral acid. 4,4-Dimethyl sterols, 4α-methyl sterols and sterols were extracted after this acid treatment in amounts *ca.* 50 per cent of those obtained after alkaline pyrogallol treatment. Organic solvent extracts of leaves of *K. blossfeldiana* contained fatty acid esters of 4,4-dimethyl sterols, 4α-methyl sterols and sterols in amounts that were much lower (*ca.* 2 per cent) than those of the bound, water-soluble forms shown in Table 1.

TABLE 1. AMOUNTS OF FREE AND BOUND, WATER SOLUBLE STEROLS AND THEIR BIOSYNTHETIC PRECURSORS ISOLATED FROM LEAVES OF VEGETATIVE AND FLOWERING *Kalanchoe blossfeldiana*

Fraction	Amounts (μg/g dry wt of leaf)*			
	Squalene	4,4-Dimethyl sterols	4α-Methyl sterols	Sterols
Free	1000 (750)	740‡ (750‡)		1100 (1350)
Water soluble (extracted after alkaline pyrogallol treatment)	0 29 (1 1)	(0 15) 0 17	0 13 (0·14)	3 0 (3·3)
Water soluble (extracted after DMSO treatment)†	0·70			3 9
Water soluble (non-dialysable, extracted after DMSO treatment)†	(0 30)			(1·2)

* Data from leaves of flowering *K blossfeldiana* are shown in parentheses. Amounts determined by GLC, approximate fractional error ± 0 2.

† Squalene and sterols only examined

‡ Determined by weighing

Significant differences were observed in the sterol composition of bound, water-soluble and free sterols from *Euglena gracilis.*[3] These sterol fractions from leaves of *K. blossfeldiana* also had different compositions by GLC analysis, the results of which are shown in Table 2. Similar GLC analysis of the free and bound, water-soluble 4,4-dimethyl sterol and 4α-methyl sterol fractions showed the presence of cycloartenol and cycloeucalenol respectively along with larger quantities of unidentified substances. Cycloartenol and cycloeucalenol are considered to be biosynthetic precursors of the phytosterols.[4] No lanosterol was detectable in any fraction. These qualitative results above were determined with leaves from vegetative *K. blossfeldiana* and were essentially the same with leaves from the flowering plant. The presence of squalene in both free and bound, water soluble form, released after alkaline pyrogallol and DMSO treatment, was determined, after TLC, by GLC analysis on four different stationary phases.

Previous work in other laboratories,[1–3] has demonstrated the presence, in two lower plants, of a bound, water soluble, and non-dialysable sterol complex which is resistant or partially resistant to acid and alkali degradation and from which sterols are released by

[4] P. BENVENISTE, M. J. E. HEWLINS and B. FRITIG, *European J. Biochem* **9**, 526 (1969)

TABLE 2. COMPOSITION OF FREE AND BOUND, WATER SOLUBLE STEROL FRACTIONS FROM LEAVES OF VEGETATIVE *Kalanchoe blossfeldiana*

	Composition (%)*				
Sterol fraction	β-Sitosterol	Campesterol	28-Isofucosterol	Stigmasterol	Cholesterol
Free	79	13	7	0	<1
Water soluble (extracted after acid treatment)	70	1	8	6	14
Water soluble (extracted after alkaline pyrogallol treatment)	57	0	26	9	7
Water soluble (extracted after DMSO treatment)	49	0	26	16	9
Water soluble (non-dialysable, extracted after DMSO treatment)†	55	0	23	15	7

* Determined by GLC peak area measurements, approximate fractional error ± 0·1.
† From leaves of flowering *K blossfeldiana*

alkaline pyrogallol treatment, silica gel chromatography, solution in DMSO and digitonin precipitation. The present results demonstrate the occurrence of a similar bound, water soluble non-dialysable sterol complex in a higher plant together with similarly bound, water-soluble sterol biosynthetic precursors, squalene, 4,4-dimethyl sterols and 4α-methyl sterols. To the author's knowledge this is the first report of any such bound, water-soluble form of squalene, 4,4-dimethyl sterols and 4α-methyl sterols in plants or animals. The nature of these water soluble forms is uncertain, although Adams and Parks[2] suggest that they might be large non-covalent saccharide complexes.

Adams and Parks[1,2] found that the water-soluble sterol complex from yeast was partly decomposed by acid. Brandt *et al.*[3] have suggested the possibility of two distinct water-soluble forms of sterols from *E. gracilis*, one from which sterols are extracted after acid treatment and the other from which sterols are extracted after alkaline pyrogallol treatment. The results presented here (Table 2), which show a significantly different sterol composition for water solubilized sterols liberated after these two different treatments, also suggest the presence of two distinct bound, water-soluble sterol forms in leaves of *K. blossfeldiana*. In addition, it seems likely from the compositions in Table 2, that the bound, water-soluble form that is broken down by alkaline pyrogallol is the same as that attacked by DMSO.

With regard to any possible role that bound, water-soluble components of sterol biosynthesis might have in plants, several possibilities exist. They could, for instance, represent a completely water solubilized and separate sterol biosynthesis or act as reversible and transportable extraparticulate reserves for sterol biosynthesis associated with cell particles A recent study[5] of sterol biosynthesis with fractionated plant cell particles has indicated that, as in animals, plant sterol biosynthesis is associated mainly with the microsomes and the aqueous supernatant after microsome precipitation, that is a soluble cell fraction There exists also the possibility that bound, water-soluble squalene and its metabolites could be concerned with their intercellular transport and metabolism.

[5] F F KNAPP, R. T. AEXEL and H J. NICHOLAS, *Plant Physiol.* **44**, 442 (1969).

As with the photosynthetic *E. gracilis*,[3] leaves of *K. blossfeldiana* contain much larger amounts of cholesterol in bound, water soluble form than in its free state. One interpretation of this finding is that the water-soluble complex contains a pool of the sterols needed by the plant in a form translocatable either inter- or intracellularly. Cholesterol, for example, is a known biosynthetic precursor of plant progesterone[6] and ecdysterone,[7] both animal hormones. It could equally well be the precursor of oestrogens[8-10] which have been found in plants and may have some hormonal role therein.[11,12] Cholesterol could also serve as the biosynthetic precursor for various other plant steroids.

Further investigations into the occurrence and nature of bound, water solubilized terpenoids are in progress.

EXPERIMENTAL

Chromatography

Columns of alumina (Grade III) were eluted with PE (PE = light petroleum, b p 40–60°) containing increasing amounts of ether. Fractions were monitored by TLC. Squalene was eluted with PE, sterol esters with 25–40% ether, 4,4-dimethyl sterols and 4α-methyl sterols 45–70% ether, and sterols 70–90% ether.

TLC plates of silica gel (250 μ thick) were eluted twice with $CHCl_3$. Separated components were visualized under u v light after spraying with 0 1% ethanolic berberine hydrochloride. Approximate R_f values were; squalene 0 95, sterol esters 0·85, 4,4-dimethyl sterols 0·65, 4α-methyl sterols 0·55 and sterols 0·45

GLC was carried out with a Pye 104 dual column chromatograph fitted with dual flame ionization detectors. Separated (TLC) 4,4-dimethyl sterol, 4α-methyl sterol and sterol fractions were quantitatively and qualitatively analysed on two different columns, 1% OV-17 and 1·5% XE-60 at 248° and 238° respectively. Squalene was analysed on four different columns, 1% OV-17, 1·5% XE-60, 2% SE-30 and 2% DEGS at 234°, 205°, 234° and 200° respectively Silanized glass columns (1 5 m × 4 mm i.d.) were packed with liquid phases adsorbed on Gas-Chrom Q (80–100 mesh) except in the case of 2% DEGS which was adsorbed on Chromosorb W (80–100 mesh) and packed into a silanized glass column (2 m × 4 mm i.d) Nitrogen carrier gas flow rates were 60 ml/min in all cases. Compounds were identified by the identity of their retention times with those of authentic samples. Amounts of 4,4-dimethyl sterols, 4α-methyl sterols and sterols were determined by GLC peak area measurements using linear detector response calibrations with cholesterol. Pure cholesterol was the only standard available in sufficient quantities for this purpose but it was anticipated that all the compounds measured with it, being chemically very similar, would have a similar detector response Amounts of squalene were determined similarly, by GLC, using pure squalene for the calibration of detector response.

Extraction and Isolation from Plant Material

(a) Lyophilized leaves from vegetative (24 5 g) or flowering (19·6 g) *K blossfeldiana* were macerated with acetone (500 ml) then refluxed in the same acetone for 2 5 hr After filtration the plant material was extracted a further three times by refluxing with $CHCl_3$–methanol (2:1, 500 ml) and filtering. The combined filtrates (acetone and $CHCl_3$–methanol) were evaporated to dryness *in vacuo* then chromatographed on a column of alumina Fractions containing squalene, 4,4-dimethyl sterols, 4α-methyl sterols and sterols were collected, combined as appropriate, and in all except the case of squalene, further purified by TLC prior to GLC analysis. The combined column fractions containing sterol esters were first purified by TLC then saponified (see below) and the non-saponifiables separated by TLC prior to GLC analysis

After the extraction with organic solvents, described above, the remaining plant material was refluxed three times with water (500 ml) and filtered The combined aqueous filtrates were evaporated to dryness *in vacuo* and treated with aqueous methanolic HCl (see below), and then, after ether extraction, neutralization, and evaporation to dryness *in vacuo*, treated with alkaline pyrogallol (see below) and further extracted with PE Components of these two organic solvent extracts were separated by TLC prior to GLC analysis.

[6] J A F WICKRAMASINGHE, P C. HIRSCH, S. M MUNAVALLI and E CASPI, *Biochemistry* **7**, 3248 (1968).
[7] H H SAUER, R. D BENNETT and E. HEFTMANN, *Phytochem* **7**, 2027 (1968)
[8] A. BUTENANDT and H JACOBI, *Z Physiol Chem.* **218**, 104 (1933)
[9] E. HEFTMANN, S -T. KO and R. D BENNETT, *Phytochem* **5**, 1337 (1966)
[10] B. SKARZYNSKI, *Nature* **131**, 766 (1933)
[11] J. KOPCEWICZ, *Naturwiss.* **56**, 287 (1969)
[12] J. KOPCEWICZ, *Naturwiss.* **57**, 48 (1970).

(b) Frozen (−40°) leaves from vegetative (146·7 g fr. wt., 15·6 g dry wt., determined by lyophilization of a sample) or flowering (309·7 g fr. wt., 19·8 dry wt.) *K. blossfeldiana* were broken and allowed to thaw out and soak in 80% ethanol (500 ml) for 2 days at room temperature. After filtration the plant material was macerated with 80% ethanol (400 ml) and allowed to soak for 1 day before filtering and resoaking in 80% ethanol (300 ml, 1 day). The combined extracts were concentrated *in vacuo* to an aqueous suspension (250 ml). This was adjusted to pH 7·5 with 2 N Na_2CO_3 and extracted with ethyl acetate (4 × 50 ml), then, after adjustment to pH 3 with 2N HCl, extracted with ethyl acetate (6 × 100 ml) and with *n*-BuOH (4 × 150 ml). The aqueous remainder after all these extractions was evaporated to dryness *in vacuo*. The dry residue from vegetative leaves was treated with DMSO (see below) followed by PE extraction. The dry residue from flowering leaves was redissolved in water (25 ml) and dialysed against water (8 × 100 ml) for 3 days. After evaporation of the non-dialyzable material to dryness *in vacuo* it was treated with DMSO (see below). The mixtures of components extracted into PE after the above two DMSO treatments were analyzed directly by GLC.

Methods for Release of Bound Lipids

1 *Ester saponification* Sterol ester fractions were refluxed under nitrogen with 6% KOH in 90% aqueous methanol (5 ml) for 4 hr. The cooled reaction mixture was then diluted with water (2 ml) and extracted with PE (3 × 5 ml). Combined extracts were washed with 2N HCl, 5% $NaHCO_3$ and water then dried over Na_2SO_4 before evaporating to dryness *in vacuo*.

2 *Water soluble.* (a) *Aq methanolic HCl* Dried material from the aqueous extracts was stirred at room temperature for 5 days with conc HCl–water–methanol (1:2·3, 300 ml). After dilution with water (200 ml) the suspension was filtered and extracted with ether (3 × 100 ml) Combined extracts were washed with 5% $NaHCO_3$ and water before drying over Na_2SO_4 and evaporating to dryness *in vacuo*.

(b) *Alkaline pyrogallol.* After the ether extraction described in 2a above, the remaining acidic aqueus solution was neutralized with 4N NaOH before evaporation to dryness *in vacuo*. The dry residue was then refluxed for 2 hr with 0·5% methanolic pyrogallol solution–60% KOH–methanol (3:2·3, 250 ml). The cooled reaction mixture was extracted with PE (4 × 75 ml) and combined extracts were washed with 2N HCl, 5% $NaHCO_3$ and water then dried over Na_2SO_4 before evaporating to dryness *in vacuo*.

(c) *DMSO.* Dry residues of aqueous extracts were heated on a steam bath for 2 hr with DMSO (10 ml). The cooled and filtered reaction mixture was extracted with PE (3 × 5 ml) Combined extracts were washed with water then dried over Na_2SO_4 before evaporating to dryness *in vacuo*.

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