The Biosynthesis of Sterols in Higher Plants

By L. J. GOAD AND T. W. GOODWIN

Department of Biochemistry and Agricultural Biochemistry,
University College of Wales, Aberystwyth

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1. [2-¹⁴C]Mevalonate was incorporated into squalene and the major phytosterols of pea and maize leaves; it was also incorporated into compounds belonging to the 4,4-dimethyl and 4 α -methyl steroid groups and which may be possible phytosterol intermediates. 2. L-[Me-¹⁴C]Methionine was incorporated into the major sterols and also into the 4,4-dimethyl and 4 α -methyl steroid groups. No radioactivity was detected in squalene. 3. Under anaerobic conditions incorporation of [2-¹⁴C]-mevalonate into the non-saponifiable lipid of pea leaves was drastically decreased but radioactive squalene was accumulated. 4. Cycloartenol, 24-methylenecycloartanol, 24-methylenelophenol, 24-ethylidenelophenol, fucosterol, β -sitosterol, stigmasterol and campesterol have been identified by gas-liquid chromatography in pea leaves. 5. The significance of these results in connexion with phytosterol biosynthesis and the introduction of the alkyl group at C-24 into phytosterols is discussed.

The biosynthesis of the principal animal sterol, cholesterol, has received much attention and many of the steps in the biosynthetic pathway have been elucidated (Clayton, 1965*a,b*). An increasing amount of interest is now being shown in the study of the biosynthesis of plant triterpenes and sterols.

In plants it is to be expected that, as in cholesterol biosynthesis, squalene will be derived from mevalonic acid via several phosphorylated derivatives. Subsequent cyclization of the squalene will give either the pentacyclic or the tetracyclic triterpenes (Ruzicka, 1959). Support for these theoretical considerations has now been obtained. Mevalonate-kinase activity has been demonstrated in different plant species (Loomis & Battaile, 1963; Williamson & Kekwick, 1965; Rogers, Shah & Goodwin, 1965). Nicholas (1962*a,b,c*) observed the incorporation of [2-¹⁴C]acetate and [2-¹⁴C]mevalonate into β -sitosterol and the pentacyclic triterpenes oleanolic acid and ursolic acid in *Salvia officinalis* and *Salvia sclarea* and into squalene, sterol and acidic triterpenes in *Ocimum basilicum*. Independently Baisted, Capstack & Nes (1962) found that [2-¹⁴C]-mevalonate was incorporated into β -amyrin and β -sitosterol by germinating seeds of the pea, *Pisum sativum*. In addition Capstack *et al.* (1962) demonstrated the biosynthesis of squalene and the incorporation of [2-¹⁴C]mevalonate into this compound by germinating pea seeds. The incorporation of [2-¹⁴C]-mevalonate into phytosterols has been observed by Bennett, Heftmann, Preston & Haun (1963) in *Dioscorea spicaliflora* and by Mercer, Davies &

Goodwin (1963) and Mercer & Goodwin (1963) in maize. Also Benveniste, Hirth & Ourisson (1964*a*) have found that [1-¹⁴C]acetate is incorporated into the sterols of tissue cultures of *Nicotiana tabacum*. Battersby & Parry (1964) degraded β -sitosterol biosynthesized by *Rauwolfia serpentina* in the presence of [2-¹⁴C]mevalonate and showed that the labelling pattern was the same as that observed in cholesterol, thus indicating that phytosterols arise by a biosynthetic pathway very similar to that for cholesterol. Capstack, Rosin, Blondin & Nes (1965) have demonstrated directly the cyclization of labelled squalene to β -amyrin in homogenates prepared from germinated pea seeds. Similarly Bennett & Heftmann (1965) have shown the conversion *in vivo* of [¹⁴C]squalene into β -sitosterol by *Pharbitis nil* seedlings.

The typical phytosterols differ from cholesterol by the presence of a supernumerary alkyl group at C-24. In ergosterol this was shown to arise by transmethylation from methionine (Alexander & Schwenk, 1958). It has since been found that the ethyl group of β -sitosterol and spinasterol and the ethylidene group of fucosterol arise by a double methylation from methionine (Castle, Blondin & Nes, 1963; Bader, Guglielmetti & Arigoni, 1964; Villanueva, Barbier & Lederer, 1964).

The present work was undertaken in an attempt to demonstrate the incorporation of [2-¹⁴C]mevalonate and [Me-¹⁴C]methionine into possible intermediates between squalene and the major phytosterols such as β -sitosterol, stigmasterol and

campesterol. In particular it was hoped to obtain some information on the stage in the biosynthetic sequence at which the transmethylation steps from methionine occur. A preliminary communication of part of this work has been published (Goad & Goodwin, 1965).

MATERIALS AND METHODS

Radiochemicals. DL-[2-¹⁴C]Mevalonic acid lactone (specific activity 5.01 mc/m-mole) and L-[Me-¹⁴C]methionine (specific activity 29.1 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. DL-[2-¹⁴C]Mevalonic acid lactone was dissolved in water and converted into the potassium salt by the addition of the required amount of K₂CO₃ before use in incubations.

Solvents. Light petroleum (b.p. 40–60°) was dried over sodium wire and redistilled. Diethyl ether was dried over sodium wire and then distilled over reduced iron to remove peroxides.

Plants. Peas (variety Onward) were germinated in sterilized soil at about 25° for 8 days with illumination provided by two daylight emission lights (Cryselco, 20 w, 2 ft.) placed approx. 25–30 cm. above the surface of the soil. After 8 days the peas were about 4–5 cm. tall.

Maize was germinated in a similar manner for 7 days.

Incubation with radioactive substrates. Pea plants were excised above the first node and 1 g. portions chopped into strips about 1–2 mm. wide with a razor blade. Although these preparations are referred to as chopped pea leaves they did contain some stem material in addition. The chopped leaves were transferred to a 50 ml. conical flask and moistened with 1.5 ml. of a solution containing either 1.0 μ C of potassium [2-¹⁴C]mevalonate or 2.5 μ C of L-[Me-¹⁴C]methionine. Usually duplicate flasks were prepared. The flasks were incubated in a water bath at 25° with illumination by two daylight emission lamps (Cryselco, 20 w, 2 ft.) placed 25 cm. above the flasks. The incubation was terminated, usually after 4 hr., by the addition of 5.0 ml. of hot ethanol followed by boiling for 2–3 min. Where incubation conditions differed from the above they are detailed in the text.

Anaerobic incubations. These were performed in specially constructed Warburg-type flasks of 50 ml. capacity. A 1 g. portion of chopped pea leaves was placed in the main compartment and moistened with 0.5 ml. of water, and 1.0 ml. of potassium [2-¹⁴C]mevalonate (1.0 μ C) was placed in the side arm. The centre well contained a filter-paper wick moistened with alkaline pyrogallol. Nitrogen (oxygen-free) entered the flask via an inlet in the side arm and escaped through a no. II serum hypodermic needle inserted through the Suba-Seal rubber cap that closed the neck of the flask. The flask was flushed with nitrogen for 15 min. before tipping the potassium [2-¹⁴C]mevalonate solution on to the chopped leaves to start the incubation. The flasks were flushed with nitrogen throughout the incubation, which was at 25° with illumination as described above. The incubation was stopped by injecting 5.0 ml. of hot ethanol through the rubber cap followed by boiling for 2–3 min.; the flow of nitrogen was continued during these operations.

Saponification and extraction of non-saponifiable lipid. The contents of duplicate flasks were combined and saponified by refluxing for 1½–2 hr. with 30–40 ml. of ethanol, 10 ml. of water and 8.0 g. of KOH. The non-saponifiable lipid was

extracted into diethyl ether, which was then washed free of alkali and ethanol and dried over Na₂SO₄ in the usual manner.

Chromatography of the non-saponifiable lipid on alumina. The non-saponifiable lipid was dissolved in a small volume of light petroleum (b.p. 40–60°) and chromatographed on 5 g. of deactivated alumina [Brockmann grade III; Woelm (anionotropic)]. The column was developed by stepwise elution with increasing percentages of diethyl ether in light petroleum (b.p. 40–60°) as follows: fraction 1, 50 ml. of light petroleum; fraction 2, 50 ml. of 2% (v/v) ether in light petroleum; fraction 3, 50 ml. of 6% (v/v) ether in light petroleum; fraction 4, 40 ml. of 9% (v/v) ether in light petroleum; fraction 5, 50 ml. of 15 or 20% (v/v) ether in light petroleum; fraction 6, 50 ml. of ether. For a 10 g. alumina column these volumes were doubled.

Thin-layer chromatography. Thin-layer chromatography was carried out on layers of silica gel (Kieselgel G; E. Merck A.-G., Darmstadt, Germany). Rhodamine 6G was incorporated into the plates during their preparation and compounds were located by examination in ultraviolet light (Avigan, Goodman & Steinberg, 1963). Squalene and the various steroids appeared as pinkish spots on a paler background. For squalene chromatography hexane was used as the developing solvent. For the separation of steroids and the total non-saponifiable lipid ethyl acetate–benzene (1:5, v/v) was used.

For separation of the acetates of cycloartenol and 24-methylene-cycloartenol thin-layer plates were prepared of silica gel impregnated with Rhodamine 6G and with 10% (w/v) AgNO₃. The plates were developed with benzene–hexane (1:4, v/v).

Compounds were spotted as their solution in cyclohexane. For preparative purposes they were applied as a band with marker spots of squalene or lanosterol and β -sitosterol at each end. After location bands were scraped off and compounds eluted with dry diethyl ether. Occasionally a small amount of Rhodamine 6G was also eluted; this was removed by chromatography on 2 g. of alumina (Brockmann grade III) and eluting with 50% (v/v) diethyl ether in light petroleum (b.p. 40–60°).

Chromatography of steroids on silicic acid–Hyflo Super-Cel. A 10 g. portion of silicic acid (100 mesh; Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.) and 5 g. of Hyflo Super-Cel (Johns-Manville, New York, N.Y., U.S.A.) were mixed and a slurry in hexane was packed into a 10 mm. x 400 mm. column. Sterols were applied as their solution in hexane and the column was developed with an increasing gradient of benzene in hexane. This was obtained by having 250 ml. of 40% (v/v) benzene in hexane in the mixing flask and 250 ml. of benzene in the reservoir. Fractions of volume 4–5 ml. were collected.

Gas-liquid chromatography. A Pye Argon Chromatograph was used. Three columns were used packed with 80–100-mesh acid-washed silicone-treated Gas-Chrom P coated with (a) 3% SE-30, (b) 5% QF-1 or (c) 1% Hi-EFF8B (all supplied by Applied Science Laboratories Inc., Pennsylvania, U.S.A.). The column temperature was 219°. Cholestane was always run with the sample. The relative retention times are given as a guide (Fig. 5) but it was found that these values tended to vary with different columns and also with the age of the column. For identification purposes authentic steroids were normally chromatographed separately on the same day as the unknown compounds.

Precipitation of steroids. $\beta\beta$ -Hydroxy steroids were precipitated from the non-saponifiable lipid essentially by the method of Windaus (1909). The precipitated digitonides were washed only with diethyl ether. Washing with ethanol was avoided because of the solubility of lanosterol and cycloartenol digitonides in this solvent (Ohta & Shimizu, 1957). The steroids were recovered from the digitonides by treatment with pyridine in the usual manner (Williams & Goodwin, 1965).

Liebermann-Burchard assay of steroids. The sample was dissolved in acetic acid and 2 vol. of the reagent (19.0 ml. of acetic anhydride plus 1.0 ml. of conc. H_2SO_4) was added. Extinctions were determined after 33 min. in a Unicam SP. 600 spectrophotometer at $460\text{ m}\mu$ for lanosterol and at $620\text{ m}\mu$ for β -sitosterol.

Formation of the squalene-thiourea adduct. The squalene (107 mg.) plus the radioactive sample were dissolved in 2.0 ml. of butan-1-ol and 8.0 ml. of a saturated solution of thiourea in methanol was added. The squalene-thiourea adduct was allowed to crystallize overnight at $0-4^\circ$. Squalene was recovered from the adduct by adding water and extracting the squalene with diethyl ether.

Formation of squalene hexahydrochloride. Squalene (100 mg.) was dissolved in 5.0 ml. of dry acetone saturated with dry HCl at -5° . Dry HCl was passed through this solution for a further 60 min. at 0° and the acetone then evaporated at 0° under a vigorous flow of nitrogen. The remaining residue was first washed with about 1 ml. of cold ethanol and then dissolved in warm acetone. After centrifugation the hexahydrochloride was allowed to crystallize overnight. Recrystallizations were from acetone.

Extraction of steroids from pea leaves. Peas were germinated for 8 days as described above. The leaves (310 g. wet wt.) were homogenized in a Waring Blendor with 1250 ml. of ethanol; 200 ml. of water and 300 g. of KOH were then added and the mixture was refluxed for 2 hr. The non-saponifiable lipid (628 mg.) was extracted from the filtered mixture with diethyl ether in the usual way. Steroids were obtained by precipitation with digitonin. After the first precipitation the 'sterol-free' non-saponifiable lipid was recovered and treated a second time with digitonin. Subsequent separation of the regenerated steroid mixture (75 mg.) is given in the Results section.

Radioactive counting. Samples were assayed in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 314 EX. Samples were dissolved in 10 ml. of scintillator containing 5.0 g. of 2,5-diphenyloxazole and 0.3 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l. of toluene. Coloured samples were dissolved in 0.5 ml. of toluene in the counting vial and decolorized under ultraviolet light (Phillips Ultraviolet, 300 w) before addition of the scintillator solution.

RESULTS

Separation of steroids into structural groups. Clayton, Nelson & Frantz (1963) observed that steroid mixtures of animal origin can be separated by suitable chromatographic methods into three structural groups depending on the number of methyl groups located at C-4 in ring A of the steroid. It has been found that steroid mixtures of plant origin can be similarly separated into these three groups (Schreiber, Osske & Sembdner, 1961; Williams, Goad & Mercer, 1965). With the chromatographic system given in Table 1 authentic triterpenes with a 4,4-dimethyl grouping (e.g. β -amyryn, lanosterol or cycloartenol) are eluted in fraction 3. Compounds with a single methyl group at C-4 (e.g. lophenol) are eluted in fraction 4. The major phytosterols such as β -sitosterol, stigmasterol, campesterol and also cholesterol and ergosterol appear in fraction 5. Accordingly the incorporation of radioactively labelled mevalonate and methionine by plants into these three structural groups of steroids was first investigated.

Incorporation of $[2-^{14}\text{C}]$ mevalonate into the non-saponifiable lipid. After incubation of chopped pea leaves with $[2-^{14}\text{C}]$ mevalonate the non-saponifiable lipid was extracted and a portion separated into fractions by chromatography on alumina. The results are given in Table 1. Radioactivity was distributed throughout all the fractions collected.

Table 1. *Chromatography on alumina (Brockmann grade III) of the non-saponifiable lipid obtained from pea leaves incubated with $[2-^{14}\text{C}]$ mevalonate or L- $[Me-^{14}\text{C}]$ methionine*

Duplicate flasks were prepared as described in the Materials and Methods section and incubated for 4 hr.; other details are given in the Materials and Methods section. The total non-saponifiable lipid from the $[2-^{14}\text{C}]$ -mevalonate incubation contained 316420 disintegrations/min. and that from the $[Me-^{14}\text{C}]$ methionine incubation 132680 disintegrations/min. Portions of this material (284700 and 119000 disintegrations/min. respectively) were separated.

Fraction no.	Concn. of ether in light petroleum (b.p. $40-60^\circ$) (% v/v)	Radioactivity (disintegrations/min.)	
		$[2-^{14}\text{C}]$ Mevalonate	$[Me-^{14}\text{C}]$ Methionine
1	0	18730	0
2	2	3430	670
3	6	46190	5970
4	9	71190	17090
5	15	115450	74030
6	100	2610	1940

Table 2. *Thin-layer chromatographic separation of the non-saponifiable lipid of pea and maize leaves after incubation with [2-¹⁴C]mevalonate and [Me-¹⁴C]methionine*

Conditions for the pea experiment were as given in Table 1. Maize plants were grown for 7 days in the light. Portions (1g.) of chopped leaves were incubated with 1.5 ml. of either [2-¹⁴C]mevalonate (1.0 μ C) or [Me-¹⁴C]-methionine (2.5 μ C) for 4 hr. at 25° with illumination. Other details are given in the Materials and Methods section. Zones A-F were as defined in Fig. 1.

Zone	Percentage of total radioactivity recovered from the plate			
	Pea leaves		Maize leaves	
	[2- ¹⁴ C]Mevalonate	[Me- ¹⁴ C]Methionine	[2- ¹⁴ C]Mevalonate	[Me- ¹⁴ C]Methionine
A	7.1	0.1	8.6	0.4
B	4.5	4.2	8.7	15.2
C	14.0	4.9	43.2	6.3
D	27.1	16.5	25.2	23.9
E	40.1	66.5	8.5	37.2
F	7.2	7.8	5.8	17.0

Fraction 1, which contained squalene and also β -carotene and other polyenes, was significantly labelled. As expected, fraction 5, which contains the major pea sterol, was the most heavily labelled. However, fractions 3 and 4 also contained high proportions of the total radioactivity.

A further portion of the total non-saponifiable lipid (31 600 disintegrations/min.) from the [2-¹⁴C]-mevalonate incubation with pea leaves was separated by thin-layer chromatography on silica gel. This system will also separate sterol mixtures into the three main structural groups (Avigan *et al.* 1963). After development of the plate the zones A, B, C, D, E and F as indicated in Fig. 1 were scraped off, the lipids were eluted with diethyl ether and the radioactivity was determined. Table 2 shows the results obtained. Also included in Table 2 is the distribution of radioactivity in the non-saponifiable lipid after incubation of chopped maize leaves with [2-¹⁴C]mevalonate. In both peas and maize radioactivity was located in material that co-chromatographed with squalene and with the three main steroid groups. However, radioactivity was also present in compounds that ran ahead of the 4,4-dimethyl steroids (zone B) and also between the origin and the β -sitosterol region (zone F). These can be seen in the radioautograph of the separated maize non-saponifiable lipid shown in Fig. 1. Three radioactive bands with mobilities greater than the 4,4-dimethyl steroids and one radioactive zone more polar than β -sitosterol were revealed. Radioactivity also remained at the origin. Similar radioactive compounds were observed by radioautography of thin-layer chromatograms of the non-saponifiable lipid from pea leaves incubated with [2-¹⁴C]mevalonate.

Identification of labelled squalene. Fraction 1 (Table 1) contained an appreciable amount of radio-

activity. To a portion of this material was added 1 mg. of authentic squalene and the material separated by thin-layer chromatography with hexane for development. The squalene and β -carotene bands were eluted and the radioactivity was determined. About 85% of the recovered radioactivity was in material that co-chromatographed with the authentic squalene. Radioactivity also co-chromatographed with β -carotene. To characterize the squalene further, a fraction 1 sample from another incubation of pea leaves with [2-¹⁴C]mevalonate was chromatographed on 2g. of alumina (Brockmann grade II). Elution with light petroleum (b.p. 40–60°) gave a fraction containing squalene; β -carotene remained on the column. This material (28 100 disintegrations/min.) was added to 107 mg. of authentic squalene, giving a specific activity of 262 disintegrations/min./mg. Formation of the thiourea adduct and regeneration of the squalene gave a specific activity of 250 disintegrations/min./mg. Squalene hexahydrochloride prepared from this material and crystallized from acetone had a specific activity of 263 disintegrations/min./mg. of squalene. Two further recrystallizations from acetone gave specific activities of 222 and 224 disintegrations/min./mg. of squalene respectively. The squalene hexahydrochloride had m.p. 117–120°.

Digtonin precipitation of pea steroids labelled with [2-¹⁴C]mevalonate. The results presented in Tables 1 and 2 indicate that pea leaves incorporated [2-¹⁴C]mevalonate into compounds with similar chromatographic properties to the 4,4-dimethyl and 4 α -methyl steroids. However, non-steroidal compounds, and in particular polyisoprenoid alcohols, may have the same R_f values in the systems used. Therefore it was important to eliminate the possibility that incorporation of [2-¹⁴C]mevalonate into such compounds was being

observed. This was achieved by precipitating the 3β -hydroxy steroids with digitonin. Authentic lanosterol and β -sitosterol were added as carriers to the non-saponifiable lipid obtained from pea leaves incubated with $[2-^{14}\text{C}]$ mevalonate and the steroids were precipitated as their digitonides. Chromatography of the regenerated steroids revealed three radioactive peaks as shown in Fig. 2. The first peak co-chromatographed with the added authentic lanosterol. The last peak, which contained the bulk of the radioactivity, was associated with the added β -sitosterol. The intermediate radioactive peak was in the position expected for 4α -methyl steroids (Clayton *et al.* 1963), and we have subsequently shown that a mixture containing 24-ethylidene-phenol and 24-methylenelophenol chromatographed as a single peak in this position relative to authentic lanosterol and β -sitosterol.

Fig. 1. Radioautograph of a thin-layer chromatogram of the total non-saponifiable lipids from maize. Chopped maize leaves (1g.) were incubated for 4hr. with 1.5ml. of $[2-^{14}\text{C}]$ mevalonate ($1.0\mu\text{C}$) at 25° with illumination. L, Marker lanosterol; S, marker β -sitosterol; A, B, C, D, E and F represent the zones eluted to obtain the results given in Table 2.

The 'steroid-free' non-saponifiable lipid recovered after precipitation of the steroid digitonides was examined by chromatography on alumina by using the conditions described in the Materials and Methods section. Fraction 1 contained appreciable radioactivity (32390 disintegrations/min.) identified by thin-layer chromatography as being due predominantly to squalene. Fraction 5 contained little radioactivity (1910 disintegrations/min.) and no sterol could be detected by the Liebermann-Burchard reaction. Some radioactivity was present in fraction 4 (15060 disintegrations/min.) but fraction 3 contained a very considerable proportion (96510 disintegrations/min.) of the total radioactivity recovered from the column. Steroids, such as lanosterol, that contain a 4,4-dimethyl grouping are known to be only poorly precipitated by digitonin and under the conditions used in the present work only about 10–15% of the total lanosterol is precipitated. Assay of fraction 3 by the Liebermann-Burchard reagent revealed that about 90% of the carrier lanosterol remained unprecipitated. From these results it was concluded that some if not most of the radioactivity in fraction 3 is in 3β -hydroxy steroids of the lanosterol type. Radioactivity was also present in fraction 2 (3960 disintegrations/min.) and fraction 6 (19040 disintegrations/min.); no attempt has been made to characterize further the compounds responsible for the radioactivity.

Fig. 2. Chromatography of pea-leaf steroids biosynthesized in the presence of $[2-^{14}\text{C}]$ mevalonate. Incubation conditions were the same as those used in Table 1. Authentic lanosterol (2mg.) and β -sitosterol (8mg.) were added to the non-saponifiable lipid (10.8mg.; 621300 disintegrations/min.) and the steroids were precipitated with digitonin. The regenerated steroids (11.1mg.; 419200 disintegrations/min.) were chromatographed on a column of silicic acid-Hyflo Super-Cel (see the Materials and Methods section). Fractions were assayed for radioactivity (●) and also for sterol by the Liebermann-Burchard assay (Δ, carrier lanosterol; ○, carrier β -sitosterol).

Incorporation of [Me- 14 C]methionine into the non-saponifiable lipid. [Me- 14 C]Methionine was readily incorporated into the non-saponifiable lipid of pea leaves. The distribution of radioactivity after chromatography on alumina is given in Table 1. Fraction 1 contained negligible radioactivity, and thin-layer chromatography showed that squalene was completely unlabelled. This indicates that [Me- 14 C]methionine was not becoming randomized during the incubation and entering the general mevalonate pathway. It further supports the view that any radioactivity observed in the compounds under study was arising by a direct transmethylation reaction. Most radioactivity was found in fraction 5, in which the major pea sterols were eluted. However, fractions 3 and 4 had significant levels of radioactivity, indicating that the methyl group of methionine had been incorporated into steroids belonging to the 4,4-dimethyl and 4 α -methyl structural groups. This was further demonstrated by thin-layer chromatography of a portion (13 270 disintegrations/min.) of the total non-saponifiable lipid from peas. The results are given in Table 2; also included are similar results obtained with the non-saponifiable lipid of maize. Rechromatography

several times failed to remove radioactivity that always co-chromatographed with the three structural groups of steroids. This is illustrated by the radioautograph shown in Fig. 3. Methionine also labelled materials less polar than the 4,4-dimethyl steroids; in maize this radioactivity was shown by radioautography to be in the two uppermost of the three bands that were radioactive after incubation with [2- 14 C]mevalonate (see Fig. 1).

Precipitation of the steroid digitonides followed by chromatography of the regenerated steroids (Fig. 4) gave three radioactive peaks, which co-chromatographed with the three steroid structural groups. The first was small but analysis of the 'steroid-free' non-saponifiable lipid by chromatography on alumina showed, as in the previous experiment, that 80–90% of the carrier lanosterol remained and was accompanied by appreciable radioactivity (11 530 disintegrations/min.). Therefore the proportion of radioactivity accompanying the 4,4-dimethyl steroids recorded in Fig. 4 represents a minimal amount of radioactivity in this group of steroids. The bulk of the radioactivity was associated with the carrier β -sitosterol but there was also very appreciable radioactivity in the intermediate peak corresponding to the 4 α -methyl steroids.

Fig. 3. Tracing of radioautograph of steroids biosynthesized by pea leaves in the presence of [Me- 14 C]methionine. Experimental conditions were as described in Table 1 and in the Materials and Methods section. The non-saponifiable lipid was separated on alumina (Brockmann grade III) followed by preparative thin-layer chromatography to give compounds that co-chromatographed with (a) 4,4-dimethyl steroids, (b) 4 α -methyl steroids and (c) β -sitosterol. They were rechromatographed on silica-gel plates as shown above. L, Marker lanosterol; S, marker β -sitosterol.

Fig. 4. Chromatography of pea steroids biosynthesized in the presence of [Me- 14 C]methionine. Incubation conditions, precipitation of steroid digitonides from the non-saponifiable lipids (11.2 mg.; 196 600 disintegrations/min.) and chromatography of the steroids (9.6 mg.; 122 600 disintegrations/min.) were the same as those used for Fig. 2. Fractions were assayed for radioactivity (●) and also for sterol by the Liebermann-Burchard assay (Δ, carrier lanosterol; ○, carrier β -sitosterol).

Table 3. *Incorporation of [2-¹⁴C]mevalonate into the non-saponifiable lipid of pea leaves under aerobic and anaerobic conditions*

Incubations were in Warburg-type flasks (see the Materials and Methods section). Duplicate flasks were prepared and incubated for 2 hr. at 25° with illumination. The aerobic flasks were left open to the air for 15 min. before tipping the [2-¹⁴C]mevalonate from the side arm. The contents of duplicate flasks were pooled before saponification. Other experimental details are given in the Materials and Methods section. The compositions of the eluents for fractions 1-5 are given in Table 1.

	Non-saponifiable lipid	Radioactivity (disintegrations/min.)				
		After chromatography on alumina (Brockmann grade III)				
		Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Aerobic	148500	7780	1850	37800	28000	64400
Anaerobic	3470	2870	0	200	0	100

Incorporation of [2-¹⁴C]mevalonate into squalene under anaerobic conditions. It is established that the biosynthesis of squalene in animal systems can proceed under anaerobic conditions. Oxygen is required for the cyclization of squalene to lanosterol (Tehen & Bloch, 1957). Therefore a comparison was made of the incorporation of [2-¹⁴C]mevalonate *in vivo* into pea-leaf non-saponifiable lipids under aerobic and anaerobic conditions. The results are given in Table 3. The most striking effect was the very considerable decrease in the total amount of [2-¹⁴C]mevalonate incorporated under anaerobic conditions. The distribution of radioactivity in the non-saponifiable lipid from the aerobic incubation followed a similar pattern to that observed previously, but in the anaerobic incubation the major proportion of the radioactivity was in fraction 1. Thin-layer chromatography showed that over 90% of this radioactivity co-chromatographed with authentic squalene.

Identification of the steroids of pea leaves. The non-saponifiable lipid (628 mg.) was extracted from the leaves (310 g. wet wt.) of peas germinated for 8 days in the light. The crude steroid mixture (75 mg.) obtained by digitonin precipitation was fractionated by chromatography on 10 g. of alumina (Brockmann grade III) and the column eluted stepwise with increasing percentages of diethyl ether in light petroleum (b.p. 40-60°) as indicated in the Materials and Methods section.

Fraction 3 (1.5 mg.) was further chromatographed on thin layers of silica gel and compounds with R_f identical with that of marker lanosterol were eluted. Gas-liquid chromatography on 3% SE-30 (Shimizu, Uchimarui & Ohta, 1964) revealed two major peaks and a number of minor peaks (Fig. 5a). The major peaks, 3a and 4a, had retention times relative to cholestane identical with those of authentic cycloartenol and 24-methylenecycloartanol respectively. The retention times of these

major components were also the same as cycloartenol and 24-methylenecycloartanol on a column of 5% QF-1. Acetylation of this fraction and thin-layer chromatography on plates of silica gel impregnated with silver nitrate revealed two spots that had R_f values close to those of authentic cycloartenol acetate and 24-methylenecycloartanol acetate respectively. In this system lanosterol acetate has the same R_f as cycloartenol acetate. Of the minor components revealed by chromatography on 3% SE-30, peak 1a had a relative retention time close to that of authentic β -amyrin (3.04) and peak 2a had a relative retention time similar to that of α -amyrin (3.36) or lanosterol (3.26); a more precise identification could not be made.

Steroids (1.0 mg.) with R_f corresponding to that of the 4 α -methyl steroids were obtained from fraction 4 by preparative thin-layer chromatography on silica gel. Earlier experiments with the Liebermann-Burchard reagent indicated the presence of a Δ^7 -steroid in this fraction. Gas-liquid chromatography of this material on 3% SE-30 (Fig. 5b) showed several compounds. The major component (peak 2b) had a relative retention time corresponding to that of 24-methylenelophenol. The relative retention time of peak 4b was the same as 24-ethylidenelophenol (citrostadienol). Two other peaks (1b and 3b) were apparent but it has not yet been possible to identify these compounds. Gas-liquid chromatography on 5% QF-1 gave similar results, the two major peaks having relative retention times identical with those of 24-methylenelophenol and 24-ethylidenelophenol respectively. Mass spectrometry of fraction 4 showed that the major component had a peak at m/e 412 that corresponds to 24-methylenelophenol. A peak at m/e 426 corresponding to 24-ethylidenelophenol was also observed and the fragmentation pattern was in accord with the presence of these compounds. The mass spectrum of the fraction

Fig. 5. Gas-liquid chromatography of steroids obtained from pea leaves. (a) 4,4-Dimethyl steroids on 3% SE-30. Relative retention times: peak 1a, 3.02; peak 2a, 3.37; peak 3a, 3.65; peak 4a, 4.23. (b) 4 α -Methyl steroids on 3% SE-30. Relative retention times: peak 1b, 3.26; peak 2b, 3.61; peak 3b, 4.65; peak 4b, 4.90. (c) Major sterols on 3% SE-30. Relative retention times: peak 1c, 2.64; peak 2c, 2.88; peak 3c, 3.33. (d) Major sterols on 1% Hi-EFF8B. Relative retention times: peak 1d, 8.25; peak 2d, 9.89; peak 3d, 11.33. In all cases Ch represents cholestane.

also revealed the presence of steroids with peaks at m/e 414 and 428. These could correspond to 24-methyl-lophenol and 24-ethyl-lophenol respectively, which would not be expected to separate from the corresponding methylene and ethylidene compounds on gas-liquid chromatography on 3% SE-30 or 5% QF-1. A compound with a peak at m/e 424, which has not been identified, was also observed in the mass spectrum.

Treatment of fraction 5 (71.6mg.) with the

Liebermann-Burchard reagent gave a green colour, maximal after about 30min. and characteristic of Δ^5 -sterols. Gas-liquid chromatography of the sterol mixture on 3% SE-30 (Fig. 5c) showed that the major sterols of pea leaves are β -sitosterol (peak 3c), stigmasterol (peak 2c) and campesterol (peak 1c). β -Sitosterol and fucosterol cannot be separated on 3% SE-30 but a good separation is possible on silicone-treated Gas-Chrom P with Hi-EFF8B as stationary phase (Knights, 1964). Examination of

the pea-leaf sterols on this system (Fig. 5d) revealed the presence of a small amount of material (peak 3d) with a relative retention time very close to that of fucosterol. Peaks 2d and 1d corresponded to β -sitosterol and a mixture of stigmasterol and campesterol, which cannot be separated on this system, respectively.

DISCUSSION

By analogy with cholesterol biosynthesis it is reasonable to assume that squalene is a precursor of the phytosterols. Squalene biosynthesis has now been demonstrated in a number of plants. Nicholas (1962*a,b,c*) showed that there was a rapid turnover of squalene with a concomitant increase in the specific activity of β -sitosterol when [2- 14 C]mevalonate was administered to *Ocimum basilicum*. These facts and also the demonstration by Bennett & Heftmann (1965) that radioactive β -sitosterol could be obtained from *Pharbitis nil* seedlings when [14 C]squalene had been applied to the cotyledons appear to establish beyond doubt that squalene is a phytosterol precursor. The present observation that [2- 14 C]mevalonate is incorporated into squalene in pea and maize seedlings was therefore to be expected, but it was important to demonstrate this in our experimental material under our conditions.

Oxygen is required for the cyclization of squalene to sterol in animal tissues, and Benveniste, Durr, Hirth & Ourisson (1964) have noted an accumulation of squalene in anaerobic tissue cultures of *Nicotiana tabacum*. The accumulation of radioactive squalene in pea leaves under anaerobic conditions is in agreement with these observations. The reason for the very considerable decrease in the incorporation of [2- 14 C]mevalonate into the non-saponifiable lipid that was noted in our experiments is obscure. Possibly oxygen is required for the uptake and transport of mevalonate or for the production of necessary cofactors.

From the work on cholesterol it would be expected that lanosterol (I) would be the first cyclization product in phytosterol biosynthesis (Rowe, 1965), but this remains to be demonstrated experimentally. Indeed, Benveniste, Hirth & Ourisson (1964*b*) and Ehrhardt, Hirth & Ourisson (1965), in preliminary reports, have been unable to detect lanosterol in *Nicotiana tabacum*. However, Benveniste *et al.* (1964*b*) identified cycloartenol (II) in these tissue cultures and suggested that this compound may take the place of lanosterol in phytosterol biosynthesis. Evidence for the widespread distribution of cycloartenol in plants is becoming available (see Ourisson & Crabbe, 1961). It has been obtained from potato leaves (Schreiber & Osske, 1962) and from grapefruit peel (Williams *et al.* 1965), and gas-liquid chromatography indicates its presence in very

small amounts in leaves of lucerne and larch and in *Fucus spiralis* (L. J. Goad, unpublished work). By contrast, apparently the only reports of lanosterol in higher plants are in the latex of *Euphorbia balsamifera* (Gonzalez & Mora, 1952; Gonzalez & Toste, 1952) and in tissue cultures of Pauls Scarlet Rose (Williams & Goodwin, 1965).

In the present investigation gas-liquid chromatography conclusively showed the presence of cycloartenol in pea leaves, but a minor component that could be lanosterol was also noted. However, its identification is by no means unequivocal because cycloartenol, which has been reported to accompany cycloartenol in rice-bran oil (Shimizu *et al.* 1964), and α -amyrin have similar retention times to lanosterol on the column we used, SE-30. Addition of authentic lanosterol to a sample of the 'lanosterol' fraction from peas labelled with [2- 14 C]mevalonate followed by acetylation and crystallization failed to establish conclusively the presence or absence of lanosterol. The material could not be recrystallized to constant specific activity, and during several recrystallizations it fell progressively to a low value at which point lack of material prevented further recrystallization. Further work, preferably at the enzyme level, is clearly required to investigate the possibility that cycloartenol replaces lanosterol as a precursor in phytosterol biosynthesis, but it should be emphasized that the inability to detect lanosterol in plant tissues does not of itself rule it out as a biosynthetic intermediate. Lanosterol loses three methyl groups as carbon dioxide during its conversion into cholesterol (Olson, Lindberg & Bloch, 1957), and a 4 α -methyl steroid is considered to be an intermediate. Regardless of the identity of the immediate squalene cyclization product a similar sequential loss of methyl groups must occur during phytosterol biosynthesis. A number of 4 α -methyl steroids have been obtained from higher plants and suggested as phytosterol intermediates. Lophenol (IV) was isolated from the cactus *Lophocereus schottii* (Djerassi *et al.* 1958), 24-ethylidenelophenol (citraoladienol) (VI) from grapefruit and orange-peel oil (Mazur, Weizmann & Sondheimer, 1958*a,b*) and from potato leaves (Schreiber & Osske, 1964), and 24-methylenelophenol (V) has been obtained from potato leaves and sugar-cane leaf waxes (Osske & Schreiber, 1965) and also identified in grapefruit peel (Williams *et al.* 1965). Both compounds (V) and (VI) have now been identified in pea leaves and have also been observed in varying proportions in the leaves of lucerne and larch (L. J. Goad & T. W. Goodwin, unpublished work). The ready incorporation of [2- 14 C]mevalonate and [*Me*- 14 C]methionine into this group of compounds by pea and maize leaves (Tables 1 and 2) that are actively synthesizing the major phytosterols has now been established and strengthens the suggestion that these com-

pounds are intermediates in the biosynthetic sequence from squalene to the phytosterols.

The accumulating evidence for the widespread occurrence of 24-methylenelophenol and 24-ethylidenelophenol in higher plants and the incorporation of [*Me*-¹⁴C]methionine into them is important in a consideration of the stage in the biosynthetic pathway at which transmethylation from methionine to C-24 of the phytosterol side chain occurs. The methylation mechanism proposed by Castle *et al.* (1963) suggests that a steroid with a Δ^{24} -bond is methylated to give a 24-methylene derivative. A second methylation then produces a 24-ethylidene steroid. From a study of pollen sterols Hgel, Vetter, Audier, Barbier & Lederer (1964*b*) suggested the sequence desmosterol $\rightarrow$ 24-methylenecholesterol $\rightarrow$ fucosterol $\rightarrow$ β -sitosterol. Though such a route may be the preferred biosynthetic pathway in pollen, the characterization of 24-methylenelophenol and 24-ethylidenelophenol in a number of plants coupled with the present results establishes that methylation can occur before the loss of the 4 α -methyl group. In the latter stages of cholesterol biosynthesis there is evidence of a rearrangement of the nuclear double bonds according to the sequence $\Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$ (Dempsey, Seaton, Schroepfer & Trockman, 1964), and these steps may be irreversible (Frantz, Sanghvi & Schroepfer, 1964). If similar reactions occur in sterol biosynthesis in moulds and plants they would rule out desmosterol as the precursor of ergosterol (Lederer, 1964) or of Δ^7 -sterols such as spinasterol. Methylation must occur at an earlier point. A Δ^{24} -bond is available immediately after the cyclization of squalene, e.g. lanosterol (I), euphol or cycloartenol (II), and transmethylation can occur at this level to give compounds such as euphorbol or 24-methylenecycloartanol (III). The latter compound (III) was first reported in rice-bran oil (Ohta, 1960). Evidence for the presence of 24-methylenecycloartanol (III) and for the incorporation into it of [*Me*-¹⁴C]-methionine in pea leaves was obtained in the present investigations. Benveniste *et al.* (1964*b*) have reported similar findings with tissue cultures of *Nicotiana tabacum*. Examination of the sterol complex from larch, lucerne and *Fucus spiralis* (L. J. Goad, unpublished work) and grapefruit peel (Williams *et al.* 1965) has indicated the presence of 24-methylenecycloartanol in small amounts. There appears to be no firm evidence in the literature for the existence of a 4,4-dimethyl steroid with either an ethylidene or an ethyl group at C-24. Thus if cycloartenol (II) and 24-methylenecycloartanol (III) are indeed true phytosterol precursors (Benveniste *et al.* 1964*b*) the second transmethylation may occur at the 4 α -methyl steroid level. A possible sequence based on the assumption that lanosterol is not an intermediate is:

Cycloeucalenol is included since it may bridge the gap between the 4,4-dimethyl steroids with a cyclopropane ring and the 4 α -methyl steroids such as 24-methylenelophenol. Some evidence for the presence of all the steroids given in the above sequence, including cycloeucalenol, has been obtained in grapefruit peel (Williams *et al.* 1965; B. L. Williams, L. J. Goad & E. I. Mercer, unpublished work) and in tobacco leaves (W. W. Reid, personal communication). Clayton (1965*b*) has suggested that a cyclopropane ring may hinder the loss of a C-14 methyl group. This could lead to the accumulation of steroids such as cycloartenol (II) and also cycloeucalenol (VII) and pollinastanol (VIII) (Hgel, Barbier & Lederer, 1964*a*) after the loss of one or both 4,4-dimethyl groups. Such steroids would then have to be regarded as side products, and not on the principal biosynthetic route to the phytosterols. It is noteworthy that macdougallin (IX) contains a Δ^8 -bond and a C-14 methyl group but no methyl groups on C-4 (Djerassi, Knight & Wilkinson, 1963). This compound apparently does not conform with the results of Gautschi & Bloch (1957) in animals, which suggest the obligatory loss of the C-14 methyl group from lanosterol before either of the 4,4-dimethyl groups can be removed.

The mass-spectral data indicate that 24-methylenelophenol and 24-ethyl-phenol are present in pea leaves. Osske & Schreiber (1965) have also obtained evidence for the presence of 24-methyl-phenol in sugar-cane leaf waxes. Possibly the 24-methylene and ethylidene groups are reduced at the 4 α -methyl steroid level. Loss of the 4 α -methyl group and double-bond rearrangement would then give campesterol and β -sitosterol respectively. But the presence of fucosterol as the major sterol of the brown algae (Carter, Heilbron & Lythgoe, 1939) establishes that reduction of the 24-ethylidene group is not an essential step before the loss of the 4 α -methyl group or rearrangement of the nuclear double bond to Δ^5 . Evidence for the presence in traces of fucosterol in pea leaves has now been obtained. It has previously been demonstrated in other higher-plant sources such as coconut (Anderson & Krawack, 1957), pollens (Hgel *et al.* 1964*b*) and lettuce seed and linseed (Knights, 1964), where it appears in conjunction with 24-ethyl

sterols such as β -sitosterol. The significance of fucosterol in relation to β -sitosterol biosynthesis must await further investigations.

It is thus becoming evident that, though the broad pattern of phytosterol biosynthesis resembles that of cholesterol, it may differ radically at certain points. It is also becoming evident that, because of the prodigality of enzyme production by plants or because of the low specificity of some plant enzymes, almost every possible compound between squalene and the phytosterols has now been detected. Thus the major pathway will only be revealed unambiguously by enzyme studies.

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