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GIBBERELLIN ESTIMATION AND BIOSYNTHESIS IN GERMINATING *HORDEUM DISTICHON*

GEORGE JOHNSTONE PARK MURPHY and DENNIS EDWARD BRIGGS
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT

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Key Word Index—*Hordeum distichon*; Gramineae; barley; germination; gibberellins; identification; ^{14}C-derivative; biosynthesis.

Abstract—Gibberellic acid (GA_3) and 13-deoxy-gibberellic acid (GA_7) were identified in extracts of germinating barley as their ^{14}C-methyl esters. The maximal level of GA_3 was estimated by an isotopic dilution procedure to be 1·5 ng per grain. Germinating barley incorporated 2-^{14}C-mevalonic acid into several terpenes, whose specific radioactivities were measured, but incorporation into GA_3 could not be detected. Cell-free embryo extracts from germinating barley converted 2-^{14}C-mevalonic acid into isopentenol,* dimethylallyl alcohol, farnesol and squalene, while ^{14}C-isopentenyl pyrophosphate was incorporated into geraniol, farnesol, geranylgeraniol and squalene. There was no detectable incorporation into the gibberellin intermediate *ent*-kaurene.

INTRODUCTION

DURING germination barley embryos release a substance which initiates the secretion of enzymes from the aleurone layer,[1–4] an effect which can be simulated by gibberellic acid (GA_3).[5–9] GA_3 (I) has been identified in immature grain, and compounds chromatographing with GA_4 (II) and GA_7 (III) are present.[10] The chromatographic properties of the stimulatory substance have led some workers to believe it to be GA_3 (I),[11,12] while others believe it to be GA_1 (IV).[13,14] The scutellum is possibly the initial site of gibberellin production,[13,15,16] with subsequent production in the embryonic axis.[17,18] In the present work an

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* The following trivial names are used: copalol: (−)-labda-8(16), 13-dien-15-ol; dimethylallyl alcohol: 3-methylbut-2-en-1-ol; farnesol: 3,7,11-trimethyldodec-2,6,10-*trans*-trien-1-ol; geraniol: 3,7-dimethylocta-2,6-*trans*-dien-1-ol; geranylgeraniol: 3,7,11,15-tetramethylhexadec-2,6,10,14-*trans*-tetraen-1-ol; isopentenol: 3-methylbut-3-en-1-ol; *ent*-kaurene: *ent*-kaur-16-ene; mevalonic acid: 3,5-dihydroxy-3-methylpentan-1-oic acid; squalene: 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexane.

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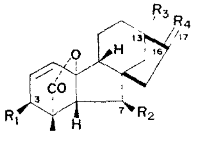
attempt was made to identify the gibberellin-like compound(s) and possible biosynthetic routes were investigated.

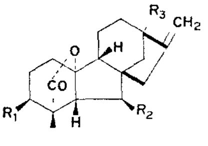
RESULTS AND DISCUSSION

Identification of Gibberellins from Germinating Barley

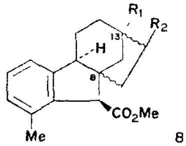
An acid fraction was prepared from barley malted for 2·5 days, when the maximum level of gibberellin occurs.[12] Materials chromatographing with the pairs GA$_1$ (IV) and GA$_3$ (I) and GA$_4$ (II) and GA$_7$ (III) were separated by TLC (solvent 1). The GA$_1$/GA$_3$ fraction was refractionated on kieselguhr G (solvent 2), the material chromatographing with GA$_3$ (I) being isolated. After methylation with ^{14}C-diazomethane the material was added to unlabelled carrier GA$_3$ methyl ester (V). The mixture was purified by preparative TLC (solvent 3) and the GA$_3$ methyl ester (V) was recrystallized from benzene–methanol to constant specific activity (about 20 dpm/mg), indicating that GA$_3$ (I) had been present in the original sample. The recovery of 60 mg of V after 5 recrystallizations was 5·5 mg. The experiment was repeated using 1 kg of barley and derivatives of the GA$_3$ ^{14}C-methyl ester were prepared. In each case the radioactive material co-chromatographed with the authentic derivative on TLC (VI, solvent 4; VII, solvent 5; VIII and IX, solvent 6), so confirming the identification of GA$_3$. The ^{14}C-methyl ester of the TLC fraction expected to contain GA$_4$ (IV) and GA$_7$ (III) was purified by TLC (solvent 3), authentic GA$_7$ methyl ester (X) was added and recrystallized from benzene. Radioactivity (about 30 dpm/mg) was retained in the crystals, so GA$_7$ (III) has been present in the initial sample of the original 32 mg of X 2·9 mg remained after six recrystallizations. Derivatives of GA$_7$-^{14}C-methyl ester were prepared. They co-chromatographed with authentic derivatives when subjected to TLC (XI, solvent 6; XIII, solvent 5; XIV, solvent 2) confirming the identity of GA$_7$.



(I)	R$_1$ = OH,	R$_2$=CO$_2$H,	R$_3$= OH,	R$_4$=CH$_2$
(III)	OH	CO$_2$H	H	CH$_2$
(V)	OH	CO$_2$Me	OH	CH$_2$
(VIII)	MeCO$_2$	CO$_2$Me	OH	CH$_2$
(IX)	MeCO$_2$	CO$_2$Me	MeCO$_2$	CH$_2$
(X)	OH	CO$_2$Me	H	CH$_2$
(XI)	MeCO$_2$	CO$_2$Me	H	CH$_2$
(XIV)	OH	CO$_2$Me	H	O



(II)	R$_1$ = OH,	R$_2$ = CO$_2$H,	R$_3$ = H
(IV)	OH	CO$_2$H	OH



			8,13-Ethano bridge
(VI)	R$_1$ = OH,	R$_2$ = CH$_2$	β
(VII)	Me	O	α
(XII)	H	Me, OH	β
(XIII)	H	Me, MeO	β

Estimation of GA$_3$ (I) in Germinating Barley

A novel method for estimating individual gibberellins, as the ^{14}C-methyl esters, can be used if sufficient unlabelled gibberellin can be obtained for use as a carrier. In principle the gibberellin (GA)-containing fraction is isolated and divided into equal portions (each containing b μg of GA). To the series of samples incremental amounts of authentic GA (x μg) are added. Each sample is independently purified by TLC, then converted to ^{14}C-methyl esters. Each sample is recrystallized with unlabelled GA-methyl ester. The amount of GA present in each sample following purification and methylation (y) is calculated from the final specific activity. At this point $y = a\,(x + b)$ where a is a factor related to the loss of gibberellin during purification and to the efficiency of ^{14}C-methylation. The factor a is expected to be approximately the same for each sample, provided that they are manipulated under identical conditions. If this is true, then a plot of y against x has a slope of a and an intercept on the y axis of ab. From this b can be calculated. This method was validated on a series of samples containing 0, 1·6, 4, 8 and 10 μg of GA_3 (I). Each sample was converted to the ^{14}C-methyl ester, was mixed with known amounts of unlabelled GA_3 methyl ester (V), and was purified and recrystallized. A plot of the final specific activity against the amount of GA_3 in the original sample gave a straight line through the origin.

The method was used to assay the level of GA_3 (I) in germinating barley. Barley (1 kg) was malted for 2·5 days. The acidic materials were extracted and divided into ten equal portions. GA_3 (0, 2, 4, 6 and 10 μg) was added to pairs of these samples. Each sample was purified, and converted to the ^{14}C-methyl esters. Then unlabelled carrier methyl ester GA_3 (V) was added and the samples were crystallized. From the final specific activity of each sample the amount of GA_3 originally present, y, was calculated. A plot of the values of y against the amounts of GA_3 added, x, gave a straight line, with a calculated slope a of 0·225 and an intercept on the y axis of 0·961 μg giving a value of $0{\cdot}961/0{\cdot}225 = 4{\cdot}27$ μg GA_3/original sample. This is about 1·5 ng of GA_3 (I) per corn, in reasonable agreement with the amount of gibberellin-like material found by bioassay (1 ng).[12] Since there is a correction for losses during purification inherent in the radioactive derivative assay a higher figure than that derived by bioassay is expected. Lack of carrier prevented GA_7 being asayed in a similar manner.

The Biosynthesis of Terpenes in Germinating Barley

Two approaches were made to achieve the incorporation of terpene precursors into *ent*-kaurene or GA_3 (I) in germinating barley. Firstly proteins were separated from cell-free extracts and tested for their ability to synthesize *ent*-kaurene, a biosynthetic intermediate, from terpene precursors. Secondly whole barley was germinated in the presence of 2-^{14}C-mevalonic acid (MVA) and incorporation into GA_3 (I) and other terpenes was determined.

Cell-free extracts from some plants actively synthesizing gibberellins are capable of converting MVA into *ent*-kaurene, the activity occurring in the supernatant fraction prepared from tissue homogenates by high-speed centrifugation.[19–24] Extracts need to be supplemented with ATP and Mg^{2+} or Mn^{2+}. The metabolism of *ent*-kaurene to *ent*-7 α-hydroxykaur-16-en-19-oic acid also occurs via the "microsomal" fraction in such preparations.[19] Comparable extracts were prepared from embryos from barley malted for 1·5 days, the time of maximal increase in the level of gibberellin-like material.[12] The incubation conditions used were those found to be optimal[25] for the production of the recognized terpene precursor, farnesyl pyrophosphate (FPP). No radioactive hydrocarbons were detected in these trials. However, about 50% of the radioactive material from 2-^{14}C-MVA was volatile at room temperature. Unlabelled carrier isopentenol, dimethylallyl alcohol, geraniol and farnesol were added to the bulk of the radioactive solution. Three radioactive materials were separated from the concentrated solution by GLC. The material eluted first was not identified but the others had the same retention times as isopentenol and dimethylallyl alcohol. Materials from four GLC separations were groupėd. Appropriate unlabelled

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alcohols were added and 3,5-dinitrobenzoyl esters were prepared. Attainment of constant specific activity on recrystallization confirmed the identities of the separated materials. With the isopentenyl ester a constant specific activity of 2·2 dpm/mg was obtained in 12 mg crystals after 4 crystallizations from ethanol, beginning with 72 mg material. With the ester of dimethylallyl alcohol four crystallizations from ethanol of 86 mg material yielded 14·5 mg with a specific activity of 3·5 dpm/mg. Portions of the non-volatile radioactive material were separated by TLC (solvent systems 7, 8 and 9), the radioactive material chromatographed like farnesol. Carrier farnesol or geraniol was added to samples of the radioactive material purified by TLC (solvent 8) and derivatives were made and recrystallized. Radioactivity was lost when the geranyl ester was recrystallized. The major non-volatile radioactive product was farnesol as four crystallizations from hexane gave 2·1 mg ester of constant specific activity (598 dpm/mg) from an original 12·3 mg of material.

Presumably the terpenols were produced by the action of a phosphatase on their pyrophosphate esters, and were side-products of the biosynthetic pathway. As the level of GA_3 (I) in intact germinating grain was low, high levels of the enzymes responsible for the production of *ent*-kaurene were unlikely to be present. It was hoped that by inhibiting phosphatase activity, incorporation into terpenes, including *ent*-kaurene, could be increased. However, such inhibitors might inhibit the enzymes of terpene synthesis, so their effects were tested on the formation of squalene. This hydrocarbon is formed from MVA, its formation could be followed easily, and it is readily formed in comparable extracts from pea seedlings.[20]

TABLE 1. INCORPORATION OF ^{14}C-ISOPENTENYL PYROPHOSPHATE INTO TERPENES USING CELL-FREE EXTRACTS OF BARLEY

Fraction	Recovery dpm	 % IPP
(a) Without iodoacetamide		
Total extract	277 000	61·5
Non-volatile extract	181 000	40·3
Material chromatographing with:		
Geraniol	42 300	9·4
Farnesol	39 300	8·72
Geranylgeraniol	22 000	4·9
(b) With iodoacetamide and farnesyl pyrophosphate		
Total extract	—	—
Non-volatile extract	52 000	11·6
Material chromatographing with:		
Geraniol	1020	0·23
Farnesol	1428	0·32
Geranylgeraniol	35 700	8·8

Lipids were extracted from incubations with 2-^{14}C-MVA and separated by TLC (solvent 7). Extracts supplemented with NADPH formed a radioactive material chromatographing with squalene (solvent 10). Levels of NADPH of 0·5 mM or more gave maximal rates of synthesis. The identity of this radioactive material was confirmed by adding unlabelled squalene, preparing the hexachloride derivative and crystallizing both acetone soluble and insoluble materials three times from ethyl acetate to constant specific activity (33 dpm/mg).

Acetone soluble material, initially 43 mg, gave 20·5 mg final crystals. Acetone insoluble material, 9·5 mg, eventually gave 4·5 mg crystals.

Various compounds shown to be inhibitory to 4-nitrophenylphosphatase (E.C. 3.1.3.2.) in cell-free extracts of barley were tested for their effects on the incorporation of MVA into squalene. Both fluoride (2–10 mM) and L-3-glycerophosphate (10–40 mM) were inhibitory while phosphate had no effect in the range of concentrations tested. Iodoacetamide, which did not inhibit 4-nitrophenylphosphatase, stimulated the incorporation at low concentrations (1–2 mM), but was a potent inhibitor at high concentrations (8–12 mM), presumably due to its effect on isopentenylpyrophosphate isomerase (isopentenyl pyrophosphate Δ^3–Δ^2 isomerase E.C. 5.3.3.2.).[26] An attempt to separate the phosphatase from the terpene-synthesizing proteins by fractionation with $(NH_4)_2SO_4$ was unsuccessful.

Two other methods were tested in attempts to reduce the effect of phosphatase on terpene synthesis. ^{14}C-Isopentenyl pyrophosphate (IPP) was used as substrate in preference to MVA to reduce the number of enzymatic steps involved. In other incubations iodoacetamide (10 mM) was added to inhibit the isopentenyl pyrophosphate isomerase, with the addition of synthetic farnesyl pyrophosphate (FPP) to condense with the ^{14}C-IPP. The iodoacetamide was expected to partially inhibit prenyl transferase (dimethylallyl pyrophosphate: isopentenyl pyrophosphate dimethylallyl transferase E.C. 2.5.1.1.) and *ent*-kaurene synthetase, but transferase from other sources is only 40% inhibited by 20 mM iodoacetamide.[27] *ent*-Kaurene synthetase from *Ricinus communis* is inhibited 65% by 10 mM iodoacetamide.[24] Cell-free extracts prepared from germinated barley were incubated, using the new conditions. Alkaline phosphatase was added to hydrolyse phosphate esters, and the lipids were then recovered. The incorporations were qualitatively similar for extracts from grain germinated for 0, 1, 1·5, 2, 2·5 and 5 days. Only results for extracts from barley germinated for 1·5 days are illustrated. Carrier geraniol, farnesol and geranylgeraniol were added to the lipid extracts and the incorporation into volatile and non-volatile lipids was measured (Table 1). The lipid was separated by TLC (system 7). No radioactive material co-chromatographed with *ent*-kaurene although traces of radioactivity ran with squalene. The polar radioactive materials remaining at the origin of the original chromatogram were separated by reverse-phase TLC (solvent 9). The incorporations into materials chromatographing with geraniol, farnesol and geranylgeraniol were determined (Table 1). The identities of these compounds were confirmed when portions of the radioactive materials were added to unlabelled carriers and 4-(4-nitrophenylazo) benzoyl esters were made, then recrystallized from hexane to constant specific radioactivity. The geranylgeranyl ester, 7·2 mg, recrystallized three times, gave 1·65 mg material of constant specific activity 190 dpm/mg. The farnesyl ester, 9·7 mg, after three crystallizations gave 1·74 mg product of constant specific activity 1320 dpm/mg. For the geranyl ester the values were 14·9 mg, 4·62 mg, specific activity 1158 dpm/mg. In incubations with iodoacetamide, geranylgeraniol was the major product, with only traces of farnesol and geraniol. In incubations without iodoacetamide, farnesol and geraniol were formed in roughly equal amounts, with only a small amount of geranylgeraniol. Since geranylgeraniol pyrophosphate cyclizes to give copalyl pyrophosphate and then *ent*-kaurene[28] the failure to find *ent*-kaurene in these tests, although the pyrophosphate appeared to be present, suggests that the enzymes for its formation are not present.

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Experiments with Intact Germinating Barley

Barley, treated with 2-^{14}C-MVA (10·7 μCi), was malted for 63·5 hr. Neutral and acidic extracts were prepared. The acidic fraction was separated by TLC (solvent 1) and the GA$_1$ (IV)/GA$_3$ (I) zone (R_f 0·05–0·25) was collected. The radioactive material was separated by PC (solvent 11). The positions to which GA$_1$ (IV) and GA$_3$ (I) would chromatograph were located respectively 25 cm and 19 cm from the origin but no increase in radioactivity above background was observed in either region, the greatest activity occurring 15 cm from the origin.

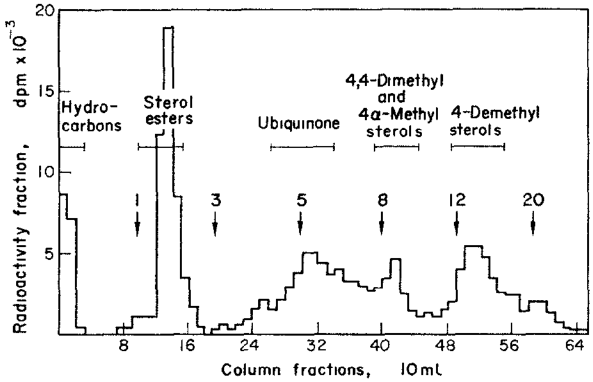

FIG. 1. THE SEPARATION BY ALUMINA COLUMN CHROMATOGRAPHY OF A SAMPLE OF NEUTRAL LIPID FROM BARLEY GERMINATED IN THE PRESENCE OF 2-^{14}C-MVA.

Solvent changed to the indicated % diethyl ether in light petrol. (b.p. 40–60°) at arrows. The tentative identification of the radioactive materials is shown above the peaks.

A small sample of the neutral lipid fraction was chromatographed on a column of alumina and the eluate was assayed for radioactivity. The elution profile and tentative identification of the separated materials is shown in Fig. 1. The MVA was incorporated into a variety of terpenes. A larger sample of the neutral lipid was separated by column chromatography (Table 2). Each column fraction was further examined (see Experimental).

TABLE 2. SEPARATION BY ALUMINA COLUMN CHROMATOGRAPHY OF NEUTRAL LIPIDS FROM BARLEY GROWN IN THE PRESENCE OF 2-^{14}C-MEVALONIC ACID

Solvent (% diethyl ether in light petrol.)	Radioactive material (dpm)	Wt eluted (mg)	Tentative identification
0·25	217 000	50 3	Hydrocarbons
1	809 000	39·7	Sterol esters
3	14 000	3 8	Tocopherol
5	192 600	13·9	Ubiquinone
20	678 000	23 1	Sterols

The hydrocarbon fraction was separated by TLC (solvent 10). Most of the radioactivity chromatographed with squalene. Identification was confirmed by adding carrier squalene, forming the hexachloride and recrystallizing to constant specific activity (1115 dpm/mg). A minor unidentified peak of radioactivity chromatographed more slowly than β-carotene.

The remaining fractions were purified (methods) and their specific activities at the various stages of purification were measured (Table 3). Exogenous 2-^{14}-C-MVA was incorporated into terpenes during germination. The specific activities of the sterol esters, sterols and ubiquinones were much higher than those of plastoquinone or α-tocopherol. The latter two were not completely purified, but the specific activities were decreasing at each stage. Since plastoquinone and α-tocopherol are plastid terpenes while the others are extra-plastidic these lower values may have important implications if GA_3 (I) is normally synthesized inside plastids.[29,30]

TABLE 3. THE SPECIFIC ACTIVITIES OF TERPENES EXTRACTED FROM BARLEY GROWN IN THE PRESENCE OF 2-^{14}C-MEVALONIC ACID

Terpene and initial specific activity (dpm/mg)	1st purification Amount (μg)	1st purification Specific activity (dpm/mg)	2nd purification Amount (μg)	2nd purification Specific activity (dpm/mg)
4-Demethyl sterol ester	2160	57 300	770	59 300
4α-Methyl sterol ester	1060	147 440	710	123 800
4,4-Dimethyl sterol ester	77	2138 000	22	292 000
Plastoquinone (1070 000)	18·5	107 800	8·5	14 100
α-Tocopherol	60	8630	35	550
Ubiquinone UQ-9	25	128 000	13	132 000
(2050 000) UQ-10	54	164 000	32	161 700
4-Demethyl sterols	7550	47 520	5930	38 700
4α-Methyl sterols	1570	46 606	700	45 700
4,4-Dimethyl sterols	60	156 200	10	568 000

The experiment was repeated twice, and the acidic fraction was purified by TLC (solvent 1). The region to which GA_3 (I) would chromatograph (R_f 0·05–0·25) was eluted and the recovered extract was added to authentic GA_3 (I). The mixture was methylated and recrystallized from benzene–methanol. No incorporation into GA_3 (I) had occurred as no radioactivity remained after four recrystallizations. The failure to obtain incorporation of 2-^{14}C-MVA into GA_3 or *ent*-kaurene, even though some terpene synthesis is occurring, suggests that in the initial stages of germination gibberellin is being formed at the expense of a stored precursor such as *ent*-kaurene, or from a bound form. Alternatively the MVA may not be able to penetrate to the synthetic site, as occurs in plastids.[29,30] Work in progress favours the first explanation.

EXPERIMENTAL

Materials. Alkaline phosphatase (Type II) from calf intestinal mucosa was obtained from Sigma, London. Farnesyl pyrophosphate was prepared from farnesol.[31,32] GA_3 (I) was obtained from Plant Protection Ltd., Yalding, Kent ^{14}C-Isopentenyl pyrophosphate was prepared from 2-^{14}C-MVA.[28] N-^{14}C-Methyl-N-nitrosotoluene-4-sulphonamide was obtained from the Radiochemical Centre, Amersham, Bucks. 4-(4′-Nitrophenylazo)-benzoyl chloride was prepared by the method of Hecker.[33] Insoluble polyvinyl-pyrrolidone

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(Calbiochem, London) was washed with 0·1 N HCl, H_2O, then EtOH and air-dried before use. GA_7 (III), geranylgeraniol and ubiquinones-9 and -10 were gifts.

Measurement of radioactivity. Liquid scintillation fluid was 2,5-diphenyloxazole (5·0 g) and 1,4-di-2-(5-phenyloxazole)-benzene (0·1 g) in toluene (1 l.). Scintillation counting employed a Nuclear Chicago Model 120. Chromatograms were initially evaluated using a Packard Model 7201 radiochromatogram scanner. Powder removed from TLC plates or strips cut from PCs were counted directly in the scintillation counter.

Column separation of lipids. The separation of neutral lipids from barley extracts was performed on Brockman grade III alumina.[34]

TLC separation of lipids. These were performed using the solvent systems listed. Unless otherwise stated the support was silica gel G. Reversephase TLC plates were prepared by preliminary development of the plate in a 10% soln. of the stationary phase in $CHCl_3$ followed by air-drying. R_fs varied in some separations and compounds were always located with reference to the indicated authentic standards. Approximate R_f are quoted in brackets after the standard. (1) EtOAc–$CHCl_3$–HOAc (40·60·5), GA_1/GA_3 (VI, I; 0·15), GA_4/GA_7 (II, III; 0·45). (2) C_6H_6–HOAc–H_2O (8·3:5; upper), GA_1 (VI, 0·67, Kieselguhr G), GA_3 (I; 0·45; Kieselguhr G), 16-oxo-17-nor-GA_7 Me ester (XIV; 0·32). (3) EtOAc, GA_3 Me ester (V; 0·50), GA_7 Me ester (X; 0·70). (4) C_6H_6–EtOAc (2·1), allogibberic acid Me ester (VI; 0·63). (5) C_6H_6–EtOAc (1:1), allogibberic acid Me ester (VI; 0·44), gibberic acid Me ester (VII; 0·71), 16-hydroxy-13-deoxy-allogibberic acid Me ester (XII; 0·55), 16-methoxy-13-deoxy-allogibberic acid Me ester (XIII, 0·78). (6) Di-isopropyl ether–HOAc (95:5), 3-O-acetyl GA_3 Me ester (VIII; 0·40; double development), 3,13-di-O-acetyl GA_3 Me ester (IX; 0·78, double development), 3-O-acetyl GA_7 Me ester (XI; 0·60). (7) Hexane, *ent*-kaurene (0·65), squalene (0·28), geraniol and farnesol (0). (8) C_6H_6–EtOAc (9:1), geraniol (0·33), farnesol (0·39). (9) MeOH–H_2O (7:3; saturated with MS-200 silicone oil, reversephase TLC), geraniol (0·63) farnesol (0·47), geranylgeraniol (0·25). (10) Light petrol (b.p. 40–60°)-C_6H_6 (19:1), squalene (0·74), β-carotene (0·30). (11) For PC: C_6H_6–HOAc–H_2O (4·2:1, upper) on Whatman No 1 paper, chromatographed for 36 hr. Gibberellin standards were detected by spraying with 0·5% $KMnO_4$, subsequently washing to remove excess (12) Light petrol (b p. 40–60°)–C_6H_6 (3:2), sterol esters (0·65). (13) $CHCl_3$ (double development), 4,4-di-Me sterols (0·60), 4 α-Me sterols (0·50), 4-deMe sterols (0·42). (14) Acetone–H_2O (19·1; saturated with liquid paraffin, reverse-phase TLC), plastoquinone (0·20), ubiquinone 9 (UQ-9) (0·45), UQ-10 (0·34) (15) Acetone–H_2O (9·1 saturated with liquid paraffin, reverse-phase TLC), plastoquinone (0·52), UQ-9 (0·85), UQ-10 (0·70). (16) $CHCl_3$–C_6H_6 (1:1), α-tocopherol (0·50), ubiquinones (0·32). (17) EtOAc–C_6H_6 (3·22), α-tocopherol quinone (0·30).

Formation of reference compounds and derivatives. Me and acetyl derivatives of gibberellins were made by standard procedures which have been used on GA_3[35] and GA_7.[36]

Allogibberic acid Me ester (VI). GA_3 (100 mg) was heated in dil. HCl (10%, 10 ml, 2·25 hr at 50–5°).[35] The mixture was cooled and filtered, and the solid residue washed with H_2O. The dried residue was extracted with boiling C_6H_6 and the undissolved GA_3 rapidly filtered. The filtrate was concentrated to dryness and the residue recrystallized from C_6H_6-m p. 203° (reported m.p. 200·5–203°) [35] The ester was prepared by reaction with CH_2N_2. Reaction of suspected GA_3-^{14}C-Me ester with acid gave rise to a compound chromatographing with VI (solvent 4).

3,5-*Dinitrobenzoyl esters.* Isopentenol, dimethylallyl alcohol and geraniol were esterified with 3,5-dinitrobenzoyl chloride The alcohol (*ca.* 100 mg) was dissolved in C_6H_6 (10 ml) and pyridine (0·2 ml) and 3,5-dinitrobenzoyl chloride (200 mg) was added The mixture was refluxed 1 hr, cooled and washed with 10% KOH (10 ml), 10% Na_2CO_3 (10 ml), 0·1 M H_2SO_4 (10 ml) then H_2O. The ester was recrystallized from MeOH.

Gibberic acid methyl ester (VII). Allogibberic acid was heated under reflux in dil. HCl (20%, 5 ml, 1 hr).[35] The product was extracted into EtOAc and recrystallized from EtOAc–light petrol. M.p. 151° (reported m p 151–3°) [35] Reaction with CH_2N_2 gave the Me ester. Treatment of suspected GA_3 ^{14}C-Me ester produced compounds chromatographing with VII and VI (solvent 5)

16-*Hydroxy*-13-*deoxy-allogibberic acid Me ester* (XII) GA_7 (5 mg) was dissolved in MeOH (0·5 ml) then H_2O (1·1 ml) and HCl (0·5 ml) were added, and held at room temp for 5 days, then extracted into EtOAc.[36] The EtOAc soluble materials were methylated and the mixture of 16-hydroxy- and 16-methoxy-13-deoxy-allogibberic acid Me esters (XII and XIII) were used directly as TLC markers. Suspected GA_7 ^{14}C-Me ester treated in this way gave products chromatographing with XII and XIII (solvent 5).

16-*Keto*-17-*nor*-*GA_7 Me ester* (XIV). GA_7 (10 mg), dissolved in tetrahydrofuran (5·6 ml) and H_2O (4·4 ml), was stirred while OsO_4 (0·1 ml, 2% solution) was added, followed by $NaIO_4$ (30 mg) and then stirred overnight [36] The mixture was diluted with H_2O (7 ml) and HCl (0·1 ml) then extracted into EtOAc. The EtOAc soluble material was methylated with CH_2N_2 and was not further purified. Suspected GA_7 ^{14}C-Me ester gave a product chromatographing with XIV (solvent 2).

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^{14}C-Methylation. N-^{14}C-Methyl-N-nitrosotoluene-4-sulphonamide(1·7 mg, 28·8 × 10^6 dpm) dissolved in diethyl ether (25 ml) at 0° was treated with NAOH (20 mg) in methyl digol (2 ml) for 15 min. The $^{14}CH_2N_2$ was distilled with the ether at 55°. The GA_3 or GA_7 containing fraction purified by TLC was dissolved in MeOH (0·2 ml) and treated with $^{14}CH_2N_2$ (1 ml ethereal solution, 512 000 dpm). The mixture was left overnight at 4°, the solvent removed by evaporation and the residue dissolved in fresh Et_2O (2·0 ml) before re-evaporating. After the addition of carrier GA-methyl ester and purification by TLC (solvent 3) the ester was recrystallised from C_6H_6–MeOH.

4-(4-Nitrophenylazo) benzoyl esters. Farnesol, geraniol and geranylgeraniol were esterified with 4-(4-nitrophenylazo) benzoyl chloride. The alcohol (10 mg) in C_6H_6 (4 ml) and anhyd. pyridine (0·1 ml) was treated with the reagent (20 mg) and left 2 days at room temp. After washing with 0 5 M H_2SO_4 (2 × 2 ml) and H_2O the dried product was applied in C_6H_6 to a column of Brockman grade II alumina (10 × 2 cm) and eluted with C_6H_6, (100 ml).[33] The eluate was concentrated and the residue separated by TLC (solvent 12) the main orange–red band being retained. The esters were recrystallized from hexane, and the amount present determined by measuring the extinction at 465 nm in C_6H_6. The following concentrations of derivatives gave an extinction of 1 at 465 nm (1 cm light path); geraniol 0·672 mg/ml, farnesol 0·778 mg/ml, geranylgeraniol 0·889 mg/ml.

Squalene hexachloride. The method of Langdon and Bloch was used.[37] The product insoluble in acetone was recrystallized from warm EtOAc.

α-Tocopherol quinone. This was prepared from α-tocopherol by the modified method of Dilley and Crane.[38]

Estimations. The following estimations were also used: 4-Nitrophenyl phosphatase.[39] Protein-biuret reaction,[40] Ubiquinone,[41] Plastoquinone,[42] α-Tocopherol quinone,[38] α-Tocopherol,[43,44] sterols- 'fast-reacting' and 'slow-reacting' sterols,[45] 4,4-dimethyl sterols.[46]

Germination of barley. The selection, preparation and decortication of barley has been described.[12] Germination of sterile grain (usually 200 g) was under aseptic malting conditions. 2-^{14}C-MVA solutions were sterilized by a Millipore filtration, added to the barley after the second draining and spread evenly throughout the grain by agitation.

Lipid extraction from intact grain. Grain (200 g dry wt) was homogenized with *iso*PrOH (400 ml, 2 min) at top speed in a Waring blender, left for 30 min with occasional agitation, then filtered. Debris was re-extracted with *iso*PrOH (400 ml), then *iso*PrOH–$CHCl_3$ (1:1, 400 ml), then $CHCl_3$ (400 ml).[47] The final $CHCl_3$ extract was colourless. The extracts were pooled, concentrated under reduced pres. to *ca.* 500 ml, then diethyl ether (200 ml) was added. The organic layer was extracted with $NaHCO_3$ (5%, 50 ml × 4), then with dist. H_2O (50 ml × 3) before concentrating to dryness. The residue constituted the neutral lipid fraction. The $NaHCO_3$ extracts were acidified (pH 2) and extracted with EtOAc (50 ml × 5). The extracts were pooled, washed with 0·01% HCl (50 ml × 2) and H_2O (50 ml × 1) then evaporated to dryness. The residue was the acidic fraction.

Preparation of proteins from cell-free extracts. Germinated grain (30 g) was homogenized with a sucrose solution (70 ml, 710 g sucrose/l.) for 30 sec, half maximum speed, MSE top-drive homogenizer. The suspension was centrifuged (1000 *g*, 5 min) when separated embryos, which floated, were collected. Yield was 2–5 g of embryos from 200 g of dry grain. The embryos were added to buffer (40 ml 0 1 M Tris–HCl, pH 7·1 containing $MgCl_2$, 5 mM and 2-mercaptoethanol, 10 mM), and polyvinylpyrrolidone (0·5 g/g embryos). The mixture was lightly ground with pestle and mortar, then at 800 rpm in a Potter homogenizer with 0·15 mm radial clearance. The homogenate was filtered through two layers of nylon mesh (15 denier) then centrifuged (10 000 *g*, 15 min). The supernatant was decanted, recentrifuged (100 000 *g*, 1 hr). The material precipitating between 20 and 80% saturation with $(NH_4)_2SO_4$ from the supernatant was collected, dissolved in buffer (15 ml) and dialysed against buffer. Typical preparations had a protein content of about 6 mg/ml.

Lipid Extraction from cell-free preparations. Acetone (equal vols) was added to incubation mixtures to stop the reactions. Diethyl ether (1·5 × original incubation vol.) was added and the lipids thoroughly mixed, then centrifuged to form 2 layers. The organic phase was collected. The Et_2O extraction was repeated 2×. The pooled Et_2O extracts were washed with H_2O ×3, then dried with Na_2SO_4 (1 g/4 ml Et_2O).

Incubations with proteins from cell-free extracts. The incubation conditions used were those found to be optimal for the formation of FPP. These were: buffer 0·1 M Tris–HCl pH 7·1, containing ATP (5 mM),

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$MgCl_2$ (5 mM) and mercaptoethanol (10 mM) In initial trials with cell-free extracts 2-^{14}C-MVA (2 μCi) was incubated with protein (10 mg) in buffer of the above composition (5 ml) at 25° for 5 hr. A portion of the extracted lipid was assayed for radioactivity, dried under N_2 at room temp. and re-assayed. Half of the radioactive material was volatile under these conditions. Carrier isopentenol, dimethylallyl alcohol, geraniol and farnesol (2 mg of each) were added to the remainder of the extract which was concentrated to *ca.* 0·2 ml. Portions (10 μl) were separated by GLC (column 5% polyethylene glycol —1500 on celite, 1·52 m, 80°, N_2 flow rate 80 ml/min). Isopentenol was eluted at 19 min dimethylallyl alcohol at 27 min, and the unidentified material at 3 min Samples of the eluate from the column, collected in traps cooled in a solid CO_2-acetone mixture, were assayed for radioactivity. In experiments in which the formation of squalene was studied, incubations (70 min at 30°) contained 2-^{14}C-MVA (0·2 μCi), protein (30 mg) in buffer (5 ml) and NADPH in the range 0–4 mM. Under optimal conditions incorporation of R-MVA into squalene was around 2% Where the effect of potassium fluoride, iodoacetamide, potassium phosphate and L-3-glycerophosphate on 4-nitrophenyl phosphatase was investigated, 4-nitrophenyl phosphate (6 mM) and protein (0·4 mg) were incubated in buffer (1·0 ml), with the appropriate inhibitor. In an attempt to fractionally precipitate the phosphatase with $(NH_4)_2SO_4$ in the ranges 0–20, 20–40, 40–60 and 60–80% saturation the specific activity of the enzyme was found to be similar in each fraction In the tests of these inhibitors on squalene formation incubations (30 min at 30°) contained MVA (0·1 μCi), NADPH (1 mM) and protein (1·2 mg) in buffer (1·0 ml). In incubations with ^{14}C-isopentenyl pyrophosphate the mixture was: ^{14}C-IPP (450 000 dpm), $MgCl_2$ (5 mM), mercaptoethanol (5 mM) and protein (21·2 mg) in 0·1 M Tris–HCl pH 7·1 (5·0 ml). One set of incubations contained in addition iodoacetamide (10 mM) and FPP (1 mM). Incubation was for 150 min at 25° then alkaline phosphatase (15 mg) was added and the incubation continued for 120 min at 37°. After lipid extraction carrier geraniol, farnesol and geranylgeraniol (2 mg of each) were added before TLC.

Experiments with intact germinating grain. In the initial experiment on the conversion of 2-^{14}C-MVA into terpenes, the incorporations were: neutral lipid 4·04 g, 7·47 × 10^6 dpm, 62·7% of the R-MVA added; acidic fraction 63 000 dpm, 0·53% of the R-MVA added. A sample of the neutral lipid (220 000 dpm, 118 mg) was chromatographed on a column of Brockman grade III alumina (15 × 1·2 cm) Fractions (10 ml) were assayed for radioactivity. A larger lipid sample (1·13 g, 2·1 × 10^6 dpm) was separated on a similar column (32 × 1·6 cm). A step-wise gradient of diethyl ether in light petrol. (b p. 40–60°) was employed in 1 l. steps. The sterol ester fraction was purified on rhodamine 6 G impregnated TLC plates (solvent 12), with a marker of cholesterol acetate. The esters were saponified and reseparated (solvent 13) using markers of lanosterol and sitosterol After measurement of their specific activities the 4 α-Me and 4,4-di-Me sterols were subjected again to TLC (solvent 13) The 4-deMe sterols were purified as their digitonides. The plastoquinone fraction was recovered from the TLC plate used in the initial separation of the sterol esters, the zone between the esters and the solvent front being recovered. After assay the quinone was purified by reverse-phase TLC (solvent 14 and 15), being detected by spraying leucomethylene blue[48] onto a marker sample. The presence of plastoquinone in the purified extract was confirmed by measuring the difference spectrum between oxidized and reduced samples, when a peak of 256 nm was observed[49] The α-tocopherol fraction, located with the aid of an authentic marker, was purified by TLC (solvent 16), then was converted to the quinone.[38] This was separated by TLC (solvent 17) and its specific activity measured. It was then reduced, separated by TLC (solvent 16) and assayed colorimetrically.[43,44] No forther purification was attempted. The ubiquinone fraction underwent preliminary purification by TLC (solvent 16) using UQ-9 as a marker. A portion of the quinone fraction was assayed, and separated by reverse-phase TLC (solvent 14) with markers of UQ-9 and UQ-10. Each samples was rechromatographed by reverse-phase TLC (solvent 15) and reassayed. The sterol fraction was purified as described for the sterol esters after saponification. In the two experiments in which the acidic lipid fraction was recrystallized with GA_3 as the Me ester, barley (200 g) was germinated for 64·5 hr with 2-^{14}C-MVA (10 μCi) In one experiment the GA_3 fraction purified by TLC (7380 dpm) was added to GA_3 (60 mg) before methylation. In another experiment these values were 630 dpm and 31 mg. In this experiment 11·7 mg of the crystals from the fourth recrystallization contained no detectable radioactivity.

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