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BIOSYNTHESIS OF KAURENE, SQUALENE AND PHYTOENE FROM MEVALONATE-2-^{14}C IN A CELL-FREE SYSTEM FROM PEA FRUITS*

J. E. GRAEBE

Pflanzenphysiologisches Institut der Universität, 34 Göttingen, Germany

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Abstract—Kaurene, squalene and phytoene were identified as products from mevalonate-2-^{14}C in a soluble system from young pea fruits. All three hydrocarbons were obtained when the incubation mixture contained ATP, Mg^{2+}, Mn^{2+} and pyridine nucleotides. Only squalene required pyridine nucleotides and only phytoene required Mn^{2+}. The omission of pyridine nucleotides therefore leads to a specific exclusion of squalene synthesis and the omission of Mn^{2+} to a specific exclusion of phytoene synthesis. In addition, the formation of squalene was inhibited over a certain range of Mn^{2+}-concentrations, while the formation of phyto ene was stimulated. Therefore the ratio of these two products was strongly dependent on the Mn^{2+}-concentration. The formation of kaurene was optimal at a lower pH than that for the formation of the other products. Only kaurene synthesis was inhibited by the plant growth inhibitor AMO 1618. A mixed plastid and mitochondrial fraction greatly increased the formation of squalene, farnesol and geranylgeraniol but not the formation of phytoene and kaurene. One or more unidentified acids were also formed in the system.

INTRODUCTION

THE ISOPRENOID pathways in plants lead to the formation of gibberellins, steroids, carotenoids, the side-chains of quinones and the phytyl side-chain of chlorophyll. These are all physiologically significant compounds[1] the amounts and ratios of which vary during the development. A knowledge of the mechanisms of their formation would therefore be valuable to an understanding of plant development. The present work is part of current efforts to produce cell-free systems from higher plants in which the terpenoid biosynthesis and its regulation can be studied.

A cell-free system from young pea fruits is described in which kaurene, squalene and phytoene are produced simultaneously from mevalonate-2-^{14}C. These hydrocarbons represent the first or, in the case of squalene,[2] almost the first members of the individual pathways leading from common intermediates to the gibberellins, steroids and carotenoids, respectively (Fig. 1). These reactions, by which the first members are formed, would be very likely points of regulation of the individual pathways. Farnesol and geranylgeraniol are also formed in the system. A preliminary report of some of the results has been presented.[3]

* This work was supported by the Deutsche Forschungsgemeinschaft.

[1] T. W. GOODWIN, in *Terpenoids in Plants* (edited by J. B. PRIDHAM), p. 1, Academic Press, London and New York (1967).
[2] H. C. RILLING, *J. Biol. Chem.* **241**, 3233 (1966).
[3] J. E. GRAEBE, *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 6 (1968).

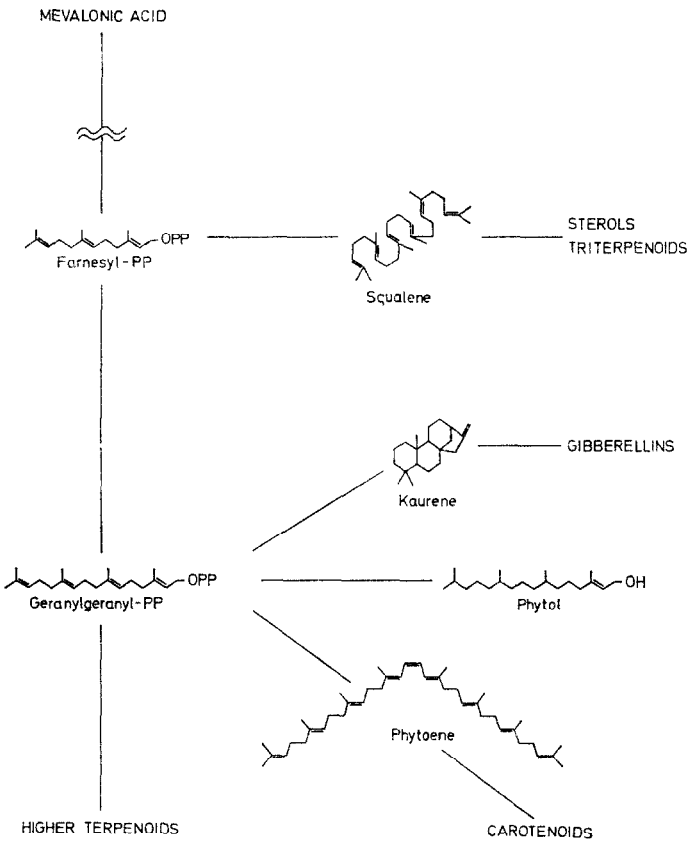

FIG. 1. BIOGENETIC RELATIONSHIPS OF THE TERPENOIDS.

RESULTS

General Pattern of Mevalonate Incorporation

When cell-free extracts consisting of the particle-free, concentrated proteins from various parts of pea plants were incubated with mevalonate-2-^{14}C, ATP with regenerating system, pyridine nucleotides, $MgCl_2$, $MnCl_2$ and buffer, the label became incorporated into neutral and acidic lipids. The neutral lipids were extracted at pH 9·5 with petroleum ether and separated by thin-layer chromatography. Scanning the plates for radioactivity and comparison with the co-chromatographed reference substances revealed that the label had become associated with fractions migrating like phytoene, squalene and—in extracts from certain developmental stages—kaurene (Fig. 2(*a*)). Some radioactivity was also associated with fractions migrating like geranylgeraniol and farnesol (Fig. 2(*b*)). The acidic fraction was obtained by acidification of the alkaline water phase and extraction with ethyl acetate. Separation of this fraction by chromatography resulted in three major radioactive areas (Fig. 2(*c*)). The peak at 3·5 cm was caused by the portion of unreacted mevalonic acid that distributed into ethyl acetate. The peak at 8 cm was due to one or more acidic components that were formed in substantial amounts by the extracts. The radioactivity at the solvent front was due to neutral components migrating like squalene, farnesol and geranylgeraniol upon rechromatography in solvent systems 1 and 2.

To find the most suitable material for a characterization of the factors influencing hydrocarbon formation, preparations from pea plants at different developmental stages were incubated with the same mixture as described in Fig. 2. Shoot tips from 8-day-old seedlings, shoot tips from flowering plants including closed buds, very young pods including minute seeds, and immature seeds from almost fully grown pods were used for the preparations in

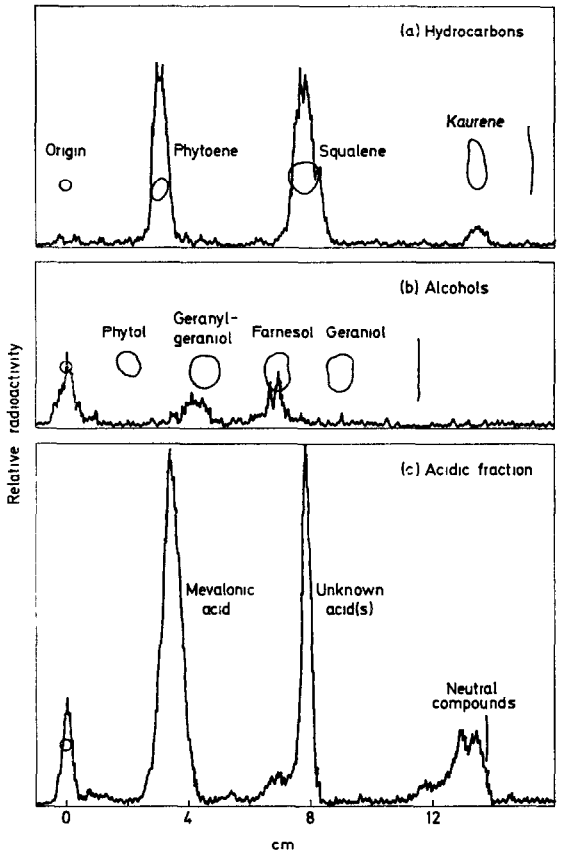

FIG. 2. CHROMATOGRAPHIC SEPARATION OF PRODUCTS OBTAINED FROM MEVALONATE-2-^{14}C.

The reaction mixture consisted of 0·2 mM D,L-mevalonate-2-^{14}C (173×10^3 cpm of active isomer), 6 mM $MgCl_2$, 3 mM $MnCl_2$, 5 mM ATP, 25 mM phosphoenol pyruvate, pyruvate kinase (10 μg), 1 mM NAD, 1 mM NADP, 1 mM NADH, 1 mM NADPH, 50 mM tris-maleate buffer, pH 6·5, and soluble extract (6 mg protein) from very young pea fruits in a total volume of 0·2 ml. The mixtures were flushed with nitrogen and incubated for 3·5 hr at 30°. The lipids were extracted and chromatographed as described under "Experimental". The plates were scanned for radioactivity with a slit width of 2 mm and a scale setting of 1800 cpm. The full scale is shown in (*c*).

this screening. The extracts were not adjusted to equal concentration of protein, since it was desirable to keep them as concentrated as possible. Table 1 shows the results of the incubations. The extract from vegetative shoots was the most active with respect to squalene and phytoene production, but it hardly affected any significant incorporation into material migrating like kaurene. The other extracts all incorporated radioactivity into the kaurene area, the extract from very young fruits most actively. This extract also very actively incorporated label into materials migrating like squalene and phytoene. The extract from

flowering shoots showed relatively good incorporation into squalene and phytoene, but it was considerably less active than the extract from young fruits. The extract from immature seeds gave poor incorporation into the phytoene area. On the basis of these results, the extract from very young fruits was chosen for further characterization of the system. This material was, however, rather difficult to obtain and therefore extracts from other stages were sometimes used to verify or expand the results.

TABLE 1. INCORPORATION OF MEVALONATE-2-^{14}C INTO HYDROCARBONS BY CELL-FREE EXTRACTS FROM DIFFERENT DEVELOPMENTAL STAGES

Source of extract	Protein (mg)	Incorporation (cpm/mixture)		
		Kaurene-area	Squalene-area	Phytoene-area
Vegetative shoots	4·0	28	11,650	8,740
Flowering shoots	4·5	179	4,066	2,087
Very young fruits	6·0	532	8,090	6,460
Immature seeds	5 1	168	9,232	339

Reaction mixtures and incubation conditions as in Fig. 2, except for the extracts (as indicated). Extraction of the neutral lipids was followed by separation of the hydrocarbons in solvent system 1 and liquid scintillation counting.

Identification of the Products

Kaurene was identified as one of the radioactive products from mevalonate-2-^{14}C in a larger incubation with extract from young pea fruits. The identification was accomplished by mixing the extracted and chromatographed material from the kaurene zone with purified authentic (−)-kaurene and recrystallization three times. Weight and radioactivity were determined at the beginning of the experiment and after each recrystallization. Table 2 shows

TABLE 2. IDENTIFICATION OF KAURENE-^{14}C THROUGH RECRYSTALLIZATION WITH AUTHENTIC (−)-KAURENE

Step	Total amount (mg)	Specific radioactivity (cpm/mg)
Original material	5·44	790
First crystals	2·62	787
Second crystals	1·68	785
Third crystals	1·04	782

that the specific radioactivity remained constant through the three crystallizations and equal to the specific activity before the recrystallizations. It is therefore concluded that the radioactive material that was formed in the cell-free system and co-chromatographed with (−)-kaurene also was (−)-kaurene. The materials in the kaurene zones from the flowering shoots and the immature seeds systems were not identified in this work. It would be reasonable to suppose that also the material in the system from immature seeds was kaurene, since the

formation of kaurene in such a system has recently been reported by others.[4] The identity of the corresponding material from the system from flowering shoots must be considered unknown at the present, since the chromatographic pattern of radioactive products in the neighbourhood of the kaurene area was different from that of the other systems. The system from flowering shoots gave at least one more peak in this area and located immediately behind kaurene, while the radioactive scans from the other systems showed no radioactivity between the peaks of kaurene and squalene (Fig. 2(*a*)).

Squalene was identified as a product of the same incubation mixture as kaurene. After extraction and chromatography, the material of the squalene zone was mixed with purified squalene and the hexahydrochloride mixture was formed with gaseous HCl. The specific radioactivity of the isomerides soluble in hot acetone was determined by weighing and counting. Table 3 shows that it remained constant through four recrystallizations. The slight difference in specific activity between squalene and the first crystals of hexahydrochloride is a sign that some impurity still remained in the carrier squalene at the time of mixing. Squalene was also identified by catalytic reduction followed by gas chromatography of the resulting

TABLE 3. IDENTIFICATION OF SQUALENE-^{14}C THROUGH RECRYSTALLIZATION OF THE HEXAHYDROCHLORIDE

Step	Total amount (mg)	Specific radioactivity (cpm/μmole)
Original material*	75·29	134
First crystals†	27·84	146
Second crystals	19·57	150
Third crystals	9·40	147
Fourth crystals	2·99	155

* Squalene.
† Squalene hexahydrochloride.

squalane. Squalane emerged as a single peak with a mean retention time of 2·7 min. In the best experiment, 53 per cent of the injected radioactivity or 2143 cpm out of 4031 cpm was recovered with the mass peak of squalane. No radioactivity was obtained before or after this material. It is concluded that the radioactive material that was formed in the incubation and chromatographed like squalene also was squalene.

Radioactive phytoene was identified through repeated TLC on different adsorbents followed each time by a determination of the u.v. spectrum and the radioactivity. The combination of chromatographic systems clearly separated phytoene, phytofluene, β-carotene and squalene. Table 4 shows that the specific radioactivity remained constant through chromatography on alumina, silica gel G, alumina again and silica gel G impregnated with silver nitrate. After this latter chromatography, the u.v. spectrum was slightly altered. The same results were obtained with material generated in the vegetative shoot system. Phytoene was also identified through catalytic hydrogenation with carrier phytoene followed by gas–liquid chromatography of the resulting lycopersane. In the best experiment, 32 per cent of the injected radioactivity (419 cpm out of 1297) or 97 per cent of the total recovered radioactivity was obtained with the peak of mass. The retention time was 17 min, the peak showed

[4] J. D. ANDERSON and T. C. MOORE, *Plant Physiol.* **42**, 1527 (1967).

trailing. On the basis of the thin-layer and gas chromatographic evidence, it is concluded that the radioactive material that was formed in the incubation mixtures and chromatographed like phytoene was indeed phytoene.

Farnesol and geranylgeraniol were not identified again in the young fruit system, since they had been identified as the main components of the corresponding chromatographic zones after incubation with extract and plastids from vegetative shoots of the same plant.[5] As will be shown later, these alcohols accumulated in substantial amounts only when plastids were included in the system. The low amounts of label incorporated into material of these fractions in the all-soluble system may have been due to other, unidentified compounds, since the peaks of radioactivity often did not centre exactly over the reference spots (Fig. 2(*b*), geranylgeraniol). The neutral components of the acidic fraction probably arose from allylic pyrophosphate intermediates, which would remain as such in the water phase during the petroleum ether extraction but would become hydrolysed into neutral components and inorganic pyrophosphate upon acidification. Thus geranylgeranyl pyrophosphate is known to give rise to geranylgeraniol and geranyllinalool while farnesyl pyrophosphate gives rise to farnesol and nerolidol, which would then appear in the ethyl acetate fraction. Phosphorylated squalene precursors of the type suggested by Rilling[2] may have given rise to the components

TABLE 4. IDENTIFICATION OF PHYTOENE-^{14}C THROUGH RECHROMATOGRAPHY ON DIFFERENT ADSORBENTS

Adsorbent	R_f	Material recovered (μg)	Specific radioactivity (cpm/μg)
Alumina G	0·8	159	35 0
Silica gel G	0·4	89	35 8
Alumina G	0·7	53	34·6
Silica gel G/$AgNO_3$	0·1	21	35·1

migrating like squalene. The peaks of neutral compounds in the acidic fraction were never large, which indicated that the pyrophosphate intermediates were used up to a great extent in the incubations.

Requirements of the System

The general requirements for the incorporation of mevalonate-2-^{14}C into kaurene, squalene and phytoene by the pea fruit system are shown in Table 5. The entire system had an absolute requirement for ATP. Pyridine nucleotides were required for the formation of squalene but not for the formation of kaurene and phytoene, although these were somewhat stimulated. The omission of pyridine nucleotides therefore lead to a specific exclusion of squalene from among the products. (Mn^{2+}) ions were required for the production of phytoene. Although Mg^{2+} alone did sustain some phytoene production, they did not replace the requirement for Mn^{2+}. Less than 8 per cent of the production at 6 mM $MgCl_2$ and 3 mM $MnCl_2$ was therefore obtained with 9 mM $MgCl_2$ and no $MnCl_2$. A second effect of Mn^{2+} was to lower the production of kaurene and strongly inhibit the formation of squalene. The omission of Mn^{2+} therefore leads to an exclusion of phytoene and an increase in kaurene and squalene. Magnesium ions stimulated the formation of both kaurene and squalene in the

[5] J. E. GRAEBE, *Science* **157**, 73 (1967).

absence of Mn^{2+}. No attempt was made to demonstrate an absolute and specific requirement for Mg^{2+}, but it is seen from the second last item of the Table that over 80 per cent of the phytoene production of the control was maintained with as little as 1 mM Mg^{2+} at equal concentration of divalent ions.

TABLE 5. GENERAL REQUIREMENTS FOR INCORPORATION OF MEVALONATE-2-^{14}C INTO HYDROCARBONS BY THE YOUNG PEA FRUIT SYSTEM

Incubation	Incorporation (cpm/mixture)		
	Kaurene	Squalene	Phytoene
Complete system			
(6 mM $MgCl_2$, 3 mM $MnCl_2$)	532	8,090	6,460
ATP, PEP, PK omitted	0	0	0
Pyridine nucleotides omitted	379	61	5,833
9 mM $MgCl_2$, no $MnCl_2$	888	13,269	476
1 mM $MgCl_2$, 8 mM $MnCl_2$	582	2,870	5,389
1 mM $MgCl_2$, no $MnCl_2$	651	4,167	7

Complete system and incubation conditions as in Fig. 2, except for mevalonate-2-^{14}C (0·25 mM, 216×10^3 cpm of active isomer). Separation and counting as in Table 1.

ATP

The effect of different concentrations of ATP is shown in Table 6. The optimal concentration was 3 mM for the production of squalene and 3–6 mM for the production of kaurene.

TABLE 6. EFFECT OF ATP ON INCORPORATION OF MEVALONATE-2-^{14}C INTO HYDROCARBONS

ATP (mM)	Incorporation (cpm/mixture)		
	Kaurene	Squalene	Phytoene
0	0	0	0
1	778	18,463	312
3	1,029	20,025	301
6	1,208	17,993	187
9	943	14,441	89
12	224	4,375	20

Reaction mixtures and incubation conditions as in Fig. 2, except for $MgCl_2$ (3 mM), $MnCl_2$ (1 mM) and ATP (as indicated). Separation and counting as in Table 1.

The experiment was done at a low level of Mn^{2+} (1 mM) to encourage the formation of kaurene. This explains the low production of phytoene. Because of this low production, the optimal concentration of 1–3 mM ATP found for the formation of phytoene may not be typical. Table 7 shows the effect of phosphoenol pyruvate, which was used together with pyruvate kinase as a regenerator of ATP. The experiment was done with extract from immature pea seeds, but it is assumed that, with respect to kaurene and squalene, the same

TABLE 7. EFFECT OF PHOSPHOENOL PYRUVATE ON INCORPORATION OF MEVALONATE-2-^{14}C INTO HYDROCARBONS

Phosphoenol pyruvate (mM)	Incorporation (cpm/mixture)		
	Kaurene	Squalene	Phytoene
0	203	4017	90
5	234	5602	85
10	343	7649	117
15	306	8755	73
20	298	8859	86
25	288	9931	82

Reaction mixtures and incubation conditions as in Fig. 2, except for $MgCl_2$ (3 mM), $MnCl_2$ (1 mM), phosphoenol pyruvate (as indicated) and the enzyme preparation (6 mg protein) which was from immature seeds. Separation and counting as in Table 1.

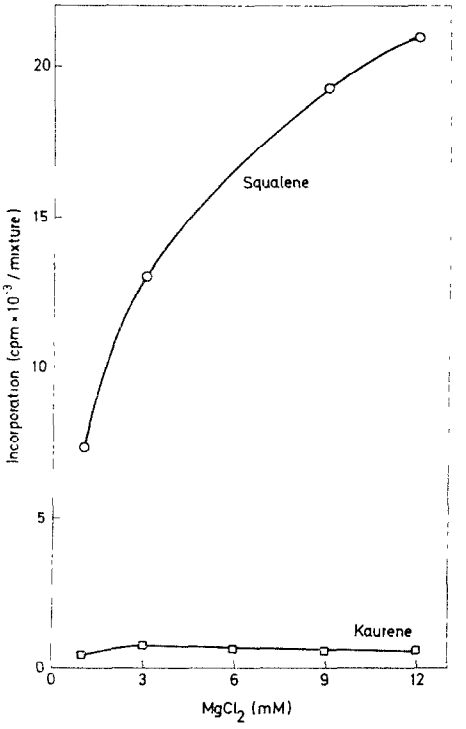

FIG. 3. EFFECT OF $MgCl_2$ ON PRODUCTION OF KAURENE AND SQUALENE IN THE ABSENCE OF Mn^{2+}.

The reaction mixtures and incubation conditions were as in Fig. 2, except for $MgCl_2$ (as indicated and $MnCl_2$ (absent). Separation and counting as in Table 1.

results would be obtained with extract from young fruits. Phosphoenol pyruvate increased the incorporation of label into both kaurene and squalene. A concentration of 10 mM was optimal for kaurene, whereas maximum production of squalene was still not reached with 25 mM. The high, and for the production of kaurene, slightly over-optimal concentration of phosphoenol pyruvate that was used throughout this study (25 mM) had been chosen on the basis of preliminary experiments in which the incorporation of mevalonate into geranylgeraniol in the presence of plastids was measured. The more active incorporation into squalene and, in the presence of plastids, into geranylgeraniol is the most probable reason for their higher requirement for phosphoenol pyruvate. The very low production of phytoene in Table 7 is in accord with the use of 1 mM Mn^{2+} and the extract from immature pea seeds (cf. Table 1).

Mg^{2+} *and* Mn^{2+}

Figure 3 illustrates the effect of Mg^{2+} on the system in the absence of Mn^{2+}. The production of squalene was strongly stimulated by Mg^{2+} and did not reach maximum within the concentrations tested (12 mM). The production of kaurene was somewhat stimulated by Mg^{2+} with a maximum at 3 mM. Phytoene was not produced at the lower concentrations of Mg^{2+} in the absence of Mn^{2+}, and even at 12 mM Mg^{2+} less than 500 cpm were obtained. This low amount of phytoene is omitted from the figure for clarity.

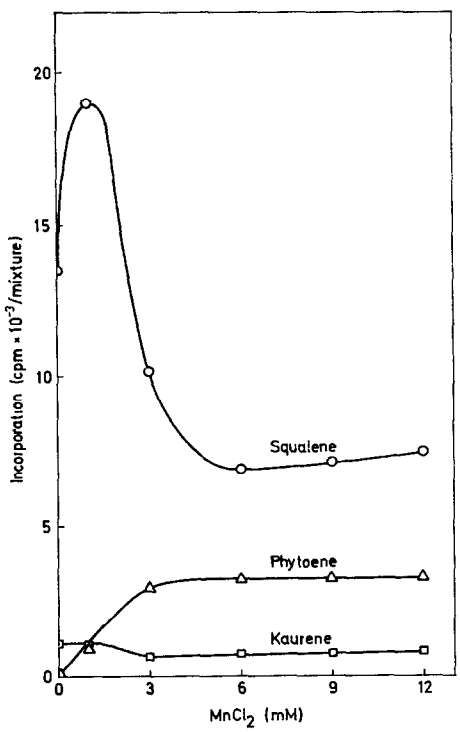

FIG. 4. EFFECT OF $MnCl_2$ ON PRODUCTION OF HYDROCARBONS.

Reaction mixtures and incubation conditions as in Fig. 2, except for $MgCl_2$ (3 mM) and $MnCl_2$ (as indicated). Separation and counting as in Table 1.

Figure 4 expands the effect of Mn^{2+} in the presence of 3 mM Mg^{2+}, the concentration that gave the best formation of kaurene. The synthesis of squalene was stimulated by 1 mM Mn^{2+} but strongly inhibited by higher concentrations. The inhibition levelled off at 6 mM Mn^{2+}. Since the stimulation of squalene at 1 mM Mn^{2+} follows from a single point on the curve, it may be added that it has been confirmed in a whole series of experiments with the vegetative shoot system.[6] Phytoene increased with Mn^{2+} until it reached saturation between 3 and 6 mM. The kaurene production was not stimulated by Mn^{2+} but was slightly decreased by concentrations above 1 mM. It remained constant from 3–12 mM Mn^{2+}. The inhibitory effect of Mn^{2+} on kaurene production was small, but it was consistently observed in numerous experiments.

pH

The effect of pH on the system is shown in Fig. 5. The optimum for both squalene and phytoene production was between 6·5 and 7·5 units. Above pH 7·5, squalene production rapidly decreased, whereas phytoene production was relatively unchanged still at pH 8·6. Due to the small amounts of enzyme used in this experiment and the presence of Mn^{2+}, the kaurene production was very low. It was, however, clearly highest at acid pH-values and decreased above pH 7·5. A pH of 6·5 was the most favourable compromise for the production of all three hydrocarbons.

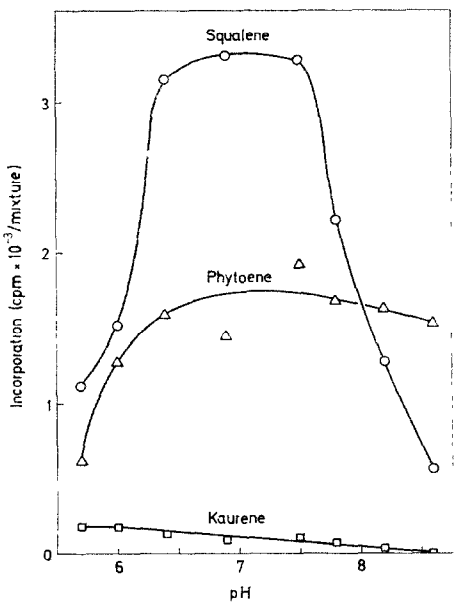

FIG. 5. EFFECT OF pH ON THE PRODUCTION OF HYDROCARBONS.

Reaction mixtures and incubation conditions as in Fig. 2, except for the buffers (100 mM tris-maleate, pH as indicated), the amounts of extract (2 mg protein per mixture) and the total volumes (0·1 ml). The pH-values were determined on 0·5 ml model mixtures containing the same ingredients but with unlabelled mevalonate instead of labelled and buffer instead of plant extract. Separation and counting as in Table 1.

[6] Unpublished data.

Particle Fraction

Table 8 shows that small amounts of the fraction that had been sedimented at 20,000 *g* and suspended in distilled water greatly increased the incorporation of mevalonate into squalene, farnesol and geranylgeraniol. The production of geranylgeraniol was stimulated 37-fold, the production of farnesol 11-fold and the production of squalene 6-fold by an amount of particulate fraction corresponding to only 20 μg of chlorophyll. Kaurene and phytoene were, however, decreased by 70 and 35 per cent, respectively. The neutral and acid components of the acidic fraction never accumulated in the presence of the 20,000 *g* sediment (not illustrated). The 20,000 *g* sediment contained fragments of both chloroplasts and mitochondria. It may be added that the same results were obtained with purer chloroplast preparations sedimented at 4000 *g* from extracts of vegetative shoots, provided they were included in amounts giving the same chlorophyll concentrations. The 4000 *g* sediments were not collected separately from the extracts of young fruits for speed of preparation.

TABLE 8. EFFECT OF PLASTID FRACTION ON INCORPORATION OF MEVALONATE-2-^{14}C INTO HYDROCARBONS AND ALCOHOLS

Plastids* (μg chlorophyll per mixture)	Incorporation (cpm/mixture)				
	Kaurene	Squalene	Phytoene	Farnesol	Geranyl-geraniol
0	374	5,820	4,300	1,210†	1,551†
2	238	19,472	3,602	4,591	16,379
10	123	32,757	2,431	12,654	40,247
20	120	37,008	2,871	14,021	57,885

* This cellular fraction consisted of chloroplast and mitochondrial fragments.

† Farnesol-^{14}C and geranylgeraniol-^{14}C have only been identified in mixtures containing plastids. The radioactivity in these chromatographic fractions from the mixture without plastids may be due to other compounds (cf. p. 2008). Reaction mixtures and incubation conditions as in Fig. 2, except for mevalonate-2-^{14}C (0·25 mM, 216 $\times$ 10^3 cpm of active isomer). The products were separated by two-dimensional chromatography in solvents 1 and 2 and measured by liquid scintillation counting.

TABLE 9. EFFECT OF THE PLANT GROWTH INHIBITOR AMO 1618 ON INCORPORATION OF MEVALONATE-2-^{14}C INTO HYDROCARBONS

AMO-1618 (mg/ml)	Incorporation (cpm/mixture)		
	Kaurene	Squalene	Phytoene
0	369	2179	2044
0·1	104	2504	2344
1·0	42	2315	2138
100	0	1922	2505

Reaction mixtures and incubation conditions as in Fig. 2, except for $MgCl_2$ (3 mM) and the amount of extract (3 mg protein per mixture). Separation and counting as in Table 1.

Inhibition by AMO 1618

Table 9 shows that only the production of kaurene was affected by the plant growth inhibitor AMO 1618. Neither the production of phytoene nor that of squalene was significantly affected even by a 100-fold greater concentration of AMO 1618 than was needed to cause an almost complete inhibition of kaurene synthesis.

DISCUSSION

The cell-free system from young pea fruits incorporated mevalonate-2-^{14}C into kaurene, squalene and phytoene. On the assumption that the usual route of isoprenoid biosynthesis was in operation, at least ten enzymatic steps were active in the extracts.[7] The characterization was done with the knowledge that the system was both crude and complicated. It was carried to a point where the effects of the incubation variables were predictable and reproducible, which the author believes to be essential for comparisons of enzymatic activities in extracts from different developmental stages as well as an aid in further purification. The investigation was done with the fruit system in order to include the formation characteristics of kaurene. Extracts from vegetative shoots are more convenient sources of the other activities described.

The production of kaurene has been reported in cell-free systems from three other sources, all involving seeds. Thus systems from endosperm of *Echinocystis*[8,9] and germinating seeds of castor bean[10] incorporated mevalonate or geranylgeranyl pyrophosphate, and a system from immature pea seeds[4] incorporated mevalonate into kaurene. A comparison between the characteristics of kaurene production in the present system and in the *Echinocystis* system[9] shows that both have the activity located in the particle-free supernatant, both have optimal activity at low pH, and both show Mg^{2+} to be a better cofactor than Mn^{2+}. This latter point is in contrast to the pea seed system, in which Mn^{2+} was a better cofactor than Mg^{2+}.[4]

Squalene is a common product from mevalonic acid in cell-free systems from animals, higher plants and micro-organisms. Cell-free systems of higher plants in which it has been identified include extracts from tomato and carrot plastids,[11] *Echinosystis* endosperm,[8] pea seeds[12] and pea seedlings.[5] The incorporation of free geraniol into squalene was recently demonstrated with a homogenate from germinating pea seeds.[13] In addition to these established cases, the formation of squalene can be inferred from published data in a number of systems where it was not directly identified. In spite of this commonplace—or because of it—few studies have been devoted to the production characteristics of squalene in plants. The general requirements for pyridine nucleotides and Mg^{2+} in the solubilized tomato plastid system were described by Beeler *et al.*[11] These requirements are in agreement with the present work, in which pyridine nucleotides, 3 mM ATP with 25 mM phosphoenol pyruvate, more than 12 mM $MgCl_2$, 1 mM $MnCl_2$ and a pH of 6·5–7·5 were optimal conditions for squalene production. It was further shown that the production of squalene as a function of Mn^{2+} had a very sharp optimum at 1 mM Mn^{2+}, higher concentrations being strongly inhibitory. Since Mn^{2+} at the same time increased the production of phytoene, its presence

[7] For example: T. W. GOODWIN, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 143, Academic Press, London and New York (1965).
[8] J. E. GRAEBE, D. T. DENNIS, C. D. UPPER and C. A. WEST, *J. Biol. Chem.* **240**, 1847 (1965).
[9] C. D. UPPER and C. A. WEST, *J. Biol. Chem.* **242**, 3285 (1967).
[10] D. R. ROBINSON and C. A. WEST, *Fed. Proc.* **26**, 454 (1967); C. A. WEST, M. OSTER, D. ROBINSON, F. LEW and P. MURPHY, Paper presented at the Sixth Internat. Conf. Plant Growth Subst., Ottawa (1967).
[11] D. A. BEELER, D. G. ANDERSON and J. W. PORTER, *Arch. Biochem. Biophys.* **102**, 26 (1963).
[12] E. CAPSTACK, JR., N. ROSIN, G. A. BLONDIN and W. R. NES, *J. Biol. Chem.* **240**, 3258 (1965).
[13] R. T. VANALLER and W. R. NES, *Phytochem.* **7**, 85 (1968).

and concentration had a drastic effect on the relative amounts of squalene and phytoene produced. This effect, which was also observed in the earlier presented system from vegetative pea shoots,[5] will be more extensively dealt with in a forthcoming publication.

Phytoene is a much less common product in cell-free systems than squalene. Anderson and Porter[14] obtained phytoene from terpenoid pyrophosphates in systems from tomato and carrot plastids. A purified and very active version of the tomato plastid system was recently presented by Jungalwala and Porter.[15] Charlton *et al.*[16] obtained phytoene synthesis in bean leaf chloroplasts isolated in non-aqueous medium. Suzue[17] reported the formation of phytoene in an extract from mutant *Staphylococcus* blocked in the carotenoid pathway at a step subsequent to phytoene formation. The formation of phytoene in a pea seedling system was observed by Graebe[5] but the product was not identified in that work.

In the present system, phytoene was dependent on Mn^{2+}, slightly stimulated by pyridine nucleotides, and not very influenced by pH between the values of 6·5 and 8·5 units. Although there was a slight production of phytoene in response to Mg^{2+} alone, it was less than one-tenth of its production in the presence of Mn^{2+}. This almost absolute requirement for Mn^{2+} is in agreement with the findings for the tomato plastid systems[14, 15] and the bacterial system.[17] In the bean leaf chloroplast system, it was mentioned that replacement of Mg^{2+} by Mn^{2+} did not enhance incorporation of mevalonate into unsaponifiable matter,[16] but this does not seem to exclude the possibility of a specific effect on phytoene production. The solubilized and purified tomato plastid system had a requirement for Mg^{2+} in addition to Mn^{2+}.[15] This point was not investigated in the present work, but lowering the concentration of Mg^{2+} to 1 mM while the sum of Mg^{2+} and Mn^{2+} was kept at 9 mM had relatively little effect on the production of phytoene. The slight effect of pyridine nucleotides on phytoene production may be an artefact of crude systems, since it is not observed in more purified systems.[15, 16] This effect has been observed before as discussed by Jungalwala and Porter.[15] The same artefact may be responsible for the slight stimulation of kaurene production by pyridine nucleotides. Perhaps intermediates or side products of squalene biosynthesis that accumulate in the absence of pyridine nucleotides have an inhibitory effect on other reactions of the isoprenoid complex. The addition of pyridine nucleotides to the system would then have the apparent effect of a stimulation of these reactions.

The accumulation of geranylgeraniol and farnesol as free alcohols or as pyrophosphate esters has been observed in a number of cell-free systems from higher plants,[5, 8, 15, 18, 19] yeast,[20] and bacteria.[21] The free alcohols are generally considered to arise from the corresponding pyrophosphates through the action of phosphatases. An alternate explanation would be the association of the pyrophosphate esters under loss of the pyrophosphate moiety with enzymes that would normally catalyse further transformations but now, some condition being unfavourable, release the alcohol parts of the molecules instead. Such a binding of farnesyl pyrophosphate to protein under loss of pyrophosphate has been demonstrated in a squalene-synthesizing system from pig liver in the absence of pyridine nucleotides.[22]

[14] D. G. Anderson and J. W. Porter, *Arch. Biochem. Biophys.* **97**, 509 (1962).
[15] F. B. Jungalwala and J. W. Porter, *Arch. Biochem. Biophys.* **119**, 209 (1967).
[16] J. M. Charlton, K. J. Treharne and T. W. Goodwin, *Biochem. J.* **105**, 205 (1967).
[17] G. Suzue, *J. Biochem.* (*Japan*) **51**, 246 (1962).
[18] D. L. Nandi and J. W. Porter, *Arch. Biochem. Biophys.* **105**, 7 (1964).
[19] C. J. Pollard, J. Bonner, A. J. Haagen-Smit and C. C. Nimmo, *Plant Physiol.* **41**, 66 (1966).
[20] K. Kirschner, Doctoral dissertation, University of Munich (1960).
[21] A. A. Kandutsch, H. Paulus, E. Levin and K. Bloch, *J. Biol. Chem.* **239**, 2507 (1964).
[22] G. Krischna, H. W. Whitlock, Jr., D. H. Feldbruegge and J. W. Porter, *Arch. Biochem. Biophys.* **114**, 200 (1966).

Unless the unidentified acid turns out to be a phytol derivative, no evidence for an incorporation of lable into phytol was obtained in over 500 incubations with or without plastids and under widely varied conditions (the vegetative system included). This is remarkable, since geranylgeraniol, which was produced in great amounts, serves as a precursor of phytol in maize seedlings.[23] Chromatographic evidence for the formation of phytol from mevalonate-2-^{14}C in isolated chloroplasts was presented by Charlton *et al.*[16] but the data did not show whether the chromatographic system employed would permit a distinction between phytol and geranylgeraniol. If phytol does not form in cell-free systems, the reason may be the lack of a specific cofactor or that the reduction from geranylgeraniol does not occur until after esterification with chlorophyllide.[24]

Even though all the enzymes needed to catalyse the sequence from mevalonate to kaurene, squalene and phytoene were located in the particle-free supernatant of the present system, they may partly have originated from various sub-structures of the intact cell. By the use of non-aqueous solvents for the isolation, it has been demonstrated by others that chloroplasts contain mevalonate-activating enzymes[25] and indeed all the enzymes necessary for the incorporation of mevalonate into phytoene.[16] The mevalonate-activating enzymes are, however, lost to the supernatant fraction during isolation in aqueous media, which explains why plastid fractions thus prepared poorly catalyse the transformations of mevalonic acid alone.[5, 14, 16] The enzymes that catalyse the sequence from isopentenyl pyrophosphate to phytoene appear to be less easily lost, since plastids of tomato fruits are able to catalyse this sequence even after initially aqueous isolation.[14, 15] It therefore seems most likely that the strong stimulation of the synthesis of geranylgeraniol and farnesol by the plastid fraction in the present system is due to structure- or lipid-bound enzymes required for the steps subsequent to isopentenyl pyrophosphate.

Since an increased synthesis of geranylgeraniol in the presence of plastids presumably reflects an increased synthesis of geranylgeranyl pyrophosphate, it was expected that both phytoene and kaurene synthesis would be increased by the plastid fraction. This was not the case, which may indicate that the enzymes catalysing the transformations of geranylgeranyl pyrophosphate into phytoene and kaurene are present in very low amounts and are already saturated with substrate in the absence of plastids. The inhibition of phytoene and kaurene formation by the plastid fraction may be due to phosphatase or other disturbing activity associated with chloroplasts or mitochondria.

The production of kaurene in the soluble fraction is in agreement with the location of kaurene synthase activity in the particle-free supernatants of *Echinocystis* endosperm[9] and extracts of castor bean seeds.[10] Further transformations of kaurene to kaurenol, kaurenal and kaurenoic acid requires the microsomal fraction.[26]

The location of squalene-synthesizing enzymes in the soluble fraction is of interest. A structure-bound origin of these enzymes seems unlikely in view of the high activity and brief extraction although squalene synthase can be solubilized from tomato plastids (by the use of lauryl sulphate).[11] Except for brief mentions by Goodwin[27] and West *et al.*,[10] no report was found of squalene formation in the conventional supernatant fraction of plant extracts. The 6-fold stimulation of squalene synthesis by the plastid fraction in the present work may indicate

[23] C. COSTES, *Phytochem.* **5**, 311 (1966).
[24] See the discussion by D. R. THRELFALL, W. T. GRIFFITHS and T. W. GOODWIN, *Biochem. J.* **103**, 831 (1967).
[25] L. J. ROGERS, S. P. J. SHAH and T. W. GOODWIN, *Biochem. J.* **99**, 381 (1966).
[26] D. T. DENNIS and C. A. WEST, *J. Biol. Chem.* **242**, 3293 (1967).
[27] T. W. GOODWIN, in *Biosynthetic Pathways in Higher Plants* (edited by J. B PRIDHAM and T. SWAIN), p. 57, Academic Press, London and New York (1965)

a main structure-bound portion of squalene synthase, but it may equally well be due to a stimulation of the formation of farnesyl pyrophosphate. In animal and yeast systems, the formation of squalene from farnesyl pyrophosphate normally requires microsomes, from which the activity can be solubilized only with difficulty.[22]

Since kaurene is a precursor of gibberellins,[28, 8] it has been assumed that the production of kaurene in cell-free systems represents part of the gibberellin synthesis of the intact plant. If this is true for the present system, it is a point of interest that the largest production of kaurene was found in extracts from young fruits which had been harvested at the onset of pod growth. Corcoran and Phinney[29] found little gibberellins in seeds of *Echinocystis*, *Lupinus succulentus* and *Phaseolus* at this stage. The main increase of gibberellins in the seeds was correlated with their own great growth period, which occurred after the fruits had reached almost mature size. The kaurene production of the present system may therefore reflect an active synthesis of gibberellins in the pod rather than in the minute seeds, a point which is presently under investigation. On the other hand, Ogawa[30] found the greatest increase of gibberellins in seeds of *Lupinus luteus* during the pod development.

AMO 1618 is a plant-growth inhibitor, which inhibits the production of gibberellins in the fungus *Fusarium moniliforme*[31, 32] and in peas cultured *in vitro*.[33] It also inhibits the formation of the kaurenoic acid derivative steviol from acetate-^{14}C in *Stevia*.[34] In cell-free systems, AMO 1618 inhibits the formation of kaurene from mevalonate[4, 35] at the specific step of geranylgeranyl pyrophosphate cyclization.[35] Since geranylgeranyl pyrophosphate is the most likely immediate precursor of phytoene as well as of kaurene, the present work adds to our knowledge about the specificity of AMO 1618 in showing that it does not affect the production of phytoene even at a hundredfold greater concentration than is needed to inhibit the formation of kaurene almost completely in the same mixture.

EXPERIMENTAL

Chemicals

Divergan SZ 9012P, a water-insoluble polyvinylpyrrolidone, was a gift from the Badische Anilin- & Soda-Fabrik A.G., Ludwigshafen/Rhein, Germany. It was purified by the method of Loomis and Battaile.[36] Kaurene was a gift from Dr. T. P. C. Mulholland of the Imperial Chemical Industries Ltd., Alderley Park, England. Synthetic geranylgeraniol was a gift from F. Hoffmann-LaRoche & Co., Basle, Switzerland. Purified phytol was a gift from E. Merck A.G., Darmstadt, Germany. Phytoene was isolated from tomato paste by the method of Jungalwala and Porter.[37] Biochemicals from Boehringer, Trizma Base from Sigma, AMO 1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride) (90%) from Calbiochem, neutral alumina from Woelm and silica gel G and aluminum oxide G for TLC from Merck were used. Petroleum ether refers to the fraction boiling at 40–60°. Mevalonic acid-2-^{14}C lactone, 4·82 mc/mmole from the Radiochemical Centre was treated with an equivalent amount of $KHCO_3$ at 30° for 15 min to open the lactone.

Plant Material

Pisum sativum (variety Grosshülsige Schnabel mit gedrücktem Korn) was sown in the field on 8 May. Flowering shoot tips were collected on 28 June, at which time the buds were still closed. Very young fruits were

[28] B. E. Cross, R. H. B. Galt and J. R. Hanson, *J. Chem. Soc.* **295** (1964).
[29] M. R. Corcoran and B. O. Phinney, *Physiol. Plantarum* **15**, 252 (1962).
[30] Y. Ogawa, *Plant Cell Physiol.* **4**, 85 (1963).
[31] H. Kende, H. Ninnemann and A. Lang, *Naturwissenschaften* **50**, 599 (1963).
[32] M. Ruddat, E. Heftmann and A. Lang, *Naturwissenschaften* **52**, 267 (1965).
[33] B. Baldew, A. Lang and A. O. Agatep, *Science* **147**, 155 (1965).
[34] M. Ruddat, *Nature* **211**, 971 (1966).
[35] D. T. Dennis, C. D. Upper and C. A. West, *Plant Physiol.* **40**, 948 (1965).
[36] W. D. Loomis and J. Battaile, *Phytochem.* **5**, 423 (1966).
[37] F. B. Jungalwala and J. W. Porter, *Arch. Biochem. Biophys.* **110**, 291 (1965).

collected on 6 July and 11 July. The pods, which varied in size from 15 to 25 mm, were dissected out of the wilted flowers. The styles and the minute seeds were included in the preparations. Immature seeds were collected on 13 and 18 July. The pods had then reached 80 per cent of their final length but were still flat. The seeds were small, approximately 4 mm long, and kidney shaped. Vegetative shoots were obtained from seedlings grown in a growth chamber for 8 days with a 16-hr light period (5000–6000 lux) at 21° and an 8-hr dark period at 14°. At the time of harvest, they had one developed leaf. The leaf bud and very young second leaf were used for the preparation.

Preparation of Extracts

The plant material was cut with scissors and ground with sand in a mortar. In addition to several preparations from other stages, two different preparations of the very young fruits were made. In the one, 66 g of plant material was ground with 33 ml of a medium consisting of 0·25 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA and 0·1 M tris-HCl, pH 7·2. In the other, 63 g of plant material was ground with 60 ml of the same homogenization mixture to which 11 g of polyvinylpyrrolidone and 5 mM mercaptoethanol had been added.

The homogenate was filtered through gauze and sintered glass. Starch was removed by centrifugation at approximately 200 *g* for 8 min. The mixed plastid and mitochondrial fraction was collected at 25,000 *g* for 20 min and the microsomal fraction at 200,000 *g* for 90 min. The supernatant was treated with solid ammonium sulphate to 90 per cent saturation (pH maintained at 6·8). The precipitate was dissolved in 3 ml of 0·02 M $KHCO_3$ and 1 mM $MgCl_2$ and the solution was dialysed for 5 hr against three 1-l portions of 10 mM tris-maleate buffer, pH 6·9, and 1 mM $MgCl_2$. The concentrated and dialysed solution was centrifuged again at 200,000 *g* for 60 min. The plastid fraction was suspended in homogenization medium, resedimented and taken up in 1 ml of water.

Chlorophyll concentrations were measured after extraction with 80% acetone according to the method by Arnon.[38] Protein determinations were done on trichloroacetic acid-precipitated material according to the method by Lowry *et al.*[39] The enzyme extracts were kept frozen in portions in liquid nitrogen; each portion was de-frosted only once. No activity was lost on freezing and the activity remained unchanged for at least 2·5 yr.

The pea fruit preparations with and without polyvinylpyrrolidone had protein concentrations of 20 and 18 mg/ml before and 40 and 56 mg/ml after the concentration with ammonium sulphate. The extract prepared with polyvinylpyrrolidone was clearly less brown, which indicated a lower level of polyphenol oxidation products.[36] The extract prepared without was, however, equally active with respect to the production of kaurene, squalene and phytoene. The concentration of the plant extracts, which could also be accomplished with dry Sephadex instead of ammonium sulphate,[5] was necessary for a good production of kaurene, phytoene and the unidentified acid(s).

Incubation and Assay

The incubations were done in centrifuge tubes carrying ground-glass stoppers with inlet tubes and outlet openings. After preparation of the mixtures in ice, the tubes were flushed with N_2 for 2 min, stoppered and incubated. The incubation was not strictly anaerobic by this technique, but the yields were increased. At the end of the incubation period, proteins were precipitated with acetone and lipids were extracted from the precipitate with more acetone. 0·5 M aqu. $NaHCO_3$ was added to the combined acetone extracts and supernatant, and neutral lipids were extracted with petrol. ether (× 3). The combined petrol. ether extracts were washed twice with water, sharply separated from the last drop of water by centrifugation and evaporated to dryness in N_2. If the incubation had been done in the absence of choroplast fragments, the neutral lipids were directly applied to thin-layer chromatograms at this stage. If chlorophyll was present, the mixtures were saponified at room temperature for 15 hr with 36% aqu. KOH (0·1 ml) in methanol (0·4 ml). The unsaponifiable lipids were then extracted with petrol. ether, washed with water and chromatographed.

For extraction of the acidic fraction, the pH of the bicarbonate solution after removal of the neutral lipids was adjusted to 2–3 and the acids were extracted (× 3) with ethyl acetate. The combined ethyl acetate extracts were washed once with water, sharply separated from the last drop of water by centrifugation and evaporated to dryness in N_2. The residues were applied to thin-layer chromatograms. The unidentified acid, illustrated as a peak of radioactivity in Fig. 2(*c*), could be extracted with petrol. ether instead of ethyl acetate at acid pH. It was then obtained free of mevalonic acid.

Thin-Layer Chromatography

TLC was carried out on 0·25 mm layers of silica gel G activated at 120° for 45 min. The chromatographic tanks were not saturated with solvent vapour, but the vapour phase volume was decreased by putting six

[38] D. I. ARNON, *Plant Physiol.* **24**, 1 (1949).
[39] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J Biol Chem.* **193**, 265 (1951).

20 × 20 cm plates tandem fashion and separated by short pieces of slit tubing into each 21 × 21 × 8·5 cm tank. The solvent systems were: 1. Petrol. ether/benzene (96:4) for hydrocarbons; 2. Methanol/water (80:20) saturated with liquid paraffin for the separation of alcohols on paraffin-impregnated layers; 3. Chloroform/-ethyl acetate/glacial acetic acid (30:70:1) for acids. For two-dimensional chromatography, the hydrocarbons were separated in the first direction with solvent 1 to which 5% liquid paraffin had been added. The plates were then dried for about 3 min before the alcohols were separated by solvent 2 at right angles to the first direction.[5] The separated hydrocarbons remained quantitatively in their lane, unless the plates had been exposed to air for longer time between developments. Phytoene and phytofluene then became oxidized and tended to move along in the second direction. Reference kaurene, squalene, phytoene, phytofluene, phytol, geranylgeraniol, farnesol and geraniol were added to the petrol. ether extracts before chromatography.

Location and Measurement of Radioactivity

The plates were scanned for radioactivity with a DESAGA scanner 12-2. The reference compounds were made visible with iodine vapour and lined up with the radioactive scan by means of radioactive markers on the plate. After bleaching, the silica gel of each spot was scraped into 5 ml of scintillation fluid consisting of 4 g of 2,5-diphenyloxazole and 50 mg of *p*-bis-2′-(5′-phenyloxazolyl)-benzene per litre toluene. The samples were counted in a Nuclear Chicago Mark I liquid scintillation spectrometer. The counting yield was 81 per cent as measured with internal standards. Quenching was controlled with channel ratios. The background rate was subtracted from all values.

Catalytic Hydrogenation and Gas Chromatography

The methods of hydrogenation and gas chromatography were modified from Anderson and Porter.[14] Squalene and phytoene were generated in a 0·9 ml mixture of the same composition as in Fig. 2. After extraction and chromatography on silica gel G with solvent 1, the compounds were eluted, mixed with 1 mg carrier squalene or phytoene and hydrogenated at room temperature for 36 hr in ethanol with 10% Pd on $BaSO_4$ as catalyst. After hydrogenation, water was added and the reduced hydrocarbons were extracted into petrol. ether. Samples of the petrol. ether solutions gave single peaks at the front of thin-layer chromatograms of silica gel G developed with petrol. ether, which showed that no unaltered phytoene or squalene remained. The solutions were further purified over 10-cm columns of alumina (activity grade 1) eluted with petrol. ether. The fractions containing radioactivity were concentrated to a small volume and analysed in a Perkin-Elmer F7 gas chromatograph at 270° on a 2 m × 3·5 mm column of 5% SE52 on celite 545 with a nitrogen flow rate of 64 ml/min and a stream split of 6:1, the $\frac{1}{7}$th going through a flame ionization detector. The injection block was kept at 390° and the line at 380°. The exit tap had to be cut off to leave only a few mm unheated. Fractions of the emerging gases were condensed in liquid N_2, the trapped material was dissolved in scintillation fluid and counted.

Recrystallizations

Kaurene for identification was generated in a 2 ml mixture of the same composition as in Fig. 2. After extraction and chromatography on silica gel G with solvent 1, the radioactive kaurene was eluted and mixed with 5·44 mg of unlabelled kaurene. The solvent was evaporated and the sample was dissolved in 100 μl of benzene. Fifteen μl were withdrawn for counting and, immediately, 75 μl for weighing (after careful removal of solvent). The weighed sample was then pooled with the residual 10 μl and recrystallized from absolute methanol. The procedure was repeated three times, the same micro-pipettes being used for the sampling each time.

Squalene generated in the same mixture as kaurene was extracted, chromatographed, eluted, mixed with 75·29 mg of purified unlabelled squalene and converted to hexahydrochloride isomerides according to the method by Heilbron *et al.*[40] Only the isomerides soluble in hot acetone were used for the four recrystallizations. After each crystallization, a sample of approximately 5 mg was weighed and counted by liquid scintillation.

Phytoene for the chromatographic identification was generated in a 0·2 ml mixture of the same composition as in Fig. 2. After extraction and chromatography on silica gel G with solvent 1, the radioactive phytoene was immediately eluted and mixed with 193 μg of unlabelled phytoene. The mixed sample was chromatographed on a plate of activated alumina G developed with petroleum ether. The radioactive zone was rapidly located and the material in its centre eluted. After removal of the solvent, the residue was dissolved in a known amount of petroleum ether. A 100 μl sample of the solution was diluted to 2·0 ml with petrol. ether, the u.v. spectrum was recorded with a Unicam S.P. 800 spectrophotometer and the entire sample was counted by liquid scintillation. This procedure was repeated after chromatography of the remaining material on silica gel G with solvent 1, on alumina G again with petrol. ether, and on silica gel G impregnated with a 12·5% solution of $AgNO_3$ in 60% aqueous methanol and developed with benzene/acetone (95:5). An $E^{1\%}_{1cm}$ of 1250 at 285 nm

[40] I. M. Heilbron, E. D. Kamm and W. M. Owens, *J. Chem. Soc.* 1630 (1926).

was used to calculate the amounts of phytoene in the samples.[41] The thin-layer adsorbents were washed with methanol/ether (4:1) and the solvents were filtered over alumina (grade I) for this experiment.

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[41] B. H. DAVIES, in *Chemistry and Biochemistry of Plant Pigments*, (edited by T. W. GOODWIN), p. 489, Academic Press, London and New York (1965).