The Biosynthesis of Squalene in Germinating Seeds of *Pisum sativum**

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The biosynthesis of squalene has been demonstrated in peas (*Pisum sativum*) in two different ways. The first of these involved germination of the seeds for a 5-day period in the presence of a large amount of mevalonic acid. The squalene, which was produced in a 40% yield, was identified by several physical and chemical properties. The second way in which squalene was experimentally biosynthesized was through a very short (24 hours) germination in the presence of a small quantity of 2-C^{14}-mevalonic acid. The radioactive hydrocarbon was produced in a 38% yield and was identified by conversion to the hexahydrochloride and co-crystallization with a standard sample until constant specific activity was reached. Furthermore, the radioactive squalene was incubated with a rat liver homogenate, and radioactive cholesterol (purified through the dibromide) was produced in a 7% yield. This work represents the first experimentally achieved biosynthesis of squalene in a flowering plant, and it gives credence to the previous assumption that the β-amyrin and β-sitosterol biosynthesized in peas arise by hydroxylative cyclization of this hydrocarbon. Evidence is presented which indicates that isopentenoid biosynthesis is regulated physiologically by the control of mevalonic acid formation.

Squalene has been considered on theoretical grounds to be the biosynthetic precursor of the pentacyclic triterpenoids (Eschenmoser *et al.*, 1955; Ruzicka *et al.*, 1953), and it has actually been isolated from a number of plants (Karrer, 1958). On the other hand, biosynthesis of squalene has not yet been clearly demonstrated in organisms (higher plants) which are known to contain the pentacyclic compounds.[1] One of these cyclization products which would be expected if squalene were attacked by a species acting as if it were $OH^{\oplus}$ is β-amyrin. Recently we demonstrated the biosynthesis of both β-amyrin and β-sitosterol in germinating seeds of the pea (*Pisum sativum*) (Baisted *et al.*, 1962), but little or no squalene accumulated during the experiment,[2] so that the question of the intermediacy of the hydrocarbon was left unanswered.[3]

In our previous paper we suggested that germinating seeds might recapitulate their evolutionary history at a chemical level. If this were so and if we reasonably assume that the hydroxylative cyclization of squalene is not a primitive reaction,[4] we would anticipate that the rate of formation of squalene might be faster than its conversion to β-amyrin (or lanosterol) during the period of seed development which was recapitulating an early period of phylogenesis. This means that squalene might accumulate when the seed is germinated with a large amount of mevalonic acid, because under these conditions the capacity of the cyclizing enzyme could become overloaded without overloading the enzymes which catalyze squalene formation from mevalonic acid. In addition, a short period of incubation, *i.e.*, an arrested germination, even with small amounts of mevalonic acid, might lead to accumulation of squalene. Although the extent to which these ideas are valid cannot be ascertained at this time, their application has allowed us to achieve the biosynthesis of squalene in peas.

EXPERIMENTAL

All melting points were obtained on a Kofler hot stage. Radioactivity was measured on a

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[1] After this paper was submitted for publication, a paper (Nicholas, 1962) appeared in which the biosynthesis of squalene from 2-C^{14}-mevalonic acid in *Ocimum basilicum* was reported. The radioactive hydrocarbon along with carrier squalene was converted to a hexahydrochloride and brought to constant specific activity, but no other criteria, *e.g.*, melting point, for identity or purity were given.

[2] A 5-day period of germination in the presence of *d,l*-2-C^{14}-mevalonic acid.

[3] Only 580 cpm were found in the hydrocarbon fraction in contrast to 55,000 cpm in β-amyrin. Therefore, what accumulation there was amounted to only about 1% of the material which proceeded to β-amyrin. An analogous observation was made by Arigoni (1958) with soya beans.

[4] Recently Bloch (1962), similarly pointed out that hydroxylations arising *via* O_2/TPNH are characteristic of highly developed systems rather than primitive ones.

Geiger counting device with a thin window (Nuclear-Chicago automatic low background counter). Infrared spectra were obtained on a Perkin Elmer Model 21 spectrophotometer. Nuclear magnetic resonance spectra were obtained on a Varian Associates instrument, Model V4302, by Mr. T. Wittstruck. Alumina for chromatography was Woelm, "neutral," activity grade 1, deactivated with 2% of water.

Five-Day Germination.—To 1.76 g of *d,l*-mevalonic acid lactone was added a dilute solution of aqueous NaOH until it was slightly alkaline. After exact neutralization with hydrochloric acid, the solution was brought to a volume of 150 ml with water, and 150 g of dry peas (Burpee's Blue Bantam, treated with Spergon to inhibit microbial growth) was added to it. The container was sufficiently large (a dish, *ca.* 12 × 8 in.) that none of the seeds was more than half covered with solution, and all of the seeds had ample access to fresh air, which was admitted by normal circulation through holes in a plastic covering on the container. The experiment was carried out at room temperature (*ca.* 24°), and as soon as the solution of mevalonic acid was absorbed (*ca.* 8 hours), 100 ml of pure water was added; after this was absorbed (25 hours total elapsed time), an additional 45 ml of water was added. Finally, 25 ml of water was added on the fourth day; this sufficed to maintain a moist environment. After 5.0 days the seeds were ground under acetone in a mortar and then subjected to continuous extraction with refluxing acetone in a Soxhlet apparatus for 10 hours.

The residue from the acetone extract was partitioned between 125 ml of water and 350 ml of ether, and the aqueous phase was extracted three times further with 350-ml portions of ether. The residue from the ether extracts was hydrolyzed in 60 ml of refluxing 5% ethanolic KOH during 2.5 hours, after which the ethanol was removed under reduced pressure. The resulting residue was partitioned between 300 ml of ether and 80 ml of water, and the water was re-extracted four times with 300-ml portions of ether. The organic phase was washed with 150 ml of water and evaporated to dryness; 1.3 g of partially solid material remained. The latter was adsorbed from 13 ml of ether–petroleum ether (1:4) onto 196 g of deactivated alumina. The column was eluted with the following solvents in 23-ml fractions: fractions 1–15 (20% ether in petroleum ether), 16–33 (30% ether in petroleum ether). Fractions 1–5 contained less than 4 mg. Fraction 6 contained 207 mg of a slightly yellow, mobile oil. Fractions 7–10 contained 27 mg of oil, and succeeding fractions (through no. 33) contained less than 1.0 mg each.

Fraction 6 was readsorbed from 3.0 ml of petroleum ether onto 45 g of alumina which had not been deactivated, and 3.7-ml fractions were collected. Fractions 1–10 were eluted with pure petroleum ether. Fractions 11–25 were 5% ether in petroleum ether. Fractions 26–36 were 10% ether in petroleum ether. Fractions 1–23 contained less than 0.5 mg each. Fractions 24–25 contained 1.2 mg of an oil. Fractions 26–29 contained 185 mg of clear, colorless, mobile oil which was used for physical and chemical studies. The 185 mg represents a 40% yield from one enantiomer of the mevalonic acid. This oil proved to be squalene.

When commercial fish squalene, obtained from Mann Research Laboratories and labeled 93% pure, was chromatographed in a similar fashion on alumina which had not been deactivated, several elution bands were obtained, of which the first two amounted to 97% of the eluted substance. The first band (7%) was an oil which failed to show C=C stretching near 6μ. The second band (90%), which did exhibit weak absorption near 6μ, was assumed to be pure squalene and constituted our standard material. Two smaller bands were eluted after the squalene and were not investigated further.

The physical properties (see section on Results) of the squalene were determined in the usual ways and the hexahydrochloride was prepared according to the earlier reported procedures for animal squalene (Heilbron *et al.*, 1926; Langdon and Bloch, 1953). Pea squalene yielded the hexahydrochloride, recrystallized several times from acetone, as colorless plates, m.p. 109–112°, which did not depress the melting point of an authentic sample. The pea squalene also yielded, in agreement with the earlier investigation of fish squalene, a high melting hydrochloride (m.p. 136–143°) which was insoluble in acetone and was not purified further.

One-Day Germination.—Twenty seeds (Burpee's Blue Bantam pea treated with Spergon) were incubated at 20° with 2.0 ml of water which contained the sodium salt of *d,l*-2-C^{14}-mevalonic acid (438,000 cpm) derived from 0.19 mg of the *N,N'*-dibenzylethylenediamine salt (5 μc/mg). Four 1.0-ml portions of water were added periodically (every 1–3 hours) as the liquid level fell to zero, and finally (after 14 hours total elapsed time) 2.0 ml of water was added. After a total of 24 hours the seeds were removed and washed with water. The remaining water and the wash contained 74,500 cpm, and the seeds must, therefore, have absorbed 363,500 cpm of which 182,000 was "available," *i.e.*, present in one enantiomer.

The seeds were ground in a mortar under acetone and then continuously extracted with refluxing acetone in a Soxhlet apparatus for 3 hours. The total acetone extract contained 226,000 cpm. A second continuous extraction for 7 hours yielded only an additional 4,000 cpm. The residue from the combined extracts (230,000 cpm) was partitioned between ether and water. The aqueous layer contained 69,600 cpm (30% of the activity of the acetone extract). By difference the organic layer should have contained 160,400 cpm. The residue from the ether was saponified for 1.0 hour in a small amount of refluxing 5% ethanolic KOH. The solvent was

then removed by distillation, and the residue was partitioned between ether and water. The aqueous phase contained 40,900 cpm (18% of the acetone activity), and the ether contained 113,000 cpm (49.3% of the acetone activity).

The residue (17 mg) from the ether extract of the saponification was chromatographed on 7.4 g of deactivated alumina in a 1.0-cm (I.D.) column, and 3.0-ml fractions were collected. The eluent was 30% ether in petroleum ether. A radioactive band (69,000 cpm, or 61% of the counts chromatographed) was eluted in the first six fractions, of which the second and third contained the most radioactivity (40,100 cpm and 26,900 cpm, respectively). This hydrocarbon fraction (69,000 cpm) corresponded to a 38% yield from the available mevalonic acid.

Identification of Radioactive Squalene as the Hexahydrochloride.—A portion of the hydrocarbon (57,000 cpm) from the 24-hour germination was dissolved in acetone which was maintained saturated with hydrogen chloride at −5° for 1.0 hours. The acetone and excess HCl were removed under reduced pressure, and 59.6 mg of pure squalene hexahydrochloride (m.p. 110–111°) was added. The mixture was crystallized from fresh acetone. It melted at 107.5–109° and possessed 247 cpm/mg. The melting point (109.5–111°) and specific activity (262 cpm/mg) were constant during two further recrystallizations. The total of identified radioactivity was 59.6 × 262 = 15,600 cpm.

Incubation with Rat Liver Homogenate.—Freshly excised rat liver (25 g, from two rats) was homogenized as previously described (Bucher and McGarrahan, 1956) at 0–5° in 0.1 M potassium phosphate buffer (25 ml, *p*H 7.35, 0.03 M in nicotinamide, 0.004 M in $MgCl_2$, 0.001 M in disodium ethylenediaminetetraacetic acid, and 0.01 M in glutathione). The homogenate was centrifuged at 700 × *g* for 20 minutes at −5°, and the resulting supernatant solution containing microsomes and soluble enzymes was used for incubation.

The incubation was conducted by shaking a flask in a thermostatted (37°) water bath for 3 hours. The flask (50 ml, round bottom) was open to the atmosphere and contained 5.0 ml of the liver supernatant, 5.0 μM of TPNH, and 164,000 cpm of pea hydrocarbon obtained from a 24-hour germination similar to but on a larger scale than the one described above. After 45 minutes 5.4 μM of glucose-6-phosphate and 3.0 μM of TPN were added, and the same amounts were added again after 90 minutes. The incubation was terminated by the addition of 10 ml of 10% KOH in 95% ethanol. Pure cholesterol (9.3 mg) was added, and the mixture was refluxed for 1.0 hour. The material which was afterwards soluble in petroleum ether was chromatographed on silicic acid (Mallinckrodt Analytical Reagent to which 5% of its weight of water had been added). The column used had an outside diameter of 1.8 cm and was filled to a length of 20 cm. Fractions of 5.0 ml were collected, and the column was eluted with 50 ml each of hexane, hexane-benzene (1:1), benzene, and 1, 2, and 4% ethyl acetate in benzene. Fractions prior to no. 10 contained 54,000 cpm, and fractions 10–60 contained 59,000 cpm. Fractions 41–52 contained the major eluted material (15 mg). The latter were combined, and more carrier cholesterol (9.7 mg) was added to them. The total cholesterol (24.3 mg) now contained 9,700 cpm or 400 cpm/mg. It was converted to the dibromide with bromine in ether, and the dibromide was reconverted to cholesterol with sodium iodide in ethanol according to the procedure of Schoenheimer (1935). The regenerated cholesterol (57% from crude cholesterol) was crystallized from methanol and melted at 148–149°, 376 cpm/mg. Upon recrystallization the melting point was not altered, and the specific activity (382 cpm/mg) was within experimental error of the first value.

Results

When peas were allowed to germinate at room temperature for 5 days in the presence of a large amount of mevalonic acid, the hydrocarbon content of the whole tissue increased markedly above the content of seeds germinated in a similar fashion with pure water. This is shown in Figure 1, from which it will also be obvious that the increase in hydrocarbon was essentially proportional to two different concentrations of mevalonic acid. The hydrocarbon present in the seeds germinated with pure water and with the smaller concentration of mevalonic acid was not examined further, but the hydrocarbon formed in the presence of the higher concentration of mevalonic acid was purified by chromatography under conditions which were found to separate several minor components from commercial squalene (of fish origin). The pea squalene yielded a hexahydrochloride which did not depress the melting point of and had an identical infrared spectrum (in CS_2) to that of the hexahydrochloride prepared from standard squalene. The infrared spectrum (as a film) of the pea squalene itself was also superimposable upon the spectrum of standard squalene. The pea squalene was further identified by its nuclear magnetic resonance spectrum and index of refraction.

The principal bands in the nuclear magnetic resonance spectrum are shown in Table I, together with those of standard squalene. The correspondence is clear, and the ratio of intensities is in agreement with theory. It is interesting to observe that, if such a small change as reduction or isomerization of just one of the six double bonds of the molecule had occurred, the spectrum would have been significantly altered. All of the CH_2 and CH_3 groups are joined to sp^2-carbon atoms, and reduction or isomerization would create a CH_2 and/or a CH_3 group which is not so situated; the result would be not only an alteration in the relative intensities of the bands but also the appearance of a new band for the CH_2 or CH_3 group,

TABLE I
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF SQUALENE

	Squalene[a]					
	$=C-CH_3$		$=C-CH_2-$		$=C-H$	
	Std.	Pea	Std.	Pea	Std.	Pea
Position (tau)[b]	8.33 8.42	8.34 8.42	8.00	8.00	4.89	4.89
Intensity[c]	42.8	59.8	35.0	45.2	9.2	17.2
Ratio[d]	1.0	1.0	0.82	0.76	0.21	0.29
Ratio (theory)	1.0		0.83		0.25	

[a] The samples were dissolved in $CDCl_3$. Both samples of squalene showed a band at 8.75 tau which was just above the "noise level" of the instrument but which was too weak to be quantitated; the integrated intensities did not exceed a third of the values for the bands at 4.89 tau. [b] Tau values = the displacement of the band in cycles per second from the position of tetramethyl silane divided by the applied frequency (60 megacycles per sec.) and this fraction subtracted from 10.00. [c] The values given are a function of the integrated intensity, *viz.*, the weight (mg) of paper encompassed by a tracing of the band. [d] The ratio of the intensities is calculated relative to the sp^2-methyl band. The latter was a doublet with the strongest peak at 8.42 tau.

which now would not be joined to an sp^2-carbon atom. Alterations of this sort were not observed. Consequently, we feel that the correspondence of the nuclear magnetic resonance spectrum with that of standard material is extremely good evidence for the identity of the pea hydrocarbon as squalene.

The index of refraction of the squalene was also determined for the D-line of sodium as a function of temperature. A plot of the data for standard squalene gave a straight line with a slope of -0.00027 per degree of increasing temperature. Substantially the same slope was observed for the pea squalene, and the absolute values ($n_D^{3.9}$ 1.4939, $n_D^{20.0}$ 1.4950, and $n_D^{19.1}$ 1.4955) were within 0.0004 of the standard values, as can be seen from Figure 2. The pea squalene yielded a line which was slightly displaced toward smaller indices of refraction, which probably indicates the presence of a small amount of impurity in one or the other sample. Nevertheless, at 20° the difference (< 0.0004) between the two samples is less than the difference between our standard sample (n_D^{20} 1.4954) and the values (n_D^{20} 1.4962 and 1.4965) reported in the literature (Dauben *et al.*, 1952; Heilbron *et al.*, 1926). It is interesting to observe further that the two literature values at 20° are for squalene purified by distillation, whereas our material was purified by chromatography. There is only one other report of the index of refraction of squalene purified by chromatography, but it is satisfying that the value (n_D^{23} 1.494) obtained (Farmer and Sutton, 1942) agrees well with our values (calcd. from Figure 2: pea squalene, n_D^{23} 1.4942; standard squalene, 1.4945).

The pea hydrocarbon taken for the physical and chemical studies discussed above amounted to a fraction of 185/234, or 79% of the total hydrocarbon. Therefore, most of the hydrocarbon obtained by forced biosynthesis must have been squalene. The remaining 21% probably was also

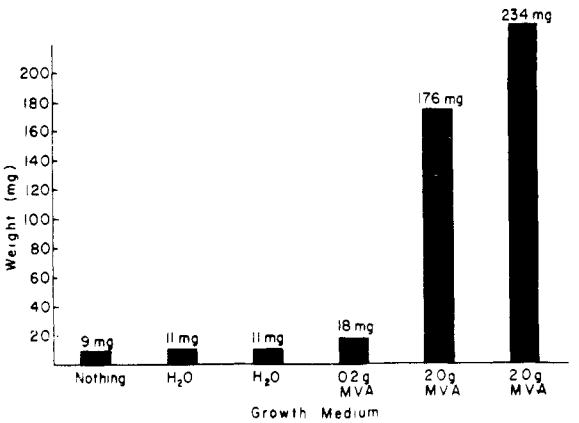

FIG. 1.—Influence of mevalonic acid on the production of hydrocarbons in germinating peas. The data are for the germination of 150 g of peas at room temperature in a manner similar to that described in the experimental section under "Five-Day Germination." The weight of total material eluted from chromatograms prior to alcoholic material, *e.g.*, β-amyrin, is given as a function of the character of the medium in which the seeds were germinated. When mevalonic acid (neutralized with NaOH) was used, it was present in about 100 ml of aqueous solution which was replaced by pure water after absorption. The column marked "Nothing" gives the hydrocarbon content of seeds which were not germinated. The results of two different experiments each are recorded for germination in pure water and for germination in the presence of 2.0 g of mevalonic acid.

predominantly squalene, since it constituted the "tail" of the chromatographic band.

When peas were germinated for only 24 hours in the presence of a small quantity of *d,l*-2-C^{14}-mevalonic acid and then extracted and chromatographed, the hydrocarbon fraction contained 38% of the available radioactivity, where "available radioactivity" is taken to mean one-half of the counts of the racemic mevalonic acid which was actually absorbed into the seeds. By con-

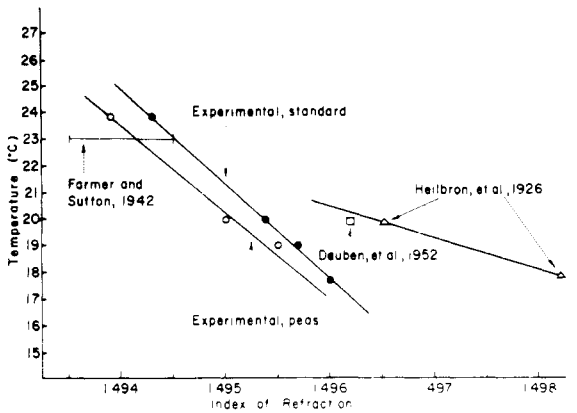

FIG. 2.—Index of refraction of squalene as a function of temperature. In our own experiments, shown by the solid (standard squalene) and open (pea squalene) circles, the data were obtained on a refractometer (Bausch and Lomb, Type 33-45-56) which was thermostatted by means of water circulating from a "Bronwill" constant temperature device, and the temperature was measured on a thermometer which had been calibrated by the National Bureau of Standards. Sufficient pea squalene was available for only three measurements.

trast only 1.2% as much (a yield of 0.47% from available mevalonic acid) was found in the hydrocarbon fraction when peas were germinated for 5 days (Baisted *et al.*, 1962). The radioactive hydrocarbon from the 24-hour germination was converted to its hexahydrochloride and cocrystallized with a standard sample of hexahydrochloride (m.p. 110–111°). The reisolated hexahydrochloride was radioactive and had a constant specific activity as indicated in the experimental section. Of the total counts, 27% were found in the hexahydrochloride. Since squalene forms at least two hexahydrochlorides as discussed in the experimental section of this paper, the yield of radioactivity does not accurately reflect the amount of squalene present, but it does give a minimum figure.

In order to confirm that the radioactive hydrocarbon was squalene, we incubated it with a rat liver homogenate under conditions similar to those used recently (Goodman, 1961; see also Bucher and McGarrahan, 1956) to convert mammalian (rat) squalene to digitonin-precipitable substances. That squalene serves as the precursor to these substances was first shown by Langdon and Bloch (1952). The material from pea squalene which was soluble in petroleum ether after saponification was chromatographed together with added cholesterol; the reisolated cholesterol was further purified through the dibromide and was recrystallized to constant specific activity. The purified cholesterol was strongly radioactive, as shown in Table II. The total recovery of radioactivity in substances which were more polar (chromatographically) than squalene amounted to 36%, which compares

TABLE II

CONVERSION OF PEA SQUALENE TO CHOLESTEROL IN RAT LIVER HOMOGENATE

Substance	Radioactivity (cpm)	Yield (%)
Substrate	164,000	100
Solubles[a] after saponification	137,000	84
Recovered hydrocarbon	54,000	33
Chromatographically polar material[b]	59,000	36
Cholesterol	11,000[c]	6.7

[a] In petroleum ether. [b] Including cholesterol. [c] Based on the specific activity of the cholesterol purified through the dibromide, *viz.*, the cpm/mg multiplied by the mg added as carrier and multiplied by 1.2 to correct for the loss of one active carbon atom of squalene.

favorably with the maximum of 48% which Goodman achieved in digitonin-precipitable materials using more highly purified enzymes.

DISCUSSION

We believe we have shown beyond any reasonable doubt that squalene is biosynthesized in the germinating pea. To our knowledge this becomes the first unequivocal demonstration of *de novo* formation of squalene in a flowering plant,[1] although its presence was suspected in radioactive fractions obtained from the germinating soya bean (Arigoni, 1958), and it has been found to be present in other plants, frequently seeds, which were not investigated from a dynamic aspect (Karrer, 1958). From the work of Langdon and Bloch (1952) and later of others (Popjak and Cornforth, 1960), the biosynthesis of squalene in mammals and in yeast is, of course, well known.

The correspondence of squalene formation and β-amyrin formation in the pea lends very strong support to the assumption that the former is a precursor to the latter, as was suggested earlier (Eschenmoser *et al.*, 1955; Ruzicka *et al.*, 1953; see also Baisted *et al.*, 1962). Further evidence in favor of the hypothesis is the fact that with time (1 day *vs.* 5 days) the radioactivity in the squalene falls (from 38% of available mevalonic acid to 0.47%), as would be expected if it were an intermediate.

It would also seem from the data we have presented that the regulation of isopentenoid biosynthesis in germinating peas is effected physiologically by regulation of the active concentration of mevalonic acid or its precursors, because it is evident that the pea has abundant capacity to utilize mevalonic acid; despite this, little or no squalene accumulates during germination in the absence of added mevalonic acid (Fig. 1), and, as we reported earlier, the triterpenoid and steroid content of the acetone extract of germinated and nongerminated peas is the same. While this

fact could be explained by metabolism which allowed a constant pool of β-amyrin and β-sitosterol to remain, it seems to us unlikely that the ratio of the two compounds would then also remain the same. We feel it is more consistent to assume that the failure of the pool to change in size is associated in a precursor-product relationship with the failure of squalene to accumulate, in which case the regulatory factor becomes the rate of production (or release) of mevalonic acid.

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