The Biosynthesis of β-Amyrin and β-Sitosterol in Germinating Seeds of *Pisum sativum**

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As part of a study of biosynthetic sequences in immature tissues which has been designed to approach the problem of evolution from the standpoint of ontogenetic recapitulation of phylogenesis, we have investigated the biosynthesis of isopentenoids in germinating seeds of the pea, *Pisum sativum*. During a 5-day period radioactivity from 2-C^{14}-mevalonic acid was incorporated into β-amyrin to an extent of 45% and into β-sitosterol to an extent of only 2.2%. The possibility is raised that this difference in apparent rates of formation results from the fact that β-amyrin arises immediately by the complete cyclization of squalene, whereas β-sitosterol is formed by the longer and more complicated process of partial cyclization of squalene, external alkylation and reduction of the terminal double bond, removal of three methyl groups, and changes in the unsaturation of ring-B.

A series of investigations has been begun in this laboratory to determine whether the problem of evolution can be approached by an examination of biosynthetic sequences in immature tissues. We believe it may be possible that a developing organism recapitulates its evolutionary history at a chemical level in a fashion analogous to the well-known recapitulation exhibited morphologically. The present report is concerned with the biosynthesis of isopentenoids in germinating seeds of the ordinary pea, *Pisum sativum*.

The pea was chosen for study because some evidence was recently presented (Ganguly and Bhattacharyya, 1957) for the presence of β-amyrin (XI) and β-sitosterol (VI) as the main polycyclic isopentenoids in the oil of the seed. From considerations of the manner in which squalene (II) cyclizes (Eschenmoser *et al.*, 1955), it is evident that these two compounds represent a major difference in the metabolism of the hydrocarbon (II), and we wished to determine whether we could detect a parallel difference in their rates of formation as measured by the relative incorporation of radioactivity from mevalonic acid into acetone-extractable samples of the two materials.

RESULTS

Since the presence of β-amyrin (XI) had not been confirmed in the earlier work (Ganguly and Bhattacharyya, 1957) by a direct comparison with an authentic sample, we first made a careful examination of the triterpenoid and sterol content of ungrown peas. We have isolated β-amyrin (XI) by chromatography of a saponified acetone extract. Professor Carl Djerassi kindly sent us a sample from *Byrsonima* species (Djerassi *et al.*, 1956) which had been proved to be identical with a sample provided by Professor F. S. Spring, in whose laboratories much of the structural work on triterpenoids had been done. Our β-amyrin (XI) was identical with that of Djerassi, as shown by a comparison of infrared spectra, melting point of a mixture, and optical rotation. The amount of the compound extractable was close to 13 mg/100 g of peas. Ganguly and Bhattacharyya had definitely established the presence of β-sitosterol (VI), which we confirmed by comparison with a commercial sample; the amount of material which we found to be extractable with acetone was close to 67 mg/100 g of peas. These two compounds were present in essentially the same amount in both ungrown and germinated (5 days) seeds and were the major components of the extractable material (155 mg/100 g of peas) which remained after saponification. These facts allowed us to proceed readily with purification of radioactive samples obtained by biosynthesis

During the germination of seeds we wished to present the tissue with 2-C^{14}-mevalonic acid (I) in a period of time which was as short as possible without interfering with the tissue by slicing or otherwise damaging it. An approximation to our objective was achieved by allowing the seeds to absorb the mevalonic acid in the water which they take up during the initial period of germination. We found, as shown in Figure 1, that seeds totally immersed in water absorb an equal weight of water in about 5 hours. By dissolving the mevalonic acid in an amount of water equal to one fifth the weight of the seeds used, we were able to effect uptake of the substrate in less than 3.5 hours. The remainder of the water which the seeds needed was then added in successive portions to insure that all of the mevalonic acid was incorporated. Since the seeds were not totally immersed, the absorption of the various portions of solution and water took slightly longer than

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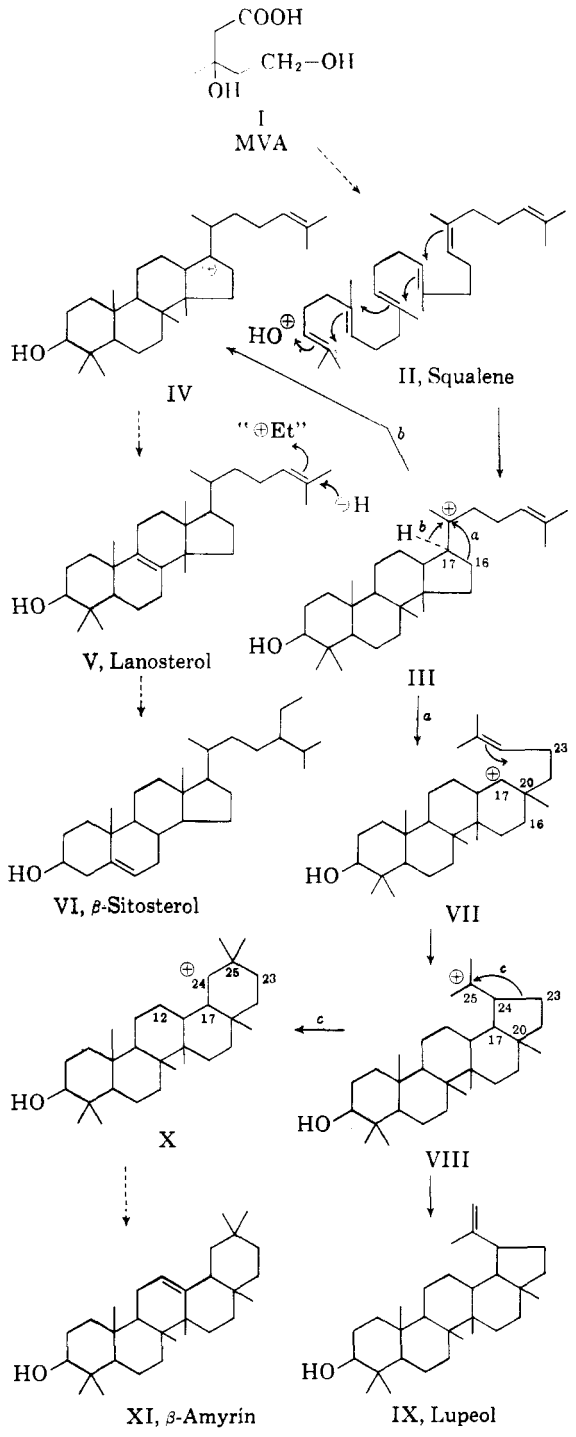

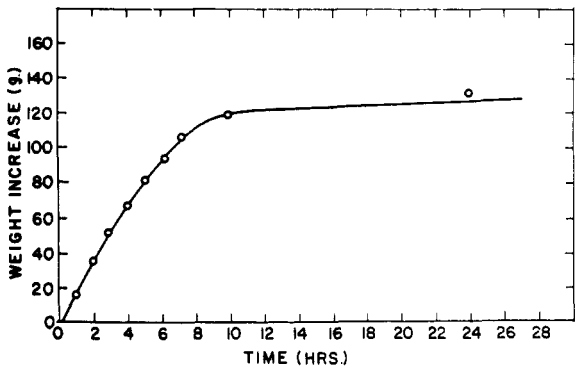

FIG. 1.—Absorption of water by pea seeds. The data are for the total increase in weight of 300 seeds (82.5 g) of *Pisum sativum* (Burpee's Blue Bantam treated with Spergon) during a period of total immersion in water at room temperature. The seeds were periodically removed, dried with filter paper, weighed, and replaced in the water.

expected from Figure 1, but the few hours required was a short period compared to the length of time during which the seeds were allowed to germinate.

We have incubated 20 peas (5.5 g) with 266,000 cpm of *d,l*-mevalonic acid (0.25 mg) in the manner just described. They were maintained wet and allowed to germinate for a total of 120 hours at room temperature (*ca.* 25°). By measurement of the radioactivity left in the water we ascertained that 246,000 cpm had actually been taken into the seeds. Assuming that only one enantiomer is active, as has been reported for a strain of mycobacterium (Lynen, 1959), there were 123,000 cpm present in available mevalonic acid.

At the end of the incubation the germinated seeds, which judging from the previous isolation experiments should have contained 0.72 mg of β-amyrin (XI) and 3.7 mg of β-sitosterol (VI), were continuously extracted with acetone, and the material so obtained was saponified. The portion (78,600 cpm; 64% of available mevalonic acid) of the hydrolysate which was soluble in ether was chromatographed on alumina together with added pure β-amyrin (XI) and pure β-sitosterol (VI) as carrier. The distribution of radioactivity in this chromatogram is shown in Figures 2 and 3. Substantially all of the elutable radioactivity was found in fractions which contained the carrier β-amyrin (54,200 cpm). That practically all of the radioactivity in this band was really associated with the triterpenoid (β-amyrin, XI) was established by recrystallizing it to constant specific activity (3520 cpm/mg). From the specific activity, the amount of material added (15 mg), and the amount already present in the tissue (0.72 mg), it was possible to calculate that the chromatographic band contained 55,300 cpm in β-amyrin. This agrees within experimental error with the total count in the chromatographic band and represents a 45% yield on the basis of the available mevalonic acid. Further identification of β-amyrin (XI) was made by converting it to the acetate, which possessed the same specific activity in cpm/mM as did the free alcohol.

Isolation of the β-sitosterol (VI) from the chromatogram and recrystallization to constant specific activity yielded the free alcohol with 60 cpm/mg, which represented 2020 cpm or a 2.2% yield

from the available mevalonic acid on the basis of the specific activity of the isolated material, the amount (30 mg) of carrier added, and the amount (3.7 mg) of endogenous material; the percentage has also been corrected for the loss of radioactivity attending the loss of labeled methyl groups at C-4 and C-14. Further identification was made by conversion of the β-sitosterol to its acetate, which had the same specific activity in cpm/mM as did the free alcohol.

Discussion

The biosynthesis of β-amyrin (XI), while not previously investigated experimentally, would be expected to follow the sequence outlined in the accompanying formulas as originally suggested by the Zurich school (Eschenmoser *et al.*, 1955). That biosynthesis does proceed along this pathway is attested to by the frequent co-occurrence of lupeol (IX) and β-amyrin (XI) (Barton, 1953). The two compounds arise through different extents of nuc ear migration in the course of cyclization of their common formal precursor, the carbonium ion (VIII). Lupeol is derived by direct elimination of a proton from one of the terminal methyl groups, while β-amyrin (XI) is derived by elimination of a proton from C-12 with concomitant transfer of hydride ions from C-13 to C-17 and from C-17 to C-24 (X to XI) and enlargement of ring E (process *c* in the scheme; VIII to X). Our finding that radioactivity from mevalonic acid is incorporated into β-amyrin (XI) is in agreement with the expectation that the biosynthetic route passes through squalene (II), which by hydroxylation at one of the terminal double bonds is cyclized.

The biosynthesis of β-sitosterol (VI) would be expected to follow the well-known route established already for cholesterol, and very recently mevalonic acid was found to be a precursor in *Salvia sclarea* (Nicholas, 1961). The difference between the expected pathways for the triterpenoids and for the steroids is that the formal carbonium ion (III) undergoes hydride ion transfer from C-17 to C-20 in the latter case (process *b* in the scheme; III to IV), while in the formation of β-amyrin and lupeol transfer of C-16 to C-20 occurs (process *a* in the scheme; III to VII).

However, the biosynthesis of β-sitosterol (VI) differs from that of β-amyrin (XI) in still another way, *viz.*, β-amyrin (XI) results immediately from the complete cyclization of squalene (II), whereas β-sitosterol (VI) requires a number of additional metabolic steps. In particular, β-sitosterol requires that the terminal double bond of squalene be alkylated by an external reagent at some stage, presumably at or after lanosterol (V). The analogous biosynthesis of ergosterol has been shown (Alexander *et al.*, 1958) to proceed by transfer of a methyl group from methionine when lanosterol was used as a substrate, and some sort of alkylation process must operate similarly not only for the sitosterol (VI) but also

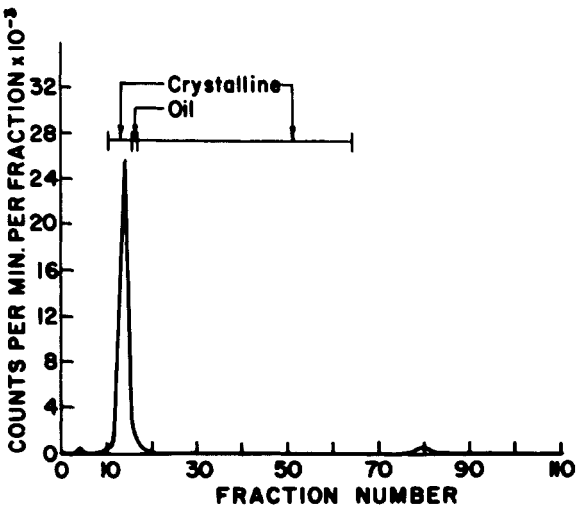

FIG. 2.—Radiochromatogram of material which was extractable into ether after saponification. The material was obtained from 5.5 g of seeds grown for 5 days in the presence of 266,000 cpm of *d,l*-2-C^{14}-mevalonic acid. Radioactivity in the peak near fraction 14 was derived from β-amyrin, as shown by further isolation. The crystalline material following the β-amyrin was β-sitosterol. The details of the solvents used for elution are given in Figure 3, which represents the same chromatogram on an expanded scale for the ordinate.

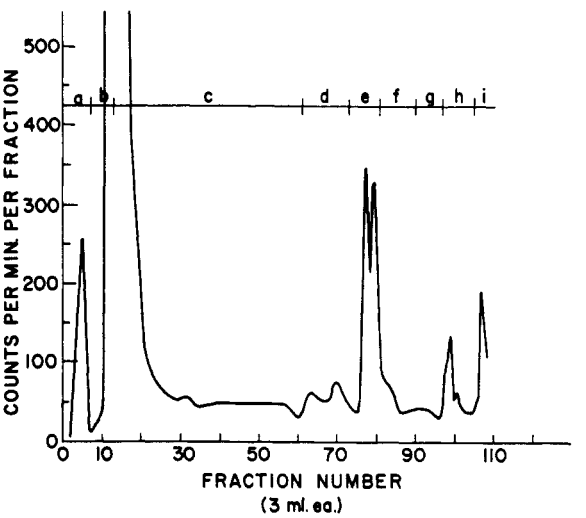

FIG. 3.—Radiochromatogram of material which was extractable into ether after saponification. The material was obtained from 5.5 g of seeds grown for 5 days in the presence of 266,000 cpm of *d,l*-2-C^{14}-mevalonic acid. In the chromatogram the following elutions were made. (*a*) 30% ether in light petroleum ether; (*b*) 40% ether in petroleum ether; (*c*) 50% ether in petroleum ether; (*d*) ether; (*e*) 1% ethanol in ether; (*f*) 2% ethanol in ether; (*g*) 5% ethanol in ether; (*h*) 10% ethanol in ether; and (*i*) 25% ethanol in ether.

for related compounds, *e.g.*, stigmasterol. In addition to the external alkylation (in contrast to internal alkylation for β-amyrin) which is necessary to arrive at β-sitosterol, a number of further steps are required to change the unsaturation from Δ^8 to Δ^5 and to remove the methyl groups at C-4 and C.14.

Thus, the sterol β-sitosterol (VI) arises by a very much more complicated route than does the triterpenoid β-amyrin (XI), and we feel that this may be the reason why mevalonic acid appears to proceed faster to the latter than to the former. We are also considering the possibility that the relative rates of biosynthesis may depend on the age of the organism, and we should like to suggest tentatively that the apparent preference for biosynthesis of β-amyrin in the germinating pea seed may be related to embryonic recapitulation of phylogenesis, assuming that a simpler sequence would have been preferred at an early stage of evolution. However, we are aware that more work is necessary before an explanation for our results can be regarded as established. A number of experiments are in progress, and we hope to report on them at a later time.

Experimental

All melting points were recorded on a Kofler apparatus. Solvents and reagents were analytical grade. The peas (*Pisum sativum*) were the Blue Bantam strain of the Burpee Seed Company; they were treated with Spergon to inhibit growth of micro-organisms. Radioactivity was measured on a thin-window counter. Chromatography was carried out with an automatic fraction collector (Gilson Medical Electronics), and the adsorbent was alumina (Woelm, "activity grade 1," "neutral") which had been deactivated with 2% of water.

Isolation of β-Amyrin and β-Sitosterol.—Sixteen hundred grams of seeds of *Pisum sativum* ground in a mortar to a fine powder were extracted in six portions with a Soxhlet apparatus with 1.5 liters of acetone for 10 hours. After evaporation of solvent, a viscous brown oil remained. This was saponified in 600 ml of 5% potassium hydroxide in absolute ethanol under reflux for 2 hours. Most of the ethanol was removed by evaporation under reduced pressure. The oil was partitioned between ether (1 liter) and water (800 ml). The aqueous layer was re-extracted with ether (1.5 liters) in four portions. The combined ether portions were washed with water (250 ml) and dried over anhydrous sodium sulfate. Evaporation of ether furnished an orange-yellow oily solid (2.49 g). This material was chromatographed on 75 g of deactivated alumina. A column of 2.5 cm (inside diameter) was used, and 15-ml fractions were collected. The material was placed on the column with light petroleum ether containing 30% of ether. Material not soluble in this solvent was removed by centrifugation. Sixteen fractions were collected with this solvent. Fractions 17 to 29 were eluted with 50% ether in light petroleum ether; 30 to 165 with pure ether; 166 to 171 with 1% ethanol in ether; 172 to 222 with 2% ethanol in ether. Material believed to be hydrocarbon, which amounted to 97 mg of a viscous yellow-brown oil, was found in fractions 4 to 9. Crude amyrin (XI) weighing 248 mg occurred in fractions 41 to 51 as a cream-colored solid. Crude sitosterol (VI) (1.15 g) appeared in fractions 85 to 188 as a pale yellow solid. The latter was recrystallized from $CHCl_3$–MeOH and then several times from pure MeOH, yielding 940 mg of colorless plates, m.p. 140.5–142°, $[\alpha]_D^{23}$ −34° ($CHCl_3$). The melting point was not depressed by mixture with an authentic sample of β-sitosterol, and the infrared spectrum (CS_2) of our sample and that of the authentic specimen were identical.

Of the β-amyrin fractions, 42 to 48 (171 mg melting at 182–188°) were combined for purification; after four crystallizations from ethanol-water, colorless needles (41 mg), m.p. 196–198°, were isolated. A sample of this material failed to depress the melting point of an authentic specimen of β-amyrin (m.p. 200–201°) (Djerassi *et al.*, 1956). Infrared spectra (CS_2) of our sample and of the authentic material were identical. The specific rotation of our sample was +84° ($CHCl_3$, 23°). Several values have been reported for the rotation of β-amyrin. The average value for our sample and those obtained from other laboratories (Djerassi *et al.*, 1956; Ganguly *et al.*, 1957; Ames *et al.*, 1951; Vesterberg, 1922) is +90° ± 6°. The melting points from the same laboratories vary from 196° to 201°.

Isolation by the above procedure of β-amyrin and β-sitosterol from peas which had been germinated for 5 days yielded substantially the same amounts of the two compounds as was obtained from the dry seeds.

Incubation.—Twenty seeds of *Pisum sativum* which weighed 5.5 g were placed in a 10-cm Petri dish with 0.25 mg of racemic 2-C^{14}-mevalonic acid containing 266,000 cpm (5 μc/mg) in 1.0 ml of water. The Petri dish was then placed in a somewhat larger evaporating dish containing a towel saturated with water, and the evaporating dish was finally closed by a watch glass. After the solution was completely absorbed (*ca.* 3.5 hours) by the peas, 1.5 ml of water was added and also allowed to be absorbed. This was followed by the addition of 3.0 ml of water, most of which was allowed to be absorbed, and then the peas were maintained wet at room temperature (*ca.* 25°) for a total of 120 hours from the beginning of the experiment. Short (*ca.* 1 cm) sprouts developed during this time.

At the end of the period of incubation, the peas were filtered from the remaining water and washed thoroughly. The combined filtrate contained 20,100 cpm. The peas (including sprouts) were ground well in a mortar with acetone, and the resulting mixture was placed into a Soxhlet thimble; the solvent filtered through and was com-

bined with more acetone (total 125 ml), which was used for Soxhlet extraction over a period of 10 hours. The acetone passed through the thimble about every 4 minutes. A small precipitate formed in the acetone.

The acetone extract, which contained 155,000 cpm, was evaporated to dryness under reduced pressure. Earlier experiments had shown that further extraction removed only a very little more radioactivity. The residue was partitioned between 10 ml of ether and 10 ml of water, and the ether was back-washed with 2.0 ml of water. The water contained 48,700 cpm, and the ether contained 88,800 cpm. The ether extract was saponified (after removal of the solvent under reduced pressure) in 2.0 ml of refluxing 5% ethanolic KOH for 1 hour. The solution was concentrated nearly to dryness, and the resulting slurry was partitioned between 10 ml of water and 10 ml of ether. The water contained 7,400 cpm, and the ether contained 78,600 cpm. The dried residue (6 mg) from the ether layer was chromatographed. Chromatography was carried out on 7.0 g of deactivated alumina. A column of 1.0 cm (inside diameter) was used, and 3.0-ml fractions were taken. The radioactive material together with 30 mg of β-sitosterol (m.p. 139.5–141.5°) and 15 mg of β-amyrin (m.p. 196–197°) was placed on the column in light petroleum ether containing 30% of ether (v/v), and six fractions with this solvent were collected. Fractions 7–11 were taken with 40% ether and fractions 12–60 with 50% ether in light petroleum ether. Pure ether was used for fractions 61–71, and this was followed by increasing percentages of ethanol in ether: fractions 72–79, 1%; fractions 80–88, 2%; fractions 89–96, 5%; fractions 97–104, 10%; fractions 105–108, 25%. All of the fractions together yielded 61,700 cpm, distributed principally in fractions 11–16, which contained 54,200 cpm, as shown in Figure 2. Figure 3 shows the distribution of the remaining radioactivity.

Early chromatographic fractions (numbers 3–6), which we regard as probably containing hydrocarbon(s) contained 580 cpm. The exact nature of the material was not studied further.

Crystalline β-amyrin was found in fractions 11–13; it was recrystallized six times from ethanol-water. The resulting needles melted at 196–198° after the first three recrystallizations, and the remaining recrystallizations failed to alter the melting point. The specific activity was 3520 ● 80 cpm/mg ($1.50 \pm 0.04 \times 10^6$ cpm/mM) for each of the samples during the last three recrystallizations. The acetate was prepared with acetic anhydride–pyridine at 100° for 1 hour; the melting point, 228–233°, was not altered by three recrystallizations from ethanol-water. The specific activity of the acetate (3105 ± 70 cpm/mg; $1.46 \pm 0.05 \times 10^6$ cpm/mM) was also unchanged during the three recrystallizations. Crystalline β-sitosterol was found in fractions 26–59. Recrystallization four times from methanol-water yielded plates melting at 140–142°; three further recrystallizations failed to alter either the melting point or the specific activity, which was 60.0 ± 1.6 cpm/mg ($2.48 \pm 0.07 \times 10^4$ cpm/mM). The acetate prepared in anhydride–pyridine at 100° for 2 hours melted at 129–131° after four recrystallizations from methanol, and a fifth recrystallization altered neither the melting point nor the specific activity, which was 53.6 ± 0.3 cpm/mg ($2.44 \pm 0.01 \times 10^4$ cpm/mM).

Highly polar material was eluted from the column in fractions 77–80 (1140 cpm), in fractions 98–99 (230 cpm), and in fractions 107–108 (300 cpm). It was not further investigated.

The experiment described in the foregoing paragraphs represents one of two carried out. The results of the other one were substantially the same.

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