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Sterol and Triterpene Synthesis in the Developing and Germinating Pea Seed

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Developing and germinating pea seeds were compared with respect to their capacity to incorporate mevalonate into sterols and triterpenes. The capacity for sterol synthesis is greatest in the least mature fruits and decreases during their development. Label is shown, by gas–liquid chromatography and counting the radioactivity of trapped fractions, to be associated with campesterol, β-sitosterol and isofucosterol. During early stages of germination sterol synthesis is insignificant. The triterpene fraction becomes heavily labelled during both development and germination. The label is associated almost exclusively with β-amyrin during germination but with cycloartenol and 24-methylenecycloartanol during development. It is only in the terminal stages of maturation that β-amyrin becomes significantly labelled. At the same time an unidentified radioactive polar compound appears. The possible significance of the appearance of this polar compound and the regulation of the synthesis of these higher terpenoids is discussed.

Variation in steroid composition during seed germination has been studied in *Phaseolus vulgaris* L. (Duperon & Duperon, 1965), in *Zea mays* L. (Kemp, Goad & Mercer, 1967), in *Happlopappus heterophyllus* Bl. (Bennett, Lieber & Heftmann, 1967), in *Pisum sativum* (Vil'yams & Krochina, 1966), in *Raphanus sativus* L., *Sinapis alba* L., *Cheiranthus cheiri* L. and in species of *Brassica* (Ingram, Knights, McEvoy & McKay, 1968). Changes in triterpenoids during germination of *Calendula officinalis* have also been examined (Kasprzyk, Sliwowski & Boleslawska-Koko, 1970).

Several years ago we studied the capacity of pea seeds to synthesize squalene, β-amyrin and sterol from [2-^{14}C]mevalonate (Scheme 1) during the first 5 days of germination (Baisted, Capstack & Nes, 1962; Capstack, Baisted & Nes, 1962). These studies showed that within the first 24h of germination the enzymes of the terpenoid pathway exhibited a considerable capacity to synthesize both squalene and the triterpene alcohol β-amyrin. It was also demonstrated that not until the third day did the synthesis of sterol become evident. A chemical examination of the dried seeds revealed that β-amyrin and β-sitosterol were present, the latter compound being present to the extent of 67mg/100g of dried seed. Clearly, for sterol to be present in the dried seed, it had to be synthesized by the seed or translocated to it during its development. If the synthesis occurred in the seed, then the biochemistry of the developing and germinating seed must be markedly different with respect to sterol synthesis. Further, as the intermediates of phytosterol biosynthesis have been shown to be cycloartenol and 24-methylenecycloartanol (Scheme 1) (Benveniste, Hirth & Ourisson, 1967), then the capacities for synthesis of individual triterpenes might also be expected to show marked differences between the developing and germinating seed.

EXPERIMENTAL

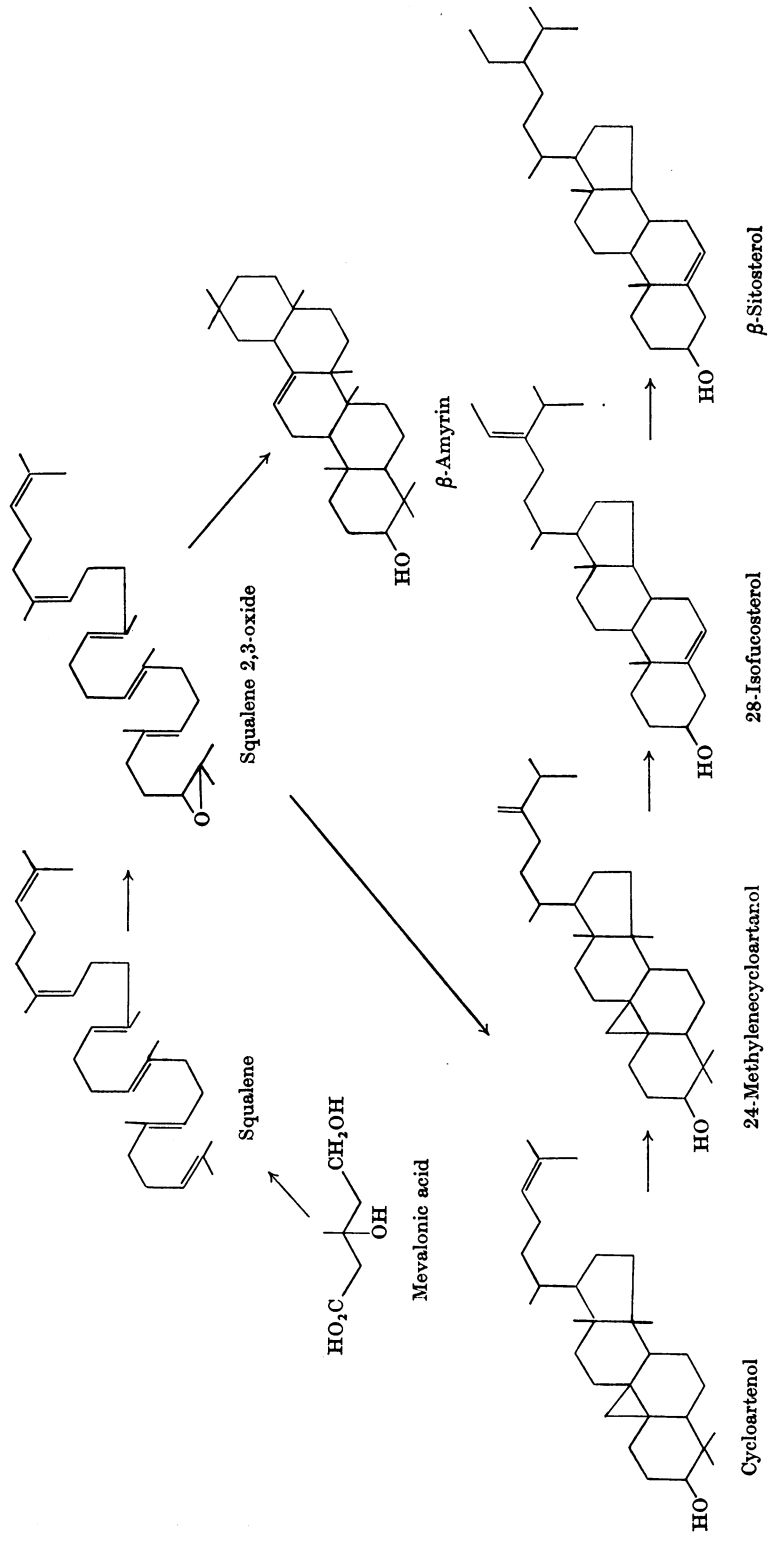
Materials

Plant material. Tall *Pisum sativum* L. cultivar Telephone (W. Atlee Burpee Co., Riverside, Calif., U.S.A.) were used in the germination studies. The developing seeds were obtained from plants grown in a greenhouse from the same seed variety and were kindly supplied by Dr T. C. Moore of the Botany Department of Oregon State University.

Radioactive material. RS-[2-^{14}C]Mevalonate (3.06mCi/mmol) was obtained as the dibenzylethylenediamine salt from New England Nuclear Corp., Boston, Mass., U.S.A. It was converted into the sodium salt before use.

Chromatographic supplies. Pre-coated t.l.c. sheets of silica gel were obtained from Sargent and Co., Los Angeles, Calif., U.S.A. and g.l.c. supplies were from Applied Science Laboratories, State College, Pa., U.S.A.

β-Amyrin was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. A gas–liquid chromatogram of the sample showed that a small amount of a contaminant was present. After three crystallizations from acetone–methanol mixtures, this impurity was removed. Cycloartenol and 24-methylenecycloartanol were gifts from Dr E. Heftmann. The sterols used as standards for t.l.c.



Scheme 1. Partial pathways of formation of higher isoprenoids in pea seeds.

and g.l.c. were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other chemicals used were of reagent-grade quality.

Methods

Seed selection and labelling. Groups of immature seeds were selected at five developmental stages representing 5%, 15%, 35%, 60% and 100% of full development. Estimation of the developmental stage was made by first grouping together freshly harvested seeds of approximately the same diameter. Seeds clearly unrepresentative of the average seeds in a pod were discarded. Each group of seeds was briefly dried on filter paper, weighed and the average weight per seed then expressed as a percentage of that of a group of seeds that had attained maximum weight (approx. 360mg). A relationship between weight per seed and the number of days the pod remains on the vine after initial opening of the flower (anthesis) has been determined by Coolbaugh & Moore (1969) for this variety of *P. sativum*. From their data the five developmental stages represent 10, 11, 12, 14 and 16 days after anthesis. A sixth stage, 24 days after anthesis, was also examined.

Groups of immature seeds, each group weighing approx. 3.0g, with the exception of the 5%- and 15%-developed groups which weighed 0.7g and 1.4g respectively, were placed in solutions of [2-^{14}C]mevalonate (100μl) in covered Petri dishes. The ratio of ^{14}C to group weight was kept at approx. 1.6μCi/g. The seeds were maintained moist with water in the dark for 24h. The seeds were then washed thoroughly with water to remove any unabsorbed substrate and the non-saponifiable lipid was isolated in the usual way (Baisted *et al.* 1962).

Thin-layer chromatography. T.l.c. of samples (10000 d.p.m.) of the non-saponifiable lipid fractions for radiochromatographic scanning purposes was conducted on layers (100μm thick) of silica gel G coated on plastic sheets. Separation was effected in ethyl acetate–heptane (3:22, v/v) and the standard compounds, squalene, β-amyrin and β-sitosterol, were located with a Rhodamine 6G spray. The sterol and triterpene fractions for further analysis were isolated by t.l.c. of the remaining non-saponifiable lipid on layers (250μm) of silica gel G spread on glass plates (8in×8in). Zones corresponding to the appropriate standards were scraped from the plate and the adsorbed compounds eluted from the scrapings with benzene–acetone (1:1, v/v). The acetates of the recovered sterols and triterpenes were made by treatment with acetic anhydride–pyridine (1:1, v/v) at room temperature overnight. Excess of reactants was removed by evaporation under high vacuum at room temperature. T.l.c. of the acetates on $AgNO_3$-impregnated sheets was carried out in benzene–hexane (2:3, v/v). Sheets were impregnated with $AgNO_3$ by passing them through a solution of $AgNO_3$ (1.58g) in ethanol–water (9:1, v/v; 45ml). The plates were air-dried before use. Standards of the acetates of cycloartenol, 24-methylenecycloartanol and β-amyrin were run as references for the triterpenes, and of β-sitosterol and isofucosterol for the sterols. The reference spots were detected by spraying with 50% (w/v) H_2SO_4 and heating at 100°C for 5min.

Gas–liquid chromatography. The sterol and triterpene acetates of representative groups were subjected to g.l.c. on a Beckman GC 4 instrument equipped with a flame ionization detector. The column was a glass U tube (8ft×$\frac{1}{4}$in) packed with 3% OV-17 on Gas-Chrom Q and was operated at 265°C. The carrier gas was argon at a flow rate of 40ml/min. Standards of sterol and triterpene acetates were co-chromatographed with the appropriate radioactive samples in a total volume of 2μl. A 7:1 stream splitter allowed one-eighth of the sample to go to the detector and the remainder to glass traps immersed in liquid nitrogen. Trapping efficiencies of 80–90% were obtained. Argon was allowed to evaporate from the traps and the residue was then washed into scintillation vials with ether. The ether was evaporated and scintillation fluor added for radioactivity counting.

Radioactivity measurements. Scanning of t.l.c. sheets was done with a Packard model 7201 instrument equipped with a disc integrator. The distribution of radioactivity on the sheets was calculated by expressing the equivalent area measurement under each peak as a percentage of the total equivalent area measured under the entire scan. As the 4-methylsterols were not sufficiently separated from sterols to permit determination of each, the equivalent area measurement under both peaks was combined. This value would then represent the proportion of radioactivity present in sterols and in compounds involved in the synthesis of sterols. Radioactivity in fractions trapped from g.l.c. and other non-aqueous samples was measured in a Packard Tri-Carb model 574 scintillation counter. Each scintillation vial contained 10ml of a mixture of 2,5-diphenyloxazole (4g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (30mg) in toluene (1 litre). For counting of radioactivity of aqueous samples, each vial contained 10ml of Bray's (1960) scintillation fluor.

Determination of specific radioactivity of β-amyrin. Samples of the benzene–acetone solutions of the isolated radioactive triterpene fraction wereind ividually evaporated with known weights of β-amyrin (30–40mg). The initial estimated specific radioactivities before crystallization ranged from 880d.p.m./mg to 14000d.p.m./mg. Each sample was repeatedly crystallized with solvent mixtures consisting of methanol–ethanol, methanol–acetone and methanol–water. After each recrystallization, the entire thoroughly dried sample was accurately weighed and transferred to 1, 2 or 5ml volumetric flasks. The sample was dissolved, made up to volume in benzene and 100μl portions were transferred to scintillation vials for radioactivity counting.

RESULTS AND DISCUSSION

Analysis of the non-saponifiable lipid fractions. The radiochromatographic traces of the non-saponifiable lipid fractions of several different groups of pea seeds are shown in Fig. 1. The distribution of radioactivity for all the groups is shown in Table 1. Several distinct changes occur with respect to the synthesis of these higher terpenoids during development. Sterol is actively synthesized during the earliest developmental stage (77% of the non-saponifiable lipid fraction) but this activity diminishes as development progresses, until at 24 days after anthesis the label associated with sterol represents only 14% of this

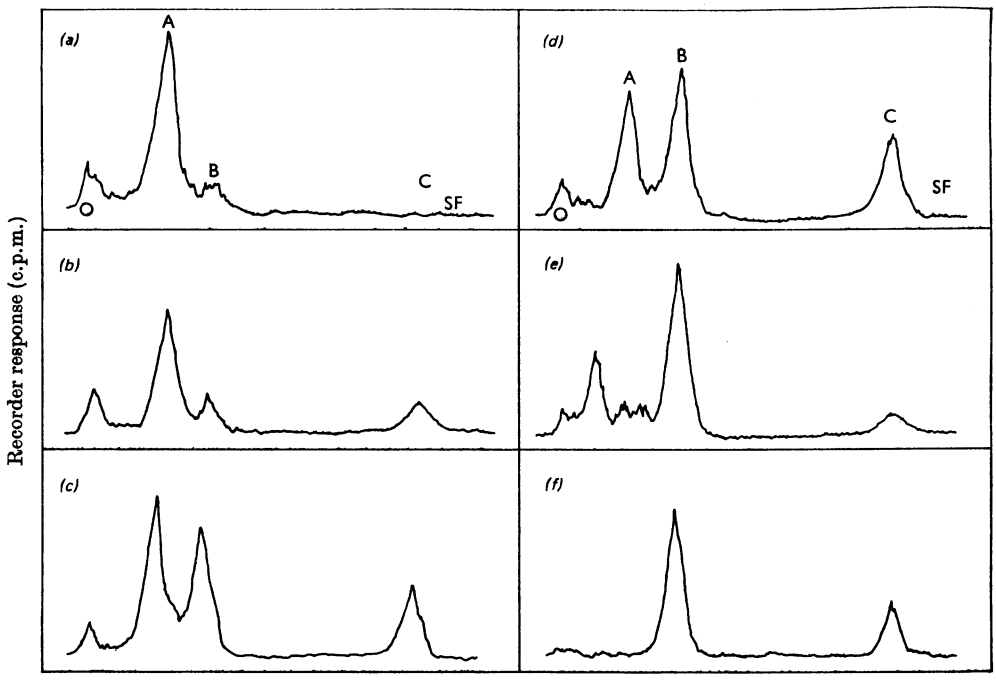


Fig. 1. Radiochromatographic scans of non-saponifiable lipid fractions from developing (*a–e*) and germinating (*f*) pea seeds incubated with [2-^{14}C]mevalonate. Samples (10000 d.p.m.) of each fraction were subjected to t.l.c. in ethyl acetate–heptane (3:22, v/v), as described in the Experimental section. The chromatograms refer to seeds allowed to develop for 10 (*a*), 12 (*b*), 14 (*c*), 16 (*d*) and 24 (*e*) days after anthesis before incubation with mevalonate. Standards of β-sitosterol, β-amyrin and squalene ran coincident with peaks A, B and C respectively. The origin is at O and the solvent front at SF.

Table 1. *Incorporation of radioactivity from* [2-^{14}C]*mevalonate into the non-saponifiable lipid fraction and its distribution between the hydrocarbon, triterpene and sterol for each stage of development and of germination*

The distribution is calculated from radiochromatographic scans of thin-layer chromatograms of the non-saponifiable lipids as described in the Experimental section. Differences between the combined percentages and 100 are due to radioactivity remaining at the origin and, for the sample 24 days after anthesis, to the presence of a compound more polar than sterol.

Time after anthesis (days)	Incorporation into non-saponifiable lipid (%)	Distribution of radioactivity in non-saponifiable lipid (%)		
		Hydrocarbon	Triterpene	Sterol
10	54	0	12	77
11	53	8	18	60
12	60	19	12	49
14	65	18	28	40
16	70	24	35	30
24	62	5	48	14
Germinating seeds	61	24	67	0

fraction. Coincident with this decrease in sterol synthesis during development an increasingly large proportion of the radioactivity appears with the hydrocarbon (predominantly squalene) and triterpene fractions. These observations might suggest that during the earliest stages of development the enzymes responsible for the transformation of squalene into sterol are so active relative

to those involved in the synthesis of squalene from mevalonate that squalene and the triterpene intermediates of phytosterol synthesis, cycloartenol and 24-methylenecycloartanol, do not accumulate to any significant extent. That the rate of conversion of mevalonate into the non-saponifiable lipid fraction is not likely to be the factor in controlling the accumulation of these compounds is supported by the fact that the percentage incorporation of mevalonate into this fraction is relatively constant for all of the experiments (Table 1). The decrease in the proportion of radioactivity associated with sterol as development progresses would then suggest that the enzymes for sterol synthesis from 24-methylenecycloartanol become less active and thus lead to the accumulation of the 4,4-dimethylsterol intermediates. During germination, sterol synthesis is not detected at all.

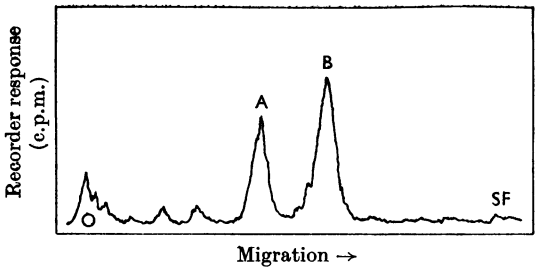


Fig. 2. Radiochromatographic scan of the thin-layer chromatogram of the sterol acetates from developing pea seeds 14 days after anthesis. The chromatogram was run on $AgNO_3$-impregnated plates. A sample (10000 d.p.m.) of the acetates was chromatographed in benzene–hexane (2:3, v/v), as described in the Experimental section. Standards of the acetates of isofucosterol and β-sitosterol ran coincident with peaks A and B respectively. The origin is at O and the solvent front at SF.

Analysis of the sterol fractions. The non-saponifiable lipid fractions were submitted to preparative t.l.c. to isolate the sterol fractions free of triterpene and 4-methylsterol. The distribution of label in these fractions was examined in two ways: by t.l.c. of the acetates on silver nitrate-impregnated sheets and by g.l.c. and radioactivity counting of trapped fractions. A scan of the thin-layer chromatogram of the sterol acetates of seeds 14 days after anthesis is shown in Fig. 2. Very little variation in the distribution of radioactivity between the four peaks was found for all stages of development. The two major radioactive peaks have mobilities coincident with those for isofucosterol and β-sitosterol. The two other peaks of lower R_F value presumably represent intermediates in phytosterol biosynthesis, e.g. the Δ^7-double bond isomer of isofucosterol and, possibly, a more unsaturated compound, the $\Delta^{5,7}$-diene. The distribution shown by g.l.c. of the sterol acetates of seeds at the same stage of development (Fig. 3) shows the label to be predominantly associated with campesterol, β-sitosterol and isofucosterol. The label emerging from the column after β-sitosterol might be expected to be associated with the Δ^7 isomer of isofucosterol acetate but might also be due in some part to degradation occurring on the column. It is important to note that whereas a steady decline in the proportion of label associated with sterol is occurring during the course of development there is no corresponding steady accumulation of radioactivity in a particular sterol intermediate. This

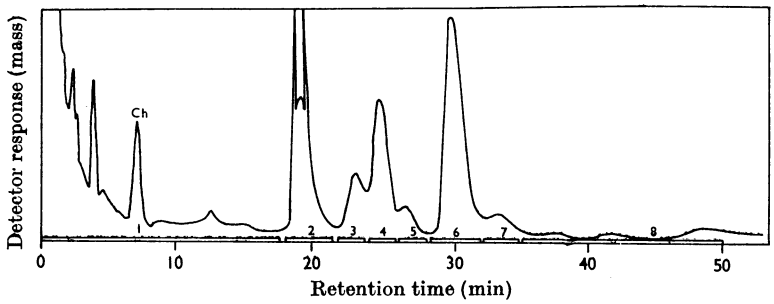


Fig. 3. Radio-g.l.c. of the sterol acetates from seeds incubated with [2-^{14}C]mevalonate 14 days after anthesis. A sample (10000 d.p.m.) of the radioactive acetates was injected with several standard acetates on to a column (8 ft × ¼ in) of OV-17 on Gas-Chrom Q operated at 265°C. Radioactivity associated with the standards was trapped and counted as described in the Experimental section. The trapped fractions are shown as numbered sections along the base line. The radioactivity in a fraction is expressed as a percentage of the total radioactivity emerging from the column: 1, unknown, 15%; 2, cholesterol acetate, 2%; 3, desmosterol acetate, 4%; 4, campesterol acetate, 10%; 5, stigmasterol acetate, 6%; 6, β-sitosterol acetate, 14%; 7, isofucosterol acetate, 22%; 8, unknown, 28%. Abbreviation: Ch, cholestane.

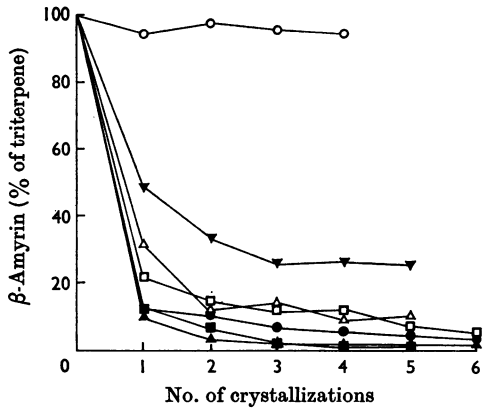


Fig. 4. Graph showing the percentage of ^{14}C remaining associated with β-amyrin after each successive crystallization of the triterpene fractions from developing and germinating pea seeds incubated with [2-^{14}C]mevalonate. Germinating seeds (○); Developing seeds, days after anthesis: 10 days (△); 11 days (□); 12 days (●); 14 days (■); 16 days (▲); 24 days (▼).

suggests that the decrease in activities of the sterol-synthesizing enzymes might be co-ordinated, or that the decrease is governed by an enzyme preceding the formation of the sterol nucleus.

Analysis of the triterpene fractions. The triterpene synthesized in the germinating seed had previously been shown to be principally β-amyrin by co-crystallization of a portion of the radioactive triterpene with non-radioactive β-amyrin (Baisted *et al.* 1962). A similar approach was taken in this study. A sample of each radioactive triterpene fraction, isolated from seeds at each stage of development and germination, was crystallized to constant specific radioactivity with non-radioactive β-amyrin. The proportion of radioactivity remaining with β-amyrin after each successive crystallization is shown in Fig. 4. It is clear that whereas 95% of the radioactivity associated with the triterpene fraction during germination is in β-amyrin, at all stages of development β-amyrin is never more than 25% of this fraction. Indeed, throughout that period when the fresh weight of the seed is increasing the synthesis of β-amyrin decreases from 10% to <2% of the triterpene fraction. After the maximum fresh weight has been attained, β-amyrin synthesis becomes relatively more active until 24 days after anthesis it represents 25% of the triterpene fraction. During germination β-amyrin is more than 95% of the total triterpene.

It was indicated above that the triterpenes, cycloartenol and 24-methylenecycloartanol, which are intermediates in phytosterol biosynthesis, are probably the major triterpene compounds labelled during development. To test this, the triterpene fractions from several experiments were isolated by preparative t.l.c., converted into the acetates and then chromatographed on silver nitrate-impregnated sheets. Radiochromatographic scans of thin-layer chromatograms of the acetates are shown in Fig. 5. As expected, most of the radioactivity during most of the development appears to be associated with the two triterpenes, cycloartenol and 24-methylenecycloartanol. A radio-g.l.c. examination was also conducted on a triterpene acetate fraction from seeds 14 days after anthesis and is shown in Fig. 6. The distribution of radioactivity again supports the contention that the cycloartenols contain most of the radioactivity in the triterpene fraction during early development. Only in the experiment using seeds 24 days after anthesis, i.e. after the maximum fresh weight has been attained, does the peak associated with β-amyrin (Fig. 5) become prominent. However, the distribution of radioactivity based on measurement of the relative areas of these three peaks indicates that the one associated with β-amyrin is 63% of the total. The crystallization to constant specific radioactivity with unlabelled β-amyrin demonstrated that only 25% of the total is this triterpene. Consequently, the remaining radioactivity cannot be all accounted for by β-amyrin. The nature of this remaining radioactivity is unknown. It is notable that at the earliest stage of development, corresponding to the period of active sterol synthesis, 24-methylenecycloartanol is the dominant triterpene. Label only begins to accumulate in cycloartenol as development progresses and sterol synthesis declines. This suggests that the enzyme catalysing the transmethylation from adenosylmethionine to the C-24 position of cycloartenol might be involved in the regulation of phytosterol biosynthesis.

General discussion. During most of development, the squalene oxide–β-amyrin cyclase is relatively inactive compared with the squalene oxide–cycloartenol cyclase. It is this latter enzyme which directs squalene oxide into the path leading to phytosterols (Scheme 1). However, during the terminal stages of development, at 24 days after anthesis not only do the enzymes responsible for transforming 24-methylenecycloartanol into sterols become less active but the relative activities of the squalene oxide cyclase enzymes change so that the direction of cyclization of squalene oxide changes from synthesis of cycloartenol to that of β-amyrin. The distribution of radioactivity in the non-saponifiable lipid fraction of seeds at 24 days after anthesis is consistent with this change in direction of cyclization of squalene oxide. Table 1 shows that during most of development the proportion of radioactivity in the hydrocarbon fraction increases

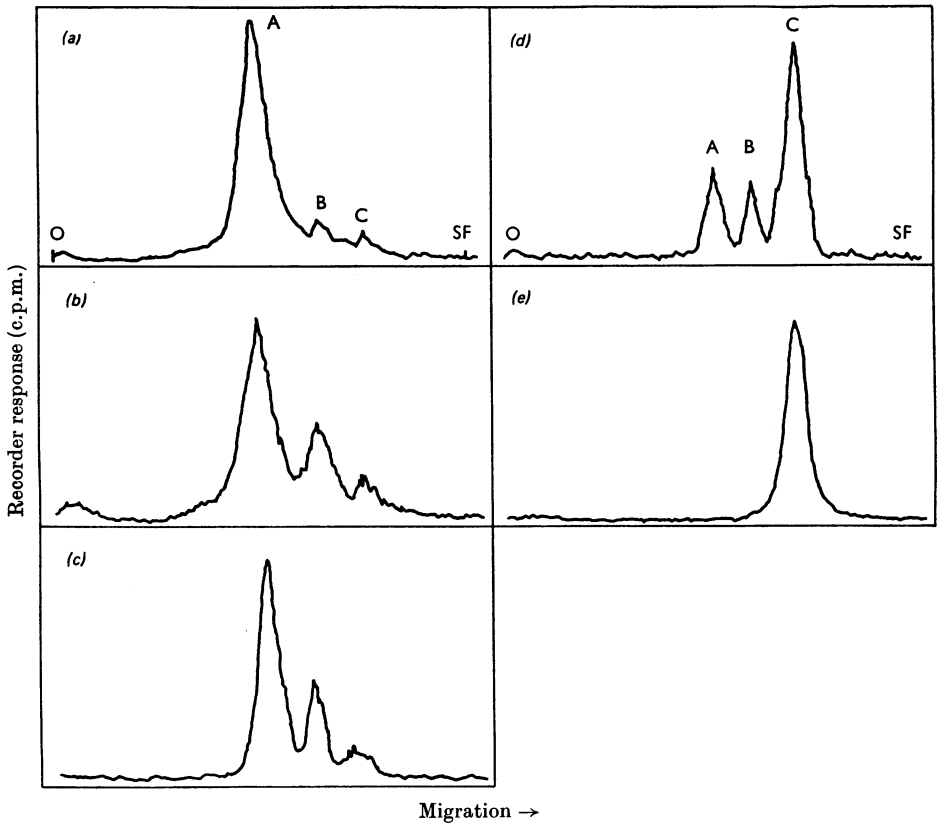


Fig. 5. Radiochromatographic scans of thin-layer chromatograms of the triterpene acetates from developing (*a–d*) and germinating (*e*) pea seeds incubated with [2-^{14}C]mevalonate. The chromatograms were developed by using the conditions described in Fig. 2. The chromatograms refer to seeds allowed to develop for 10 (*a*), 12 (*b*), 16 (*c*) and 24 (*d*) days after anthesis before incubation with mevalonate. Standards of the acetates of 24-methylenecycloartanol, cycloartenol and β-amyrin ran coincident with peaks A, B and C respectively. The origin is at O and the solvent front at SF.

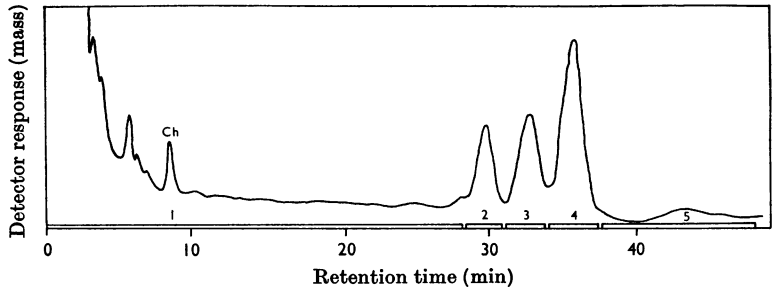


Fig. 6. Radio-g.l.c. of the triterpene acetates from seeds incubated with [2-^{14}C]mevalonate 14 days after anthesis. A sample (10000 d.p.m.) of the radioactive acetates was chromatographed under the conditions described in Fig. 3. Radioactivity associated with the standards was trapped and counted as described in the Experimental section. The trapped fractions are shown as numbered sections along the base line. The radioactivity in a fraction is expressed as a percentage of the total radioactivity emerging from the column: 1, unknown, 9%; 2, β-amyrin acetate, 4%; 3, cycloartenol acetate, 37%; 4, 24-methylenecycloartanol acetate, 42%; 5, unknown, 9%. Abbreviation: Ch, cholestane.

but at 24 days after anthesis it declines sharply. This would be expected if there were increased competition for squalene oxide by another active cyclase enzyme. During germination, when sterol synthesis cannot be observed, there is correspondingly no formation of the first cyclic intermediate in sterol synthesis, cycloartenol, and at this time β-amyrin synthesis appears to be the exclusive product of cyclization of squalene oxide. The fact that β-amyrin is actively synthesized at a time when sterol synthesis is negligible indicates a regulation of sterol synthesis at the level of squalene oxide cyclization. Clearly, if β-amyrin and phytosterol are being synthesized in the same compartment, a suggestion supported by the work of Goodwin (1965), then a regulation of either end-product must occur at the common branch point of squalene oxide cyclization.

The mechanism by which the direction of cyclization of squalene oxide is regulated is unknown, but it is interesting to speculate that, since many cellular membranes have a sterol requirement (Rothfield & Finkelstein, 1968; Korn, 1969) the decrease in sterol synthesis may reflect a decrease in the overall rate of cell division of the developing seed as development progresses. During germination it is possible that sufficient sterol is present in the seed to satisfy the initial demands made by growth on the existing sterol pool. Not until the third day of germination is sterol synthesis found to occur to any significant extent (Nes, Baisted, Capstack, Newschwander & Russell, 1967), and this may be at a time when the seed's reserves of sterol are being depleted.

The fact that the dry weight of the seed continues to increase after the attainment of maximum fresh weight (dry weight per seed corresponding to seeds 16 days after anthesis is 120mg, and for dried seeds before germination, 210mg) suggests an alternative mechanism for regulating the activity of the two cyclases. Yamamoto, Lin & Bloch (1969) found that the squalene oxide–lanosterol cyclase from rat liver required 0.4M-potassium chloride for activation, whereas the same enzyme in yeast (Shechter, Sweat & Bloch, 1970) required low salt concentration for activity. Though the catalytic activity is the same the nature of the environment is a critical factor in determining the specific activity of the enzyme. In the case of the pea seed, after attainment of the maximum fresh weight the developing seed must continue to synthesize necessary components and must therefore displace water in the process. This would lead to increased concentration of all components in the seed, including that of the electrolytes. The most effective concentrating process occurs when the fully mature seed dries out before germination. If the two cyclases in the pea seed respond differently to changes in salt concentration, then the change in direction of the cyclization of squalene oxide might be caused by this.

An alternative explanation for regulation of the terpenoid pathway at this level might be the different relative rates of synthesis and degradation of the appropriate enzymes for sterol and β-amyrin synthesis. This mechanism for regulation of enzymes in higher plants has been reviewed recently (Filner, Wray & Varner, 1969). The nature of the activation processes during germination also remains obscure, and may well depend on the regulatory mechanisms operating during development.

The origin of the polar compound(s) in the non-saponifiable lipid in the seeds 24 days after anthesis (Fig. 1*e*) is unknown. However, an indication of its identity might lie in the fact that the glycosides of sterol and triterpene aglycones (saponins) are powerful surfactive agents (Basu & Rastogi, 1967). The aglycones themselves are usually oxidized derivatives of the parent sterol or triterpene alcohol; e.g. soyasapogenols A, B, C, D and E (Willner, Gestetner, Lavie, Birk & Bondi, 1964; Smith, Smith & Spring, 1958) and medicagenic acid (Djerassi, Thomas, Livingston & Ray-Thompson, 1957) are clearly metabolites of β-amyrin. Such surfactive agents present in ungerminated seeds might bring about structural alteration of cell-membrane lipids (Bangham & Horne, 1962) and could conceivably regulate germination (Nord & Van Atta, 1960; Shany, Birk, Gestetner & Bondi, 1970) by affecting the permeability of cell and organelle membranes during the initial imbibition of water. The polar non-saponifiable lipid compound(s) synthesized during the terminal stages of development may be intermediate(s) in aglycone formation, the saponins of which may be destined to have such a role in pea seed germination.

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