2,3-Oxidosqualene Cyclase and Cycloartenol-*S*-Adenosylmethionine Methyltransferase Activities *in vivo* in the Cotyledon and Axis Tissues of Germinating Pea Seeds

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1. Axis tissues, root and shoot, of germinating pea seedlings actively synthesize sterol from [2-^{14}C]mevalonate during the first 3 days of germination. In addition to the intermediates of sterol synthesis, cycloartenol and 24-methylenecycloartanol, these tissues also form the triterpene β-amyrin. The cyclase catalysing the formation of cycloartenol from oxidosqualene is about four times as active as that for β-amyrin synthesis. 2. Sterol synthesis in the cotyledon is negligible, but cycloartenol and 24-methylenecycloartanol, as well as β-amyrin, are synthesized there. Oxidosqualene cyclase activity in this tissue is 2.6 times as active for β-amyrin synthesis as for cycloartenol synthesis. 3. Comparison of the relative amounts of ^{14}C in cycloartenol and 24-methylenecycloartanol in the axis tissues and cotyledons of 3-day-old seedlings point to relatively active cycloartenol-*S*-adenosylmethionine methyltransferase systems in both axis tissues and a poorly active system in the cotyledon. 4. The role of β-amyrin synthesis in the germinating pea seedling is discussed.

Examination of the variation in steroid and triterpenoid composition during seed germination has been reported for *Phaseolus vulgaris* L. (Duperon & Duperon, 1965), for *Zea mays* L. (Kemp *et al.*, 1967), for *Pisum sativum* L. (Duperon & Renaud, 1966), for species of the Cruciferae (Ingram *et al.*, 1968), for *Calendula officinalis* (Kasprzyk *et al.*, 1970) and for *Nicotiana tabacum* (Bush & Grunwald, 1972). Biosynthetic investigations with the seeds of *Pisum sativum* L. (Baisted *et al.*, 1962), with *Happlopappus heterophyllus* Bl. (Bennett *et al.*, 1967) and with the isolatable organs of *Corylus avellana* L. (Shewry & Stobart, 1974) have also been made.

Several years ago we reported that [2-^{14}C]mevalonic acid is incorporated into squalene and β-amyrin during the initial stages of pea-seed germination. The appearance of label in sitosterol is not evident until the third day (Nes *et al.*, 1967). It is clear that the axis tissue of the seed undergoes considerable growth within the first 2 days of germination. As such growth would demand sterol for the formation of membranes it is conceivable that such a demand may initially be met by the ample supply of sterol stored in the cotyledon. Thus sterol synthesis in the growing axis tissue or the cotyledon might be considered unnecessary during the early stages of germination. We report here that the axis tissue is indeed an active site of sterol synthesis from the onset of germination. In addition, the axis tissue is also a site of synthesis of the major triterpene of the cotyledon, β-amyrin. Although the cotyledon does not appear to synthesize sterol, the intermediates of sterol synthesis, cycloartenol and

24-methylenecycloartanol (Benveniste *et al.*, 1965), are formed in this tissue.

Experimental

Materials

Seed material. *Pisum sativum* L. cultivar Alaska (W. Atlee Burpee Co., Riverside, Calif., U.S.A.) were used.

Radioactive materials. *RS*-[2-^{14}C]Mevalonate (10.5 and 5.9 μCi/μmol) was obtained as the dibenzylethylenediamine salt from Amersham–Searle Corp., Arlington Heights, Ill., U.S.A., and New England Nuclear Corp., Boston, Mass., U.S.A., respectively. 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, Division of Biology, California Institute of Technology, Pasadena, Calif., U.S.A. The sterols used as standards for t.l.c. and g.l.c. were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sitosterol is a mixture of campesterol, stigmasterol

and sitosterol, with the latter sterol predominating. Isofucosterol was obtained from *Euphorbia peplus* (Baisted, 1969). All other chemicals used were of reagent-grade quality.

Methods

Seed germination. Germination was initiated in water for 9 or 24h depending on the experiment. Germination was continued in moist vermiculite in the dark for up to an additional 36h.

Incorporation of substrate. At the end of the germination period the seeds were blotted dry and the axis tissue was removed so that incubation of the axis and cotyledon with the substrate could be carried out separately. In one experiment in which seeds were germinated for 60h, the axis tissue was cut into two at the point of attachment to the cotyledon to give root and shoot tissue. Four separate experiments were conducted.

Expt. (1). From a batch of seeds germinated for 60h, five seeds, similar in appearance (root, 12–16mm; shoot, 4mm) were selected. The roots, shoots and cotyledons were incubated separately with [2-^{14}C]mevalonate (2μCi; 10.5μCi/μmol) in 0.1ml of water for an additional 12h. The tissue was maintained in uniformly wet condition during the incubation period.

Expt. (2). A batch of 30 seeds was germinated for 9h and the axis tissue incubated with the substrate (1μCi; 5.9μCi/μmol) for a further 15h. The cotyledons were germinated for 15h with 2μCi of the same substrate.

Expts. (3) and (4). Two sets of 30 seeds each were germinated for 48h and their axis tissue was incubated with substrate (1μCi; 5.9μCi/μmol) for a further 5 and 24h respectively.

The substrate for each of Expts. (2), (3) and (4) was dissolved in 0.5ml of water. All the incubations were conducted in small beakers at 20–22°C.

Isolation of non-saponifiable lipid. After the incubation the samples were thoroughly washed with water to remove any unabsorbed mevalonate. Each sample was macerated in 20% (w/v) KOH (3ml/g of tissue) and with sand in a pestle and mortar. The tissues were transferred to centrifuge tubes to complete the saponification by heating in a boiling-water bath for 2h. The non-saponifiable lipid was isolated by repeated extraction of the cooled samples with diethyl ether.

Thin-layer chromatography. T.l.c. of samples (10000d.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 activated at 110°C for 15min. Separation was effected in ethyl acetate–hexane (3:22, v/v) and the standard compounds, squalene, β-amyrin and sitosterol, were located with a Rhodamine 6G spray. Sterol and triterpene fractions for

further analysis were isolated by t.l.c. of the remaining non-saponifiable lipid on sheets (20cm×20cm) with the same thickness of silica gel G. The two fractions were isolated from the silica, converted into their corresponding acetates and analysed by t.l.c. on AgNO$_3$-impregnated sheets in benzene–hexane (2:3, v/v). Sheets were impregnated with 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 also run. The reference compounds were detected by spraying with 50% (v/v) H$_2$SO$_4$ and heating at 100°C for 5min.

Gas–liquid chromatography. The sterol and triterpene acetates from the 60h seedling tissues 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 (2.44m×6mm) 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 permitted one-eighth of the sample to go to the detector and the remainder to glass traps immersed in liquid N$_2$. 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 area measured under each peak as a percentage of the total area measured under the entire scan. Radioactivity in fractions trapped from g.l.c. and other non-aqueous samples was measured in a Packard Tri-Carb model 574 liquid-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 (1litre). For counting of radioactivity of aqueous samples, each vial contained 10ml of Bray's (1960) scintillation fluor.

Results and Discussion

Absorption of the *RS*-[2-^{14}C]mevalonate by the seed tissues varied from 50 to 90%. Of the *R*-mevalonate absorbed, from 25 to 60% was incorporated into the non-saponifiable fraction.

Table 1 shows the distribution of label from [2-^{14}C]-mevalonate in the non-saponifiable lipid fraction of axis and cotyledon tissue of seeds germinated for only 9h, and also that for root, shoot and cotyledon tissue for seeds germinated for 60h. At both germination stages the axis tissue is engaged in sterol synthesis. In sharp contrast, the cotyledons at each of these time-

Table 1. *Distribution of ^{14}C by t.l.c. of the non-saponifiable lipids from pea seedling tissues incubated with [2-^{14}C]mevalonate*

Details of the experimental procedure and chromatographic separation are described in the Experimental section.

Expt. no.	Germination period (h)	Tissue	Incubation period (h)	% of total ^{14}C on chromatogram*			
				Origin	Sterol†	Triterpene	Squalene
(1)	60	Shoot	12	14	33	27	21
		Root	12	13	31	21	28
		Cotyledon	12	9	<1	70	5
(2)	9	Axis	15	8	11	38	30
		Cotyledon	15	7	<1	34	33

* The remainder of the ^{14}C is distributed throughout the chromatogram.
† This represents 4-methyl sterol and sterol.

Table 2. *Distribution of ^{14}C by g.l.c. of the acetates of the sterol fractions from Expt.* (1)

Details of the conditions used for the separation are given in the Experimental section. For details of Expt. (1), see Table 1.

Tissue	% of total ^{14}C emerging from g.l.c.			
	Cholesterol	Campesterol	Sitosterol	Isofucosterol
Root	1.0	24.4	53.5	17.4
Shoot	4.3	20.1	43.0	27.2

intervals do not synthesize sterol. A limitation on sterol synthesis in the isolated axis tissue might have been expected owing to the potentially limited availability of ATP and NADPH during the incubation period. Each molecule of squalene synthesized from 6 molecules of mevalonate requires 18 molecules of ATP and 1 molecule of NADPH. The cotyledon, however, is much less likely to suffer from a scarcity of these cofactors, because they are made readily available by the breakdown of starch. The synthesis of β-amyrin in the cotyledon suggested that the absence of sterol synthesis in the cotyledon may be due to the absence or inactivation of the oxidosqualene-cycloartenol cyclase or the 24-methylenecyclo-artanol 4-demethylase system.

The reported absence of sterol synthesis from [2-^{14}C]mevalonate by whole pea seeds during the first 3 days of germination (Nes et al., 1967) is most likely a consequence of the greater competitive advantage that the cotyledons have over the axis for the substrate by virtue of their much larger surface area in contact with the substrate.

Sterol fractions free of 4-methyl sterols and triterpenes were obtained from the root and shoot tissues of the 60h germinated seeds. The distribution, shown by g.l.c. of the derived acetates (Table 2), shows the label to be associated predominantly with sitosterol, campesterol and isofucosterol. Interestingly, the proportion of label associated with cholesterol, although small in both the root and shoot, is much higher in the shoot tissue. As Treharne & Mercer (1966) found that cholesterol was one of the lipid components of chloroplasts, it is possible that the cholesterol-synthesizing machinery is activated in the shoot tissue for proplastid formation.

An analysis of the triterpene fractions of root, shoot and cotyledon tissue by g.l.c. of their acetates (Table 3) reveals two unexpected findings. The root and shoot tissue are manufacturing β-amyrin as well as cycloartenol and 24-methylenecycloartanol. Thus both oxidosqualene–cycloartenol cyclase and oxido-squalene–β-amyrin cyclase are active in the root and shoot axis tissue. Table 4 also shows this to be the case in axis tissue from two other germination stages. As the sterol fraction arises from 24-methylene-cycloartanol, and 24-methylenecycloartanol is derived from cycloartenol by methylation of the latter with S-adenosylmethionine (Heintz & Benveniste, 1972; Wojciechowski et al., 1973), the ratio of radio-activity in β-amyrin to that in cycloartenol, 24-methylenecycloartanol and total sterol gives a crude measure of the relative activities *in vivo* of the two cyclases. For the root tissue, β-amyrin represents 42.3% of the triterpene fraction (Table 3). This fraction represents 21% of the non-saponifiable fraction (Table 1). β-Amyrin is therefore 0.21×42.3% (8.9%) of the non-saponifiable fraction. Cycloartenol then represents 31% (Table 1) + (21−8.9%) of the non-saponifiable fraction. Thus in root tissue we find the ratio of β-amyrin cyclase/cycloartenol cyclase activity to be 0.21 and in shoot tissue 0.28. The cyclization of oxidosqualene in these growing and differentiating tissues clearly favours the path leading to sterol formation.

Table 3. *Distribution of ^{14}C by g.l.c. of the acetates of the triterpene fractions from Expt.* (1)

Details of the conditions used for the separation are given in the Experimental section. For details of Expt. (1), see Table 1.

	% of total ^{14}C emerging from g.l.c.		
Tissue	β-Amyrin	Cycloartenol	24-Methylenecycloartanol
Cotyledon	64.3	19.6	5.2
Root	42.3	27.7	12.4
Shoot	48.6	16.0	25.5

Table 4. *Distribution of ^{14}C by t.l.c. of the acetates of triterpene fractions from pea seedling axis tissue*

Details of the experimental procedure and chromatographic separation are described in the Experimental section.

Expt. no.	Germination period (h)	Incubation period (h)	% of total ^{14}C on chromatogram		
			Cycloartenol	24-Methylenecycloartanol	β-Amyrin
(2)	9	15	10	23	36
(3)	48	5	—	47	50
(4)	48	24	14	17	38

Analysis of the triterpene fraction of the cotyledon (Table 3) unexpectedly reveals that in addition to β-amyrin, cycloartenol and 24-methylenecycloartanol are labelled. The latter two 4,4-dimethyl sterols, intermediates of sterol synthesis, are thus produced in a tissue from which sterol synthesis is absent. On the basis of the distribution of label between these two different products of oxidosqualene cyclization, the β-amyrin cyclase/cycloartenol cyclase ratio in cotyledon tissue is 2.6, or approximately ten times that in the axis tissue. The absence of sterol, or even 4-methyl sterol, from the cotyledon is unlikely to be due to the absence or limiting effect of some cofactor, e.g. NADPH. Consequently, we are led to conclude that one or more of the enzymes involved in the transformation of 24-methylenecycloartanol (Heintz & Benveniste, 1974) is presumably absent or inactive. The fact that this tissue is also rich in sterol (Baisted et al., 1962) suggests the possibility of a feedback inactivation on the 24-methylenecycloartanol-transforming system. That the cycloartenol–S-adenosylmethionine methyltransferase system may also participate in regulating the synthesis of sterol is revealed by a comparison of the distribution of label between 24-methylenecycloartanol and cycloartenol in the three tissues shown in Table 3. This suggests that cotyledon tissue, which has a negligible capacity for sterol synthesis, is also poorly active in the transmethylation of cycloartenol. In sharp contrast, the root and shoot tissues in which sterol synthesis is active appear to have active systems for the transmethylation of cycloartenol. Similar observations have been made on sterol synthesis in maturing pea seeds (Baisted, 1971). Such variations in the distribution of label between the two 4,4-dimethyl sterols

might also be explicable on the basis of the different pool sizes of the intermediates in the isoprenoid pathway in the three tissues.

The ratio of label in 24-methylenecycloartanol/ cycloartenol can be altered by a variation in the experimental conditions. Expts. (3) and (4) (Table 4) show the distribution of label in the triterpene fraction of axis tissue germinated for 48 h. The only difference between the two experiments is the length of time for which each sample is left to metabolize the [2-^{14}C]-mevalonate substrate. For the short-duration experiment no cycloartenol accumulates, whereas for a 24 h interval the two 4,4-dimethyl sterols are in a ratio close to 1.0. Such a finding is probably due to a decline in activity of the transmethylating system as the incubation progresses, either because of depletion of S-adenosylmethionine or because of the inactivation or degradation of the transmethylase enzyme. The alternative explanation, that the rate of transformation of mevalonate into cycloartenol relative to the transformation of 24-methylenecycloartanol into sterol increases with the incubation period, seems less likely.

The synthesis of β-amyrin, a product synthesized with a very considerable expenditure of energy in the form of ATP and NADPH (see above), by pea cotyledons is difficult to explain. The generally accepted role for the cotyledon is that of a storage organ, although Henshall & Goodwin (1964) have reported that the specific activity of the amino acid-activating enzymes increases eightfold during the first 3 days of germination, suggesting the cotyledons to be capable of protein synthesis.

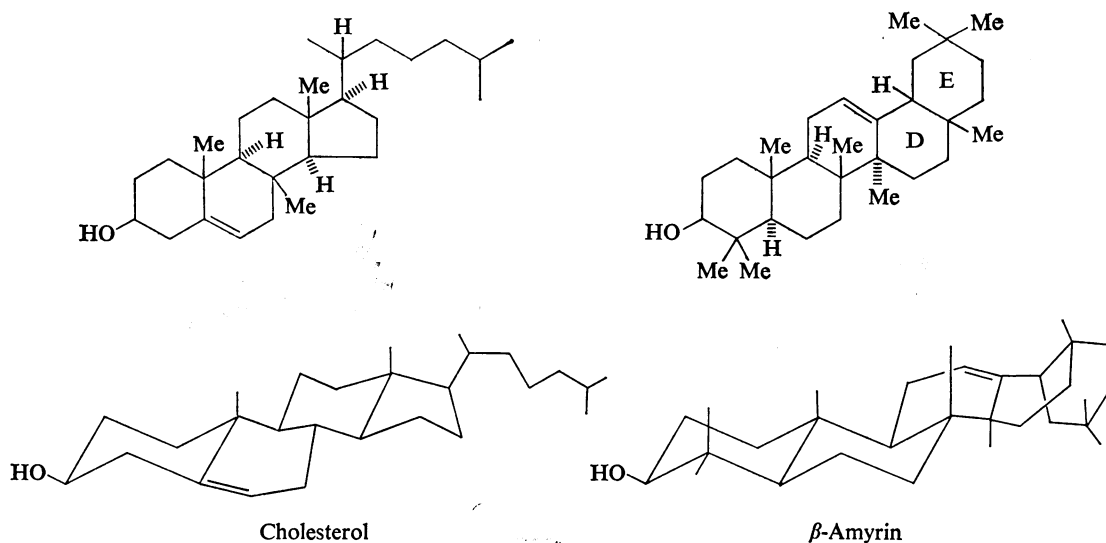The similarity of structure between triterpenes and sterols has prompted us to propose a role for them as

Fig. 1. *Structures of cholesterol and β-amyrin showing the configuration at each asymmetric carbon atom*

Only the hydroxyl group at C-3 and the methyl (Me) groups at the remaining asymmetric carbon atoms are shown in the conformational structures. For further details see the text.

modifiers of membrane function. It has often been suggested (Kemp & Mercer, 1968; Heftmann, 1970; Grunwald, 1971) that free sterols are associated with the membranes in plants as cholesterol is with the cellular membranes of animals. Chapman & Wallach (1968) have proposed a dual role for cholesterol in membranes the net result of which is to control the fluidity of the hydrocarbon chains of the phospholipids. Such control is effected through hydrophobic interactions of the large, planar cholesterol molecule (Fig. 1) with the fatty acid chains of the phospholipid component of membranes. β-Amyrin (Fig. 1) is also a flat molecule in four of its five rings. However, unlike cholesterol, in which the side-chain carbon atoms have the freedom to assume their most favourable conformation, in β-amyrin these carbon atoms are restricted by the formation of a fifth ring. Further, the stereochemistry of the D/E ring junction is *cis*, which destroys the planarity of the molecule. If the β-amyrin produced in the cotyledon is inserted into the cell membranes of this tissue, either by passive diffusion or by some active energy-linked process, interaction between the acyl side chains of the phospholipid components of the membrane would be disrupted and a much less stable membrane would result. The breakdown of these storage-tissue cells and the transport of needed substrates and cofactors from the cotyledon to the axis tissue would thereby be enhanced.

The oxidosqualene cyclase activity for β-amyrin synthesis is only one-tenth that for cycloartenol syn-

thesis in the axis, and the role of β-amyrin in this growing tissue would be expected to be quite different. Again, if it is a membrane component it may serve to bring about very localized alterations in membrane properties consistent with the function of the cell or organelle in which it resides. In general, these polycyclic isoprenoids may serve as very subtle modifiers of membrane properties in plant cells.

References

Baisted, D. J. (1969) *Phytochemistry* **8**, 1697–1703
Baisted, D. J. (1971) *Biochem. J.* **124**, 375–383
Baisted, D. J., Capstack, E., Jr. & Nes, W. R. (1962) *Biochemistry* **1**, 537–541
Bennett, R. D., Lieber, E. R. & Heftmann, E. (1967) *Plant Physiol.* **42**, 973–976
Benveniste, P., Hirth, L. & Ourisson, G. (1965) *Bull. Soc. Fr. Physiol. Vég.* **11**, 252–268
Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
Bush, P. B. & Grunwald, C. (1972) *Plant Physiol.* **50**, 69–72
Chapman, D. & Wallach, D. F. H. (1968) in *Biological Membranes: Physical Fact and Function* (Chapman, D., ed.), pp. 125–202, Academic Press, London and New York
Duperon, P. & Duperon, R. (1965) *C. R. Hebd. Séances Acad. Sci. Ser. D* **261**, 3459–3462
Duperon, P. & Renaud, M. (1966) *C. R. Séances Soc. Biol.* **160**, 1710–1714
Grunwald, C. (1971) *Plant Physiol.* **48**, 653–655

Heftmann, E. (1970) *Lipids* **6**, 128–133

Heintz, R. & Benveniste, P. (1972) *C. R. Hebd. Séances Acad. Sci. Ser. D* **274**, 947–950

Heintz, R. & Benveniste, P. (1974) *J. Biol. Chem.* **249**, 4267–4274

Henshall, J. D. & Goodwin, T. W. (1964) *Phytochemistry* **3**, 677–691

Ingram, D. S., Knights, B. A., McEvoy, I. J. & McKay, P. (1968) *Phytochemistry* **7**, 1241–1245

Kasprzyk, Z., Sliwowski, J. & Boleslawska-Kokosza, D. (1970) *Acta Biochim. Pol.* **17**, 11–18

Kemp, R. J. & Mercer, E. I. (1968) *Biochem. J.* **110**, 119–125

Kemp, R. J., Goad, L. J. & Mercer, E. I. (1967) *Phytochemistry* **6**, 1609–1615

Nes, W. R., Baisted, D. J., Capstack, E., Jr., Newschwander, W. W. & Russell, P. T. (1967) in *Biochemistry of Chloroplasts* (Goodwin, T. W., ed.), vol. 2, pp. 273–282, Academic Press, London and New York

Shewry, P. R. & Stobart, A. K. (1974) *Phytochemistry* **13**, 347–355

Treharne, K. J. & Mercer, E. I. (1966) in *Biochemistry of Chloroplasts* (Goodwin, T. W., ed.), vol. 2, pp. 181–185, Academic Press, London and New York

Wojciechowski, Z. A., Goad, L. J. & Goodwin, T. W. (1973) *Biochem. J.* **136**, 405–412