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Development of the Activities of Enzymes of the Isoprenoid Pathway during Early Stages of Pea-Seed Germination

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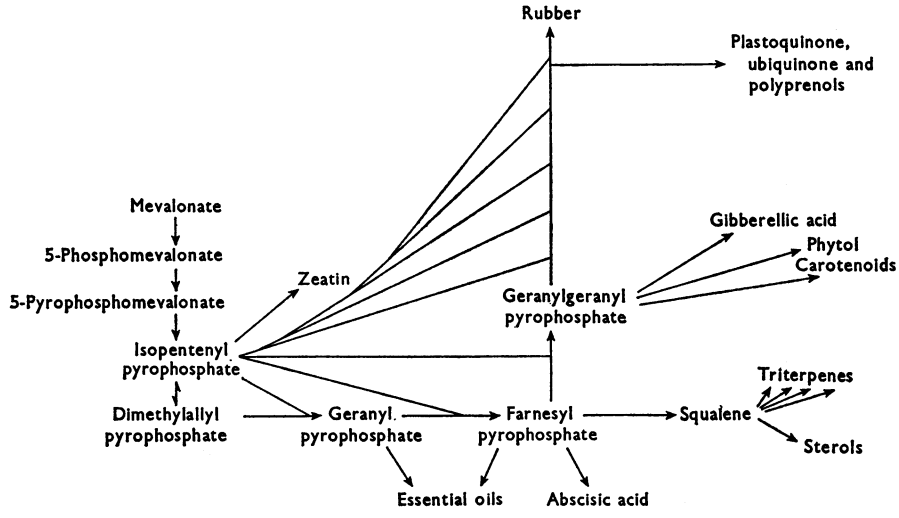
The activities of individual enzymes of the isoprenoid pathway from mevalonate kinase to squalene synthetase in homogenates of seeds germinated up to 32h were assayed. Changes in the activity of each enzyme were observed and compared with the activity at the 2h germination stage. Activities of alkaline phosphatase and fructose 1,6-diphosphate aldolase were similarly measured to provide a reference for changes in the general metabolic activity of seeds during imbibition of water. Water uptake reached a plateau after 12h. The reference enzymes almost doubled in activity between 2 and 8h and thereafter their activities steadily declined. All of the enzymes of the isoprenoid pathway increased in activity between 2 and 6h and, thereafter, with the exception of the prenyltransferase, their activities remained relatively constant. With the prenyltransferase activity the initial increase was followed by a short plateau between 6 and 9h and then a second increase to a maximum between 14 and 16h. After 16h the activity declined. The relative activities of the isoprenoid enzymes at 16h of germination were mevalonate kinase > phosphomevalonate kinase > pyrophosphomevalonate decarboxylase ≈ isopentenyl pyrophosphate isomerase > squalene synthetase > isopentenyl pyrophosphate/dimethylallyl pyrophosphate prenyltransferase. The finding that the prenyltransferase may be the rate-limiting enzyme in squalene synthesis from mevalonate is discussed in relation to regulation of isoprenoid synthesis during pea-seed germination.

The isoprenoids comprise a very large class of compounds widely distributed in Nature, and have a wide range of functions. They are of diverse structure but have one feature in common; they can be theoretically constructed of isoprene units (Wallach, 1887) or are biogenetically derived from such compounds (Ruzicka *et al.*, 1953; Ruzicka, 1959). The chemistry and biochemistry of several groups of isoprenoids has been reviewed by several authors in a volume edited by Goodwin (1971).

In Scheme 1 are shown the major routes to the various isoprenoids from mevalonic acid. The pathway between mevalonate and squalene has been thoroughly investigated with respect to the structure of the intermediates. The understanding of the stereochemistry and mechanisms of the enzyme-catalysed reactions has been immensely aided by the synthesis and use of various species of stereospecifically labelled mevalonates (Popják & Cornforth, 1966; Cornforth & Cornforth, 1970). Although most of the enzyme isolation and purification procedures have been adapted for mammalian tissues (Popják, 1969), in mammals very little branching from the main pathway leading to steroids is shown. Only the plant kingdom displays the full range of branching from the major pathway. These branch pathways must be highly regulated if biological requirements for specific end products for growth and differentiation are to be met efficiently. Regulation at the branch points within the pathway would seem a plausible means of accomplishing this objective. Examples of the end products of these branches in plants are the hormones gibberellic acid and abscisic acid and a partial isoprenoid, zeatin, which act as regulators of a wide range of physiological processes. Other isoprenoid products are involved in the photosynthetic machinery of the chloroplast, and triterpenes and sterols may be involved in membrane formation. Consequently, regulation of carbon flow through the many branches of the isoprenoid pathway is clearly of major physiological importance. The highly branched pathway of isoprenoid formation in higher plants offers a potentially fruitful area of research into regulation of the synthesis of this vast array of compounds with their equally wide range of functions.

We have observed the development of squalene-synthesizing activity from mevalonate in extracts of germinating pea seeds (Green & Baisted, 1971). The



Scheme 1. *Pathways for the conversion of mevalonate into plant isoprenoids*

present paper focuses attention on the development of activity of the individual enzymes of the same segment of the pathway during pea-seed germination.

Experimental

Materials

Plant material. Pisum sativum were the Burpee Blue Bantam variety (W. Atlee Burpee Co., Riverside, Calif., U.S.A.).

Chemicals. RS-[2-^{14}C]Mevalonate was obtained as the dibenzylethylenediamine salt from New England Nuclear Corp., Boston, Mass., U.S.A. It was used in the form of the sodium salt. Its specific radioactivity is quoted for each experiment. *RS*-[1-^{14}C]Mevalonate (6.85mCi/mmol) was obtained from Amersham/Searle Corp., Des Plaines, Ill., U.S.A. as the lactone dissolved in benzene. The solvent was evaporated and the lactone converted into the sodium salt of mevalonate before use. [4-^{14}C]-Isopentenyl pyrophosphate was biosynthesized from [2-^{14}C]mevalonate by using a cell-free preparation from yeast. [1-^{14}C]Isopentenyl pyrophosphate (5.37mCi/mmol) as the trilithium salt was purchased from New England Nuclear Corp. Glucose 6-phosphate, fructose 1,6-diphosphate, ATP, NADPH, reduced glutathione, *p*-nitrophenyl phosphate and unlabelled mevalonolactone were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. as 'Sigma Grade Products'. The mevalonolactone was converted into sodium mevalonate before use. Bovine serum albumin was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Geraniol and linalool were obtained from the Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Farnesol was purchased from International Chemical and Nuclear Corp., Burbank, Calif., U.S.A. Nerolidol was from Frinton Laboratories, So. Vineland, N.J., U.S.A. 2,5-Diphenyloxazole, 1,4-bis-(5-phenyloxazol-2-yl)-benzene and naphthalene were obtained from Packard Instrument Co., Downers Grove, Ill., U.S.A. All other chemicals used were of reagent-grade quality.

Chromatographic supplies. Chromosorb W (AW-DCMS) (60–80 mesh) and butanediol succinate polyester were both obtained from Perco Supplies, San Gabriel, Calif., U.S.A. DEAE-cellulose anion-exchanger was from Sigma Chemical Co. and the Dowex 1 (formate form) from Bio-Rad, Richmond, Calif., U.S.A.

Enzymes. Glucose 6-phosphate dehydrogenase was obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Alkaline phosphatase was from Sigma Chemical Co.

Methods

Protein determinations. The protein content of the seed extracts was determined by the biuret method of Gornall *et al.* (1949). Crystalline bovine serum albumin was used as a standard.

Paper chromatography of water-soluble isoprenoid intermediates. Descending paper chromatography was carried out on Whatman no. 1 paper in the following solvent systems: I, butan-1-ol–formic acid–water (77:10:13, by vol.); II, propan-1-ol–aq. NH_3 soln. (sp.gr. 0.88)–water (3:1:1, by vol.); III, propan-2-ol–butan-2-ol–aq. NH_3 soln. (sp.gr. 0.88)–water (40:21:1:39, by vol.).

Gas chromatography of the prenols. Samples of the hexane-extractable material from enzymic or acid hydrolysis of the water-soluble isoprenoid intermediates were diluted with carrier prenols and then chromatographed on a column (1.83m×0.64cm) of Chromosorb W coated with 20% butanediol succinate polyester. The column packing was prepared by the procedure of Horning *et al.* (1963). The column was run isothermally at 80°C for 6min and then temperature-programmed to 180°C at 20°C/min followed by isothermal operation at 180°C. The instrument used was a Beckman GC4 equipped with a flame-ionization detector. The helium flow rate was 40–50ml/min and the effluent was split 10:1 in favour of passage through a Nuclear–Chicago gas ionization chamber.

Germination conditions. Batches of 100 seeds, the seed coats of which were partly broken to enhance a uniform uptake of water, were germinated in water in the dark at 23–26°C for specified periods of time.

Preparation of the pea-seed homogenates. These were prepared essentially by the procedure of Capstack *et al.* (1965). A batch of 100 seeds was rinsed in water and transferred to a chilled Waring Blendor. The seeds were homogenized for 5s at low speed and 30s at high speed with 75ml of cold 100mM-phosphate buffer (Na^+/K^+), pH7.4, containing 10mM-$MgSO_4$, 10mM-reduced glutathione and 0.45M-sucrose. The crude homogenate was poured through four layers of cheesecloth and then centrifuged at 40000*g* for 10min in a refrigerated Spinco model L centrifuge. The supernatant was used for assays of the isoprenoid-synthesizing enzymes and of fructose 1,6-diphosphate aldolase.

For the alkaline phosphatase assay the preparation of a 40000*g* supernatant was identical except that the homogenizing medium was 100mM-tris–HCl, pH7.4, containing 10mM-reduced glutathione.

Preparation of enzymes from baker's yeast. Dry baker's yeast was a source of enzymes for the synthesis of labelled 5-phosphomevalonate, isopentenyl pyrophosphate and farnesyl pyrophosphate. Yeast powder (50g, Fleischmann's) was mixed with water (75ml) and toluene (7.5ml). The mixture underwent autolysis at 37°C for 1h and was then diluted with 100ml of water. The pH was maintained at 7.4 for an additional 3h by the addition of 5M-KOH at 30min intervals. The autolysate was centrifuged at 30000*g* for 10min. The pellet and toluene layers were discarded and the middle layer was retained for the synthesis of isopentenyl pyrophosphate and farnesyl pyrophosphate. For the synthesis of 5-phosphomevalonate 25–50ml of this layer was dialysed overnight against 3 litres of 0.05M-phosphate buffer (Na^+/K^+), pH7.4, at 6°C.

Preparation of R-[2-^{14}C]-5-phosphomevalonate. A sample (0.5ml) of the dialysed yeast extract was incubated at 37°C for 1h with 50μmol (0.099μCi/μmol) of *RS*-[2-^{14}C]mevalonate in 0.05ml of water and 0.8mmol of tris–HCl buffer, pH7.4, 0.12mmol of iodoacetamide, 0.16mmol of sodium EDTA, 0.24mmol of $MgCl_2$, 0.12mmol of ATP and 1.2mmol of KF in 25ml of water. After boiling for 5min the protein was removed by centrifugation. The supernatant was chromatographed on a 1.5cm×10cm column of Dowex 1 (formate form) (Bloch *et al.*, 1959) in a stepwise gradient of 4M-formic acid (144ml), 0.4M-ammonium formate in 4M-formic acid (160ml) and 0.8M-ammonium formate in 4M-formic acid (336ml). Fractions (8ml) were collected. Fractions 19–21 containing 5-phosphomevalonate were combined and the formic acid was removed at 45°C under vacuum, followed by sublimation of the ammonium formate. Paper chromatography in solvent system I (Bloch *et al.*, 1959; Witting & Porter, 1959) and also in system II (Shah *et al.*, 1965) of a sample of the material revealed a single radioactive peak. The R_F values were 0.30 and 0.26 for the two systems respectively. Literature values of 0.28–0.33 and 0.18–0.20 for 5-phosphomevalonate in these systems have been reported (Dugan *et al.*, 1968). The 5-phosphomevalonate was stored at −15°C at neutral or slightly alkaline pH.

Preparation of [4-^{14}C]isopentenyl pyrophosphate. A sample (0.5ml) of the undialysed 30000*g* yeast supernatant was incubated at 37°C for 1h with 1.07μmol of *RS*-[2-^{14}C]mevalonate (4.7μCi/μmol) in 0.05ml of water and 25ml of the buffer mixture used in the previous preparation. The reaction was terminated and the denatured protein removed as before. Analysis of samples of the supernatant by paper chromatography and Dowex 1 (formate form) ion-exchange chromatography as for the 5-phosphomevalonate preparation showed ^{14}C to be almost exclusively associated with isopentenyl pyrophosphate and unmetabolized mevalonate. Elution of isopentenyl pyrophosphate from the ion-exchange column occurred in fractions 55–59. Based upon the ^{14}C distribution in the supernatant the incorporation of *R*-mevalonate into [4-^{14}C]isopentenyl pyrophosphate ranged from 70 to 100% for several incubations. When needed, the product was rapidly isolated by t.l.c. Samples of the supernatant (0.3ml) were applied to plates of silica gel H (20cm×20cm; 0.25mm thick) and developed in propan-1-ol–aq. NH_3 soln. (sp.gr. 0.88)–water (6:3:1, by vol.). The radioactive material with R_F 0.17–0.23 was scraped from the plate into centrifuge tubes and the labelled isopentenyl pyrophosphate recovered by repeated extraction with 1.0–2.0ml of aq. 0.01M-NH_3.

The isolated material appeared homogeneous and with the expected mobilities for isopentenyl pyrophosphate by the paper-chromatographic and anion-exchange procedures used earlier. On incubation with calf intestinal alkaline phosphatase, [^{14}C]isopentenyl pyrophosphate was converted into a hexane-soluble

material. Careful evaporation of this extract and analysis by the g.l.c.-effluent radioactivity counting procedure revealed that all the label emerged in a single peak with R_T expected for a C_5 compound, presumably isopentenol.

[^{14}C]Isopentenyl pyrophosphate was stored at slightly alkaline pH at −15°C.

Preparation of [4,8,12-^{14}C]*farnesyl pyrophosphate.* A sample of the 30000*g* undialysed yeast supernatant was centrifuged for 3h at 45000*g* at 4°C to sediment the microsomal fraction. A sample (0.5ml) of supernatant was incubated at 37°C for 1h with 1.07μmol of *RS*-[2-^{14}C]mevalonate (4.7μCi/μmol) in 0.05ml of water and 0.8mmol of tris–HCl buffer, pH7.4, 0.16mmol of sodium EDTA, 0.24mmol of $MgCl_2$, 0.12mmol of ATP and 1.2mmol of KF in 25ml of water. The reaction was terminated by boiling the mixture for 5min and the denatured protein removed by centrifugation. The supernatant was stored at pH7.4 at −15°C and was used as the crude source of [^{14}C]farnesyl pyrophosphate. A 5ml sample of this supernatant was diluted to 15ml with aq. 0.01M-NH_3 and then applied to a 1.5cm × 10cm column of DEAE-cellulose. A stepwise gradient of $(NH_4)_2CO_3$ (0.01M, 80ml and 0.10M, 80ml) was used to separate the prenyl pyrophosphates from unmetabolized mevalonic acid. Fractions (8ml) were collected. The combined fractions (11–13) containing the prenyl esters were freeze-dried and residual traces of $(NH_4)_2CO_3$ removed by sublimation at room temperature. The residue was dissolved in aq. 0.01M-NH_3 and stored at −15°C. The composition of this material was investigated by paper chromatography and g.l.c. Paper chromatography in solvent system III revealed >90% of the radioactivity to be present in a single peak with R_F 0.80–0.93. The literature values quoted by Dugan *et al.* (1968) for farnesyl pyrophosphate are 0.85–0.90. To a second sample of the radioactive material was added 1mg each of linalool, geraniol, nerolidol and farnesol. The mixture was hydrolysed at 37°C for 30min with 1M-HCl in a tightly stoppered tube and the reaction stopped by the addition of 5M-KOH to pH10. Analysis of the hexane-extractable material by g.l.c. showed approx. 95% of the ^{14}C associated with farnesol and nerolidol in the ratio 1:4. This is the expected ratio (Popják, 1969) of products resulting from the acid-catalysed allylic rearrangement of farnesol. The remaining radioactivity was distributed between geraniol and dimethylvinyl carbinol.

Assays of the isoprenoid-synthesizing enzymes. Mevalonate kinase had been assayed (Green & Baisted, 1970) under first-order kinetics with respect to mevalonate. The prenyltransferase was also assayed under first-order kinetics with respect to isopentenyl pyrophosphate. For the other assays, the volume of homogenate and the times of reaction were varied to ensure that equal amounts of product were formed (or substrate used) in equal intervals of time. Initial experiments indicated sufficient turnover of substrate to ensure that the assay conditions were zero order with respect to substrate. All incubations were conducted at 24°C and at a pH of 7.4.

Mevalonate kinase assay. Mevalonate kinase (EC 2.7.1.36) was assayed by the procedure of Green & Baisted (1970). The 40000*g* pea-seed supernatant (1.5–3ml) was equilibrated in a water bath at 24°C for a few minutes. To this solution was added 0.18μmol of ATP in 0.1ml of water and *RS*-[2-^{14}C]-mevalonate (5.9μCi/μmol) in 0.01ml of water. The amount of mevalonate ranged from 0.05 to 0.15μmol. The incubation was terminated by the addition of 2ml of 20% (w/v) KOH 2min after the addition of substrate. Unlabelled mevalonolactone (2mg) was added as carrier and the solution transferred to a continuous ether-extractor. The solution was acidified with 6M-H_2SO_4 to pH1 and then warmed at 37°C for 15–30min to lactonize the non-metabolized mevalonate. The lactone was continuously extracted with ether overnight and a sample of this extract counted for radioactivity in Bray's (1960) solution. From the rate of disappearance of *R*-mevalonate and the initial substrate concentration the specific activity of mevalonate kinase was calculated. The kinase was measured over the first 32h of germination.

Phosphomevalonate kinase assay. Phosphomevalonate kinase (EC 2.7.4.2) was assayed by the method of Tchen (1962). [2-^{14}C]Phosphomevalonate (0.15μmol; 0.099μCi/μmol) was added to 0.30ml of the 40000*g* supernatant and 20μmol of ATP in 0.01ml of water. The reaction was terminated after 3min by the addition of an equal volume of 0.1M-EDTA. The kinase activity was determined by measuring the rate of disappearance of phosphomevalonate. The EDTA-treated incubation mixture was transferred to a Dowex 1 (formate form) column and the phosphomevalonate remaining in the incubation mixture eluted from the column in a sharp peak under the same conditions as were used to prepare [2-^{14}C]phosphomevalonate. The difference between the initial substrate concentration and the recovered phosphomevalonate represents the amount of substrate phosphorylated. Phosphomevalonate kinase activity was measured over the first 20h of germination.

Pyrophosphomevalonate decarboxylase assay. Pyrophosphomevalonate decarboxylase (EC 4.1.1.33) was assayed by measuring the rate of evolution of $^{14}CO_2$ from [1-^{14}C]mevalonate. Advantage was taken of the fact that the decarboxylation of pyrophosphomevalonate was the rate-limiting step in the formation of isopentenyl pyrophosphate from mevalonate. The apparatus used for the incubation and trapping of $^{14}CO_2$ was identical with that used by Habibulla & Newburgh (1969), which was a modification of the assembly devised by Cuppy & Crevasse (1963). The

incubation vessels were 10ml conical flasks equipped with serum caps and a removable polyethylene centre well. To each flask was added 1ml of the 40000*g* supernatant and 0.18μmol of ATP in 0.1ml of water. After this 0.1ml of 20% (w/v) KOH was pipetted into the centre well and the serum cap tightly fitted over the flask opening. The flask was equilibrated at 24°C for 3min and 0.26μmol of [1-^{14}C]mevalonate (3.8μCi/μmol) in 0.01ml of water was injected through the serum cap into the enzyme solution. The assay was terminated after 20min by injection of 0.3ml of 6M-HCl. The incubation was continued at 37°C for an additional hour to ensure complete trapping in the centre well of the liberated $^{14}CO_2$. The centre well was removed and transferred to a scintillation vial containing Bray's (1960) solution. The vial was cooled and kept in the dark for several hours before counting of radioactivity, to allow fluorescence to cease.

Evolution of $^{14}CO_2$ as a function of time of incubation shows a lag before a linear appearance of label. The lag period for the 2h and 9h stages of germination was experimentally determined as 6.5 and 3.6min respectively. The shorter lag period with the longer germination time occurs because of the increasing rate at which phosphomevalonate kinase can produce saturating concentrations of pyrophosphomevalonate as germination progresses. From the curve relating development of phosphomevalonate kinase activity with germination interval, and knowing the time-lag of $^{14}CO_2$ evolution at the 2h and 9h germination stage, time-lags for the other germination intervals were determined.

Pyrophosphomevalonate decarboxylase activity was determined through the first 24h of germination by the evolution of $^{14}CO_2$ in a time-period equal to 20min, less the calculated lag period for each stage of germination.

Isopentenyl pyrophosphate isomerase assay. Isopentenyl pyrophosphate isomerase (EC 5.3.3.2) was assayed by a procedure similar to that described by Popják (1969). To a 5ml conical centrifuge tube was added 0.1ml of the 40000*g* supernatant. The enzyme solution was equilibrated at 24°C for 3min and then mixed with 0.02ml of the solution of [4-^{14}C]isopentenyl pyrophosphate (4.7μCi/μmol) containing 1.73nmol. After a 2min incubation the reaction was stopped with 2 drops of 20% (w/v) KOH. The solution was extracted four times with 1ml volumes of diethyl ether to isolate labelled squalene. The combined extracts were washed with water, dried, and a sample was counted for radioactivity. The aqueous phase consists of unchanged isopentenyl pyrophosphate and the acid-labile isoprenoid pyrophosphates. This mixture was hydrolysed at 37°C for 15min and at pH1 by the addition of 6M-H_2SO_4. During the reaction, the centrifuge tube was kept tightly stoppered to prevent losses of the more-volatile prenols. The solution was then chilled in an ice tray, brought to alkaline pH with 5M-KOH, and extracted with 5vol. of diethyl ether. The ^{14}C content of the combined extracts was measured in a sample by radioactivity counting. The distribution of radioactivity between the C_5, C_{10} and C_{15} intermediates was determined by g.l.c. of a sample of the extract and counting the ^{14}C radioactivity of the effluent in the gas ionization chamber. The action of the isomerase on isopentenyl pyrophosphate produces dimethylallyl pyrophosphate. In addition, the dimethylallyl moiety represents one-third of the squalene molecule, one-third of the farnesol molecule and one-half of the geraniol molecule. The amount of dimethylallyl pyrophosphate formed was then determined by summing the appropriate values derived from the prenol and squalene fractions. The isomerase was measured over the first 34h of germination.

trans-Prenyltransferase assay. *trans*-Prenyltransferase (geranyltransferase, EC 2.5.1.1) catalyses the synthesis of farnesyl pyrophosphate from dimethylallyl pyrophosphate and isopentenyl pyrophosphate (Holloway & Popják, 1967). The enzyme was assayed under first-order conditions with respect to isopentenyl pyrophosphate in the 40000*g* homogenates by measuring the rate of squalene synthesis from [1-^{14}C]isopentenyl pyrophosphate after the initial lag period. The rate of squalene synthesis from farnesyl pyrophosphate, described in the next section, and isopentenyl pyrophosphate isomerase activity, exceeded the rate of squalene synthesis from isopentenyl pyrophosphate. Thus *trans*-prenyltransferase is the rate-limiting step in the sequence of the three enzymes and therefore justified such an assay.

To each of four 5ml conical centrifuge tubes was added 0.2ml of the 40000*g* supernatant, 12nmol of NADPH, 2 units of glucose 6-phosphate dehydrogenase and 660nmol of glucose 6-phosphate in 0.01ml of water, and 0.03ml of the homogenizing buffer. Each tube was equilibrated at 24°C for 3min and then mixed with 0.01ml of the solution of [1-^{14}C]-isopentenyl pyrophosphate (5.37μCi/μmol) containing 21.05nmol. The reactions were terminated with 5M-KOH at 5min intervals between 10min and 25min after the addition of substrate. Labelled squalene was extracted from each tube with five 1ml volumes of diethyl ether and the combined, washed, extracts from each fraction were evaporated to dryness in scintillation vials and their radioactivities counted with the Bray's (1960) counting fluid. Each molecule of squalene synthesized arises through a total of four condensations involving isopentenyl pyrophosphate (two in each farnesyl moiety). Thus two-thirds of the label in squalene arises from the condensation of isopentenyl pyrophosphate with dimethylallyl pyrophosphate or with geranyl pyrophosphate. *trans*-Prenyltransferase activity was measured over the first 29h and attained a peak activity between 14 and 16h of germination.

$V_{max.}$ for the prenyltransferase in seeds germinated for 16h was determined by measuring the rate at which *R*-[2-^{14}C]mevalonate was incorporated into squalene under conditions that allowed a steady-state synthesis of squalene, i.e. conditions in which the rate-limiting enzyme has become saturated with its substrate. To each of 5ml conical centrifuge tubes was added 0.2ml of the 40000*g* supernatant, 360nmol of ATP, 12nmol of NADPH, 2 units of glucose 6-phosphate dehydrogenase and 660nmol of glucose 6-phosphate in 18μl of water, and 20μl of the homogenizing buffer. The tubes were equilibrated at 24°C for 3min and then mixed with *RS*-[2-^{14}C]-mevalonate (59000d.p.m./59nmol) in 12μl of water. The reactions were terminated with 5M-KOH at 10min intervals between 15min and 55min after the addition of substrate. Labelled squalene was extracted from each tube with five 1ml volumes of diethyl ether and the combined washed extracts from each fraction were evaporated to dryness in scintillation vials and their radioactivities counted with Bray's (1960) counting fluid. The prenyltransferase activity was measured from the rate of the incorporation of ^{14}C into squalene after the lag period. As with the assay using isopentenyl pyrophosphate, two-thirds of the incorporation arises as a result of the prenyltransferase activity.

Squalene synthetase assay. Squalene synthetase is defined here as that enzyme activity catalysing the synthesis of squalene from farnesyl pyrophosphate. To 0.1ml of the 40000*g* supernatant in 5ml conical centrifuge tubes was added 100nmol of NADPH in 0.01ml of water. After equilibration at 24°C, 2.11nmol of [4,8,12-^{14}C]farnesyl pyrophosphate (4.7μCi/mol) was added and the sample was incubated for 8–16min. The reaction was stopped by the addition of 5 or 6 drops of 5M-KOH, and the squalene extracted with four 1ml volumes of diethyl ether. The combined extracts were washed, dried, and evaporated in a scintillation vial, and then counted for radioactivity in Bray's (1960) fluid. Squalene synthetase activity was measured in terms of nmol of farnesyl pyrophosphate consumed over the first 28h of germination.

Fructose 1,6-diphosphate aldolase assay. Fructose diphosphate aldolase (EC 4.1.2.7) was assayed essentially by the procedure of Jagannathan *et al.* (1956). Cleavage of fructose diphosphate was followed by observing the rate of change in the absorption at 240nm of the hydrazone produced by reaction of hydrazine sulphate and a product of the aldolase reaction, 3-phosphoglyceraldehyde. Absorbance changes were measured on a Beckman DB recording spectrophotometer.

Reagent solution (A) was 3.5mM-hydrazine sulphate–0.1mM-EDTA. The solution was adjusted to pH7.5 with a few drops of 1M-NaOH. Solution (B) was 12mM-fructose diphosphate also adjusted to pH7.5. The order of addition to a 1.0cm silica cuvette was as follows: 2.0ml of (A), 0.1ml of 40000*g* supernatant and 1.0ml of (B). Two blanks were run, one in which solution (B) was replaced with 1.0ml of water and one in which 0.1ml of enzyme solution was replaced with 0.1ml of water. The difference in initial velocity between the enzymic and non-enzymic assays at 24°C was determined as the aldolase activity. Units of aldolase activity were expressed as ΔE_{240}/min per ml of reaction mixture.

Alkaline phosphatase assay. Alkaline phosphatase was measured essentially by the method of Garen & Levinthal (1960), by using *p*-nitrophenyl phosphate.

The 40000*g* supernatant (0.2ml) containing 3.0mmol of tris–HCl buffer, pH8.4, and 15μmol of $MgSO_4$, was mixed in a 1.0cm silica cuvette with 3.0ml of an aqueous solution containing 3μmol of *p*-nitrophenyl phosphate. A blank cell contained the same solutions with the exception of the substrate. Changes in E_{410} as a function of time were measured. Phosphatase activity was determined from the initial rate at 24°C. One unit of alkaline phosphatase is defined as that activity liberating 1μmol of *p*-nitrophenol/min. The molar absorbancy index for *p*-nitrophenol under the assay conditions is 1.62×10^4.

Results and Discussion

We intended to examine the development of the enzyme activities involved in isoprenoid synthesis from mevalonate kinase to squalene oxide–β-amyrin cyclase during pea-seed germination, but although crude homogenates of pea seeds germinated for 24h synthesize squalene from mevalonate very effectively, their capacity to synthesize β-amyrin is greatly impaired compared with the system *in vivo* (Capstack *et al.*, 1965; Nes *et al.*, 1966). Clearly the squalene oxidase and/or the squalene oxide-β-amyrin cyclase is functioning very poorly in the crude homogenate preparation. Benveniste *et al.* (1970) suggested that the mixed-function oxidases required for sterol formation are inhibited in cell-free systems of plant tissues, and specifically that the transformation of squalene into squalene 2,3-oxide does not occur. For simplicity we eliminated all traces of the oxidase and cyclase activities and exclusively studied the mevalonate to squalene sequence of the pathway. The 40000*g* supernatant has all of the enzymes required to synthesize squalene from mevalonate. Analysis by t.l.c. of the ether-soluble material from incubation of the 40000*g* supernatant with [2-^{14}C]-mevalonate shows label associated with squalene and no label associated with either β-amyrin or squalene oxide. A second simplification was to carry out all the assays at the one pH of 7.4. The pH optima of these enzyme activities isolated from liver are: mevalonate kinase, 7.3; 5-phosphomevalonate kinase, 7.3; 5-pyrophosphomevalonate decarboxyl-

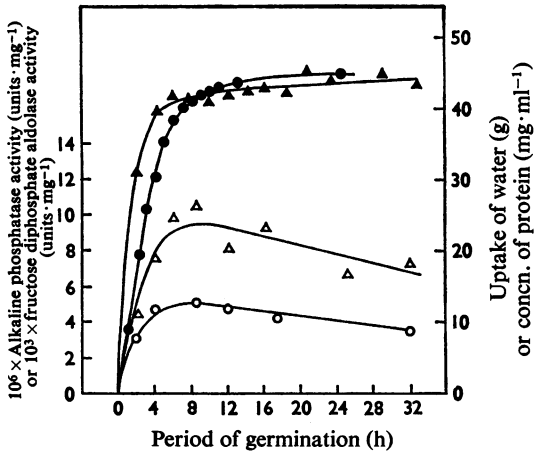


Fig. 1. *Uptake of water by* 100 *pea seeds* (28*g*) *during* 24*h and the protein concentration of the* 40000*g supernatants prepared from the imbibed seeds*

The procedures used are described in the Experimental section. ●, Uptake of water; ▲, concn. of protein. Variation in the content of alkaline phosphatase (○) and fructose 1,6-diphosphate aldolase (△) in the supernatants is also shown.

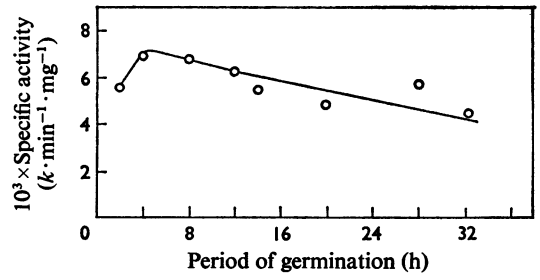


Fig. 2. *Variation in mevalonate kinase activity in the* 40000*g supernatant of pea seeds germinated for* 32*h*

Conditions for the assay are described in the Experimental section. k is the first-order rate constant for the consumption of *R*-mevalonate from the initially racemic substrate, the calculation for which has been described (Green & Baisted, 1970).

ase, 5.1; isopentenyl pyrophosphate isomerase, 6.0; prenyltransferase, 7.9 (Popják, 1969); squalene synthetase, 7.4 (Krishna *et al.*, 1966).

The capacity of pea-seed homogenates to synthesize squalene from mevalonate changes during germination (Green & Baisted, 1971). Two distinct phases of increasing activity appear: the first between 0 and 6h, followed by an interval of several hours when the activity appears to be constant, and then a second burst of activity between 12 and 16h. We have suggested that these changes occur as a consequence of the compartmentation of two sites of isoprenoid synthesis from mevalonate, which develop at different rates during early germination. These compartments may be different organelles within a single cell but clearly, as we are using whole seeds, these compartments may be different tissues of the seed. In the present work we have attempted to determine which is the rate-limiting enzyme in this pathway during germination by direct measurement of the activity of each enzyme between mevalonate kinase and squalene synthetase. This approach also permits observation of each of these enzymic activities as germination progresses.

Fig. 1 shows the change in fresh weight of seeds during germination. Also shown is the protein content of the 40000*g* supernatants from each batch of seeds and the specific activities of alkaline phosphatase and fructose 1,6-diphosphate aldolase. These two enzymes are involved in degradative reactions closely linked to energy production. Consequently it might be expected that they would be widely distributed and develop rapidly during germination. In both cases they reach peak activities about 8h after the onset of water imbibition, which corresponds closely to the time of maximum water uptake. Both specific activities increase 100% between 2 and 8h of germination and thereafter decline steadily. The protein obtained is relatively constant after about the sixth hour of germination.

The mevalonate kinase assay was developed with germinating pea seeds as the source of enzyme (Green & Baisted, 1970), and it makes use of first-order kinetics with respect to mevalonate; Fig. 2 shows the change in specific activity of this enzyme measured at each stage of germination under these conditions. The activity reaches a maximum after 4h but the increase from 2h is very slight (approx. 20%). Again the activity declines steadily during the next 28h of germination. The decline in specific activity of this enzyme, a decline observed also for alkaline phosphatase and fructose 1,6-diphosphate aldolase, cannot be due to dilution of the enzymes with inactive protein present in the extracts from the seeds germinated for a longer time because the protein concentration of these extracts is relatively constant after the sixth hour of germination. In absolute terms, mevalonate kinase is the most-active enzyme in the pathway even as measured under first-order kinetics with respect to mevalonate (Table 1).

The measurement of 5-phosphomevalonate kinase was done under substrate-saturating conditions. The activity was determined by measuring the rate of disappearance of substrate by chromatography of the terminated reaction mixtures on Dowex 1 (formate form) columns. Although alkaline phosphatase activity is present in these extracts it does not

interfere in this assay. No labelled mevalonate was observed to accumulate even though unchanged 5-phosphomevalonate was clearly present. At the assay pH of 7.4 the phosphatase activity may be functioning poorly. Fig. 3 shows the change in specific activity of this enzyme during the first 20h of germination. The peak activity is reached after 12h and represents about a 400% increase over the value at 2h.

5-Pyrophosphomevalonate decarboxylase was assayed by measuring the rate of evolution of $^{14}CO_2$ from [1-^{14}C]mevalonate after the initial lag period. The activity of this enzyme was much less than either of the first two enzymes in the pathway at all stages of germination measured. Consequently, as the decarboxylase is the rate-limiting enzyme for the first three transformations it may be measured by using [1-^{14}C]mevalonate as the substrate. Fig. 4 shows plots of $^{14}CO_2$ evolution in homogenates from seeds germinated for 2 and 9h. Corrected rates of evolution of $^{14}CO_2$ are determined by subtracting the calculated lag periods from the 20min interval used for the remaining assays. Lag periods of 6.5 and 3.6min for the 2 and 9h germinations respectively are obtained by extrapolation of the linear part of each curve back to zero $^{14}CO_2$ evolution as shown. The lag period presumably represents the time needed for 5-pyrophospho[1-^{14}C]mevalonate to be synthesized from [1-^{14}C]mevalonate. This will depend almost exclusively on 5-phosphomevalonate kinase, as the mevalonate kinase is more active than the phosphomevalonate kinase (Table 1), and phosphomevalonate is absent at zero time. Thus the lag period to be subtracted from the 20min assay time for the remaining germination stages can be deduced from the curve showing the development of phosphomevalonate kinase with germination stage (Fig. 5).

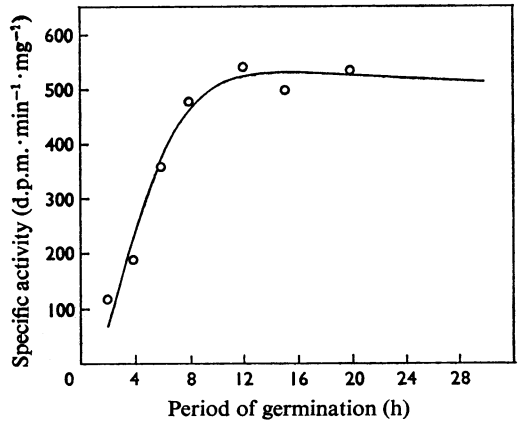


Fig. 3. *Variation in 5-phosphomevalonate kinase specific activity in the 40000g supernatants of pea seeds germinated for 20h*

Conditions for the assay are described in the Experimental section.

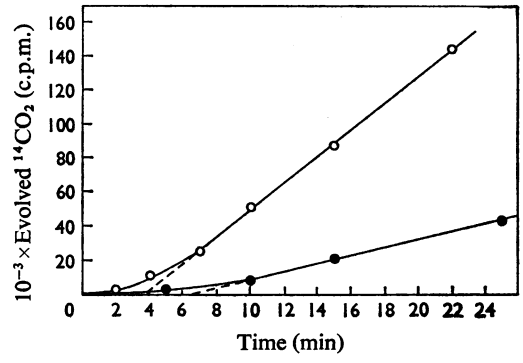


Fig. 4. *Kinetic assay of $^{14}CO_2$ evolution from [1-^{14}C]-mevalonate in 40000g supernatants of pea seeds germinated for ●, 2h and ○, 9h*

Conditions for the assay are described in the Experimental section. The lag periods before linearity in $^{14}CO_2$ evolution are determined by extrapolation of the lines to the abscissa.

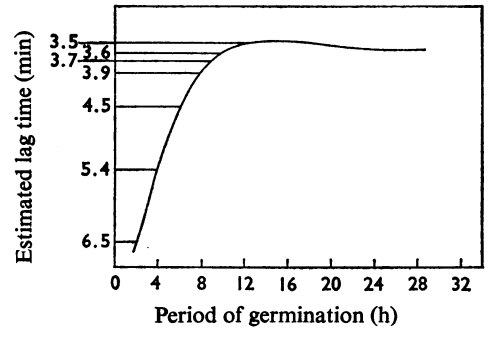


Fig. 5. *Graphical estimation of the lag period before linearity in $^{14}CO_2$ evolution from [1-^{14}C]mevalonate in 40000g supernatants of pea seeds*

The lag period occurs as a result of the time it takes to saturate 5-pyrophosphomevalonate decarboxylase with its substrate. This time-period is almost exclusively dependent on the activity of 5-phosphomevalonate kinase. The curve used for the estimation is therefore that of the variation of 5-phosphomevalonate kinase with germination period. The lag times for the 2 and 9h germination periods, determined from Fig. 4, are used as reference points to construct a linear scale for the remaining lag times.

The increase in specific activity of 5-pyrophosphomevalonate decarboxylase is shown in Fig. 6. An increase in activity of 150% over the 2h value is reached after 10h, after which there is little change through 24h.

Isopentenyl pyrophosphate isomerase has been measured in ammonium sulphate fractions of pig liver extracts by a coupled assay procedure (Popják, 1969). The method requires the addition of excess of prenyltransferase to the protein solution in the presence of the labelled isopentenyl pyrophosphate and the isomerase activity is then measured as being one-third of the amount of ^{14}C-labelled farnesyl pyrophosphate synthesized in unit time. The pea-seed homogenate contains the enzymes necessary to convert isopentenyl pyrophosphate into squalene. In our assay, the complete distribution of label in the reaction mixture was investigated at the end of the incubation period. Labelled squalene was extracted with ether and its radioactivity counted. The distribution of residual label in dimethylallyl pyrophosphate, geranyl pyrophosphate and farnesyl pyrophosphate was determined by acid hydrolysis of the remaining aqueous phase, ether extraction of the solution after it was made alkaline and g.l.c. of the ether extract. At each stage of germination no ^{14}C label emerged from the g.l.c. other than that with a retention time corresponding to dimethylvinylcarbinol, the product resulting from the acid-catalysed rearrangement of dimethylallyl alcohol. Isomerase activity is then expressed as one-third of the ^{14}C label in squalene plus the total label in dimethylvinylcarbinol.

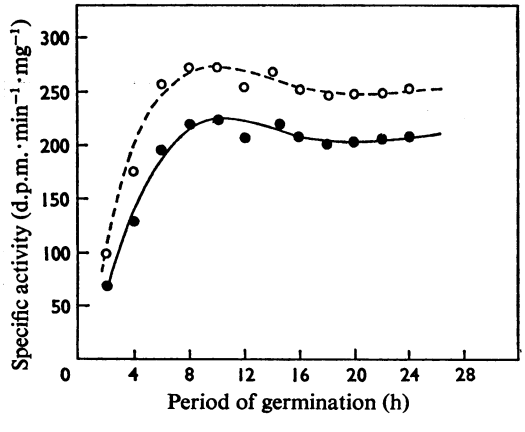


Fig. 6. *Variation in 5-pyrophosphomevalonate decarboxylase specific activity in the 40000g supernatants of pea seeds germinated for 24h*

Curves are shown for the specific activity values corrected (o) and uncorrected (●) for the lag period.

Fig. 7 shows the change in specific activity of the isomerase as germination progresses. A maximum isomerase activity is reached after about 10h, after which the activity remains constant through 34h. This represents about a 70% increase in activity from the 2*h* value. Importantly, the fact that no label appeared in geraniol or farnesol suggests that the prenyltransferase is the rate-limiting step in squalene synthesis, provided that the distribution of label between the intermediates in the pathway is a result of a kinetically controlled rather than a thermodynamically controlled pathway.

The assay of prenyltransferase utilized the previous observation that indicated prenyltransferase to be the rate-limiting step in squalene synthesis. Prenyltransferase activity was measured by coupling it with the endogenous squalene synthetase activity present in the homogenate. The rate of squalene synthesis from isopentenyl pyrophosphate was measured after labelled squalene appeared linearly with time of incubation. As the label appearing in squalene results from two labelled farnesyl pyrophosphate molecules, each of which arises by two successive isopentenylations of dimethylallyl pyrophosphate, the prenyltransferase activity is expressed as two-thirds of the amount of labelled isopentenyl pyrophosphate converted into squalene/min.

The change in specific activity of prenyl transferase with germination is shown in Fig. 8. This activity profile is similar to that observed for the development of squalene-synthesizing activity in these homogenates when assayed with mevalonate (Green & Baisted, 1971) and supports the finding that the prenyltransferase is the rate-limiting enzyme in squalene synthesis from mevalonate when assayed under these conditions. Assay of the remaining enzyme in the pathway,

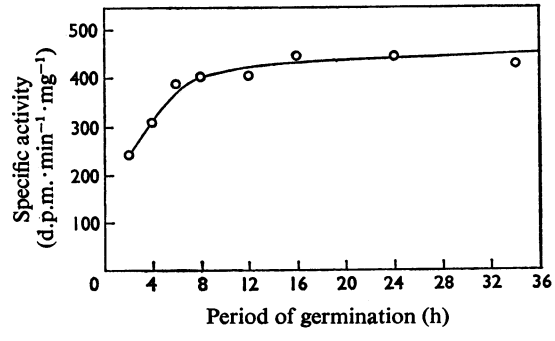


Fig. 7. *Variation in isopentenyl pyrophosphate isomerase specific activity in the 40000g supernatants of pea seeds germinated for 34h*

Conditions for the assay are described in the Experimental section.

squalene synthetase, confirmed this conclusion. The activity of the prenyltransferase reaches a peak between 14 and 16h but achieves this peak activity in two stages. There is an approximately 250% increase between 2 and 7h followed by a short plateau and then an additional 500% increase to a maximum activity between 14 and 16h. This overall increase from 2h to 16h represents the greatest change in activity for any enzyme in the pathway. The $V_{max.}$ for this enzyme at the 16h germination stage is calculated from the rate of formation of [^{14}C]squalene from *R*-[2-^{14}C]mevalonate (Fig. 9). The assay conditions were chosen to be optimum for mevalonate, ATP and NADPH. Under these conditions the linear portion of the curve represents the maximal rate at which the rate-limiting enzyme is functioning. Two-thirds of the ^{14}C in squalene results from prenyltransferase activity as rationalized in the previous paragraph.

The last enzyme activity measured, squalene synthetase, has received very much attention recently (Epstein & Rilling, 1970; Edmond *et al.*, 1971, and references therein; Shechter & Bloch, 1971). Two steps have been shown to be involved in the synthesis of squalene from two molecules of farnesyl pyrophosphate. The first of these is independent of NADPH (Rilling, 1966); the product of the reaction, presqualene pyrophosphate, is transformed into squalene in a second reaction which requires NADPH. For the present study we took squalene synthetase to be the activity catalysing the synthesis of squalene from farnesyl pyrophosphate. The development of squalene synthetase activity during germination is shown in Fig. 10. A 75% increase over the activity at 2h occurs by the fifth hour. Thereafter

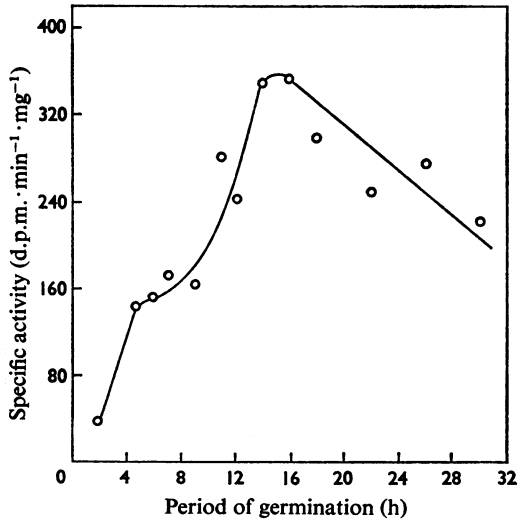


Fig. 8. *Variation in prenyltransferase specific activity in the* 40000*g supernatants of pea seeds germinated for* 32*h*

Conditions for the assay are described in the Experimental section.

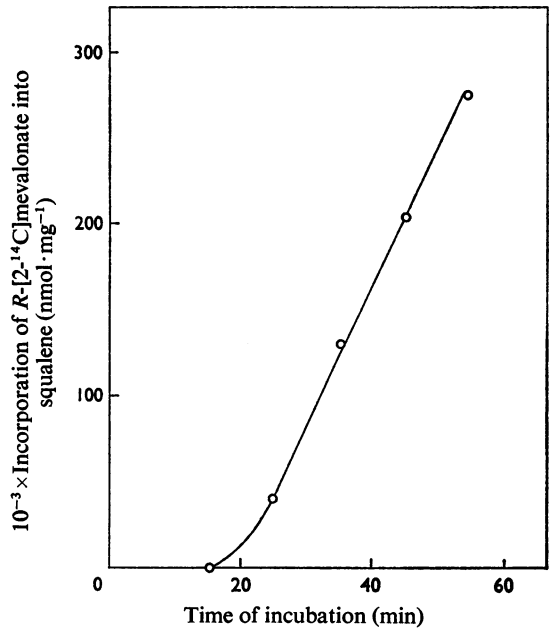


Fig. 9. *Formation of* [^{14}*C*]*squalene from* [2-^{14}*C*]-*mevalonate in the* 40000*g supernatant of seeds germinated for* 16*h as a function of incubation time*

Conditions for the reaction are described in the Experimental section.

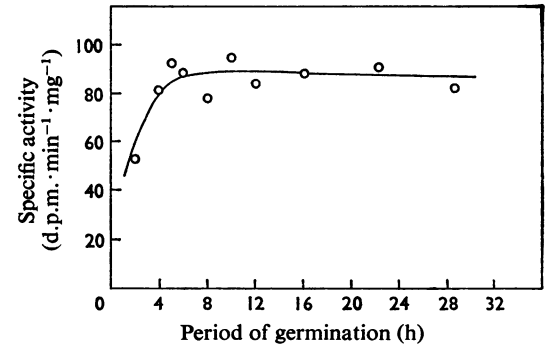


Fig. 10. *Variation in squalene synthetase specific activity in the* 40000*g supernatants of pea seeds germinated for* 28*h*

Conditions for the assay are described in the Experimental section.

Table 1. *Comparison of the enzymic activities of the isoprenoid pathway in homogenates of seeds germinated for* 16*h*

Enzyme	Specific activity (nmol of substrate consumed·min^{-1}·mg^{-1})	Relative activities
Mevalonate kinase	0.44*	88
Phosphomevalonate kinase	0.14	28
Pyrophosphomevalonate decarboxylase	0.03	6
Isopentenyl pyrophosphate isomerase	0.04	8
Isopentenyl/dimethylallyl pyrophosphate transferase	0.005	1
Squalene synthetase	0.008	1.6

* Estimated from the first-order rate constant at 16h (5.9×10^{-3} min^{-1}·mg^{-1}) and the amount of *R*-mevalonate (75nmol) in the assay volume.

little change in activity occurs during 29h of germination.

A comparison of the specific activities at 16h for these isoprenoid-synthesizing enzymes is shown in Table 1. At 16h the prenyltransferase is at peak activity and is the rate-limiting enzyme in squalene synthesis under the conditions used for the assays. With the exception of the prenyltransferase, the relative activities of the enzymes do not suffer large changes after the initial increase. Consequently, the prenyltransferase must be rate-limiting throughout the entire germination period investigated. It is specially interesting that the relative levels of activity of each of the first five enzymes favour the rapid establishment of a pool of isopentenyl pyrophosphate and dimethylallyl pyrophosphate. These two intermediates are isomeric and the activities of the decarboxylase, isomerase and prenyltransferase maintain the two pyrophosphates in near-equilibrium. These two pyrophosphates are the fundamental units for the elaboration of all the isoprenoids (Scheme 1). The existence of a pool of these isomers permits the synthesis of the higher isoprenoids to be regulated by the chain-extending enzyme, the prenyltransferase. These conclusions must at present be tentative because we do not know that the enzyme activities measured *in vitro* reflect the relative activities of the enzymes under physiological conditions. In this regard, Popják (1969) has stated that the liver prenyltransferase is inhibited by phosphate.

The presence of diverse products arising from branching of the isoprenoid pathway is a feature common among plants. Branching from this pathway is uncommon in non-plant systems. The major exception is at the C_{15} level, where the pathway may be diverted toward squalene synthesis or, by chain extension, to the formation of polyprenyl pyrophosphates. Of special interest is the fact that chain extension may occur in two ways; one of the two hydrogen atoms at C-2 of isopentenyl pyrophosphate may be stereospecifically removed to give a prenyl unit that has either *trans*- or *cis*-configuration. Rubber in the latex of *Hevea brasiliensis* is composed of *cis*-prenyl units (Archer *et al.*, 1966). Polyprenyl pyrophosphates of mixed stereochemistry function as sugar carriers in mammalian systems (Richards *et al.*, 1971) and have a similar function in cell-wall biosynthesis in bacterial systems (Higashi *et al.*, 1967; Scher *et al.*, 1968). The prenyl units are all-*trans* in the polyprenyl pyrophosphate precursors of the lipid side chain in the ubiquinones (Daves *et al.*, 1966; Threlfall & Whistance, 1969).

Thus, in a system in which branching may be extensive, the amount of product resulting from a branch of the pathway may be governed by the relative activities of the chain-branching enzymes and the two chain-extending enzymes.

Scheme 1 shows the prenyltransferase to function at each branch point, that is, at the level of C_{10}, C_{15}, C_{20} .... The chain may be extended at each of these levels by isopentenylation. Alternatively, by other reactions at the same level involving cyclizations, rearrangements, eliminations and hydrolyses, the carbon flow may be diverted into mono-, sesqui-, di-, tri- and tetra-terpene synthesis. Consequently, the prenyltransferase activity may fluctuate in a developing system where differential demands for products of several paths are required at different stages of development. It should be emphasized that if this mechanism for the regulation of the synthesis of these different end products is used by plants then it is not an absolute requirement that the prenyltransferase be rate-limiting. Anomalous behaviour of prenyltransferases has been observed by Kandutsch *et al.* (1964). They have isolated a geranylgeranyl pyrophosphate synthetase from *Micrococcus lysodeikticus*. The enzyme possesses isopentenylating activity with dimethylallyl, geranyl and farnesyl pyrophosphates as co-substrates. The three activities, and in particular that activity catalysing the isopentenylation of farnesyl pyrophosphate, were extremely sensitive to protein concentration, being optimum over a fairly narrow range.

Goad (1970) has reasoned that the relative activities of the enzymes catalysing the synthesis, isomerization and utilization of isopentenyl pyrophosphate can

explain some of the low ^3H contents found in sterols synthesized from [2-^{14}C]mevalonate stereospecifically labelled with ^3H at the C-2 position. If the relative activities of the enzymes permit the isomerase to allow isopentenyl pyrophosphate and dimethylallyl pyrophosphate to approach an equilibrium then ^3H on C-2 of mevalonate (C-4 of isopentenyl pyrophosphate) will undergo exchange with protons from the surrounding medium. The relative activities satisfying these conditions would have the isomerase > prenyltransferase. However, these were long-term (4–6 days) experiments *in vivo* with the protozoan *Ochromonas malhamensis* and the mould *Aspergillus niger*. Shorter-term (4–12h) experiments *in vitro* with rat liver and yeast have given sterols that do not show any appreciable exchange of ^3H.

Evidence showing the isomerase to be rate-limiting has been provided by Rilling & Bloch (1959). They found that squalene synthesized from mevalonate by yeast extracts in the presence of heavy water incorporated one ^2H atom into each terminal isopropylidene group, the ^2H incorporated at the centre of the molecule being disregarded. The fact that the label is confined to that moiety derived from dimethylallyl pyrophosphate and not on appropriate methylene carbon atoms derived from isopentenyl pyrophosphate indicates that the isomerase, or reactions leading to isopentenyl pyrophosphate, must be rate-limiting. Lynen *et al.* (1959) have provided experimental support by showing considerably more synthetase than isomerase activity in yeast extracts.

Although regulation of the pathway may be effected through the prenyltransferase, an alternative and important means of regulation is one involving the compartmentation of enzymes. Compartmentation would permit the formation of an isoprenoid product at a site physically separated from the formation of a different isoprenoid. This would clearly provide a means for uninterrupted synthesis of sterols, which are presumably required at most stages of the life cycle of the plant if such compounds are membrane constituents, and also synthesis of hormones such as abscisic acid, gibberellic acid and zeatin, which may be required only at specific periods of the cycle. The compartmentation of intermediary metabolites in plants has been reviewed by Oaks & Bidwell (1970). Evidence for compartmentation at the subcellular level has been offered by Rogers *et al.* (1968) for the isoprenoids synthesized within the chloroplast and the cytoplasm of cells of green plants. In this case the chloroplast membrane serves as the physical barrier separating the two compartments. Loomis (1967) and Loomis & Croteau (1972) suggested that the enzymes of monoterpene synthesis are compartmented within the oil glands of essential oil-bearing plants. They have also convincingly demonstrated that the sites for monoterpene and sesquiterpene synthesis are remote from one another in *Mentha piperita* and that each site is separated from squalene synthesis. In studies with germinating pea seeds we found that there is a compartmentation of squalene 2,3-oxide cyclase activities. The axis tissue actively synthesizes sterols whereas the non-axis tissue does not (T. Y. Fang & D. J. Baisted, unpublished work).

Regulation of isoprenoid synthesis may thus be effected by compartmentation, and also by modulation of enzyme activities. In the latter case changes in activity may arise through the influence of specific small molecules on a regulatory enzyme in the pathway or by protein synthesis, which would produce changes in the amount of enzyme present. Enzyme induction in higher plants has been reviewed by Filner *et al.* (1969).

Finally, the physical state of these enzymes should also be considered. In cell-free extracts from mammals the steps from mevalonate kinase to prenyltransferase are catalysed by soluble enzymes whereas squalene synthetase is a microsomal enzyme. Clearly then, the degree of organization of this multi-enzyme system will be of considerable importance in the overall activity for squalene synthesis from mevalonate. Multi-enzyme systems have been reviewed by Ginsburg & Stadtman (1970), and although the isoprenoid pathway has not been viewed in the usual sense of a multi-enzyme system there is evidence for organization among the soluble enzymes of another lipid-synthesizing system: fatty acid synthetase of *Escherichia coli* (Van den Bosch *et al.*, 1970). Of particular interest in the pathway from mevalonate to squalene is the fact that the substrates and intermediates are water-soluble but the product is a lipid. A protein in liver that functions as a carrier of water-insoluble cholesterol precursors and which stimulates their metabolism (Ritter & Dempsey, 1971; Scallen *et al.*, 1971) has also been found to stimulate the conversion of farnesyl pyrophosphate into both presqualene pyrophosphate and squalene (Rilling, 1972). Rilling (1972) also found a protein in yeast with sterol carrier protein activity. The existence of a similar protein in higher plants might be predicted. The activity of an enzyme system in which the water-insoluble product, squalene, was formed would clearly be influenced by the rate at which it could be removed from the active site of the synthetase.

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