Intracellular Localization of Mevalonate-Activating Enzymes in Plant Cells

By L. J. ROGERS, S. P. J. SHAH AND T. W. GOODWIN

Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth

(Received 7 December 1965)

Mevalonate-activating enzymes are shown to be present in the chloroplasts of French-bean leaves. The chloroplast membrane is impermeable to mevalonic acid. Mevalonate-activating enzymes also appear to be found outside the chloroplast. These results support the view that terpenoid biosynthesis in the plant cell is controlled by a combination of enzyme segregation and specific membrane permeability.

It has been shown (Goodwin, 1958*a,b*; Mercer & Goodwin, 1962) that $^{14}\text{CO}_2$ is actively incorporated into β -carotene and phytol in illuminated maize seedlings whereas $[2\text{-}^{14}\text{C}]\text{MVA}^*$ is insignificantly incorporated under the same conditions. $[2\text{-}^{14}\text{C}]\text{MVA}$ is, however, effectively incorporated into sterols and related products but not markedly into chloroplast terpenoids such as plastoquinone and tocopherolquinone (Mercer & Goodwin, 1963). A similar situation exists in illuminated seedlings of a variety of plant species (Treharne, Mercer & Goodwin, 1964). Further experiments by Griffiths, Threlfall & Goodwin (1964) showed that during chloroplast formation in maize seedlings $[2\text{-}^{14}\text{C}]\text{MVA}$ was more effectively incorporated into ubiquinone than into plastoquinone, whereas the reverse was true when $^{14}\text{CO}_2$ was the substrate.

These results led Goodwin & Mercer (1963) to suggest that the regulation of terpenoid biosynthesis in developing seedlings was achieved by a combination of intracellular enzyme segregation and specific membrane permeability (see also Goodwin, 1965). First, both chloroplastidic and extrachloroplastidic sites of synthesis are regarded as having a common biosynthetic 'backbone' by which acetate is converted, via MVA, MVA-5P, MVA-5PP, IPP, geranyl pyrophosphate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, through to C_{45} and C_{50} isoprenoid pyrophosphates (Scheme 1). It was proposed further that as well as this common biosynthetic pathway each 'compartment' has certain specific enzymes, the substrates of which can be members of the common biosynthetic pathway. Thus, for example, squalene

synthetase would be extrachloroplastidic whereas phytoene synthetase would be chloroplastidic. Secondly, the scheme required the chloroplast membrane to be impermeable to the specific intermediates of the biosynthetic pathway. Such a scheme explained the experimental observations made by these workers. Germinating seeds form MVA from food stores and this is used to produce sterols required for the formation of cellular organelles. The MVA cannot enter the undeveloped plastids to a significant extent and so chloroplast terpenoids are not synthesized until carbon dioxide fixation occurs on illumination of the seedling on its emergence from the soil. At this juncture MVA is rapidly produced inside the chloroplast from carbon dioxide and converted into the plastid terpenoids now needed; little or none leaks out to form extrachloroplastidic sterols, whose synthesis is, at this time, unnecessary.

On this view the chloroplast membrane should be essentially impermeable to mevalonic acid, the first specific terpenoid precursor, and mevalonate kinase (ATP-mevalonate 5-phosphotransferase) and phosphomevalonate kinase (ATP-5-phosphomevalonate phosphotransferase) should exist both inside and outside the chloroplast. The experimental demonstration of this situation forms the subject of the present paper. Part of this investigation has already been briefly reported (Rogers, Shah & Goodwin, 1957).

EXPERIMENTAL

Plant material. *Phaseolus vulgaris* (French bean) plants were grown from seeds in a warm room for 6–8 days and then placed in a dark cabinet in the warm room for 24–36 hr. This treatment depletes the plants of starch; if this is not done, then, on centrifugation of homogenates, starch grains in the

* Abbreviations: MVA, mevalonic acid (3,5-dihydroxy-3-methylvaleric acid); MVA-5P, mevalonic acid 5-phosphate; MVA-5PP, mevalonic acid 5-pyrophosphate; IPP, isopentenyl pyrophosphate.

Scheme 1. Suggested scheme for 'compartmentation' of terpenoid biosynthesis in germinating seedlings.

* Leaf tissue is cut into small pieces, then frozen and freeze-dried. It is then kept under vacuum at -25° for 2 days, then over P₂O₅ in a vacuum desiccator for 24 hr., also at -25° .

Scheme 2. Chloroplast preparation in non-aqueous media.

chloroplasts would be pulled through the lamellae and bounding membranes and break up the chloroplasts. Leaves of *Pelargonium kewensis* and *Hibiscus* sp. were obtained from the College greenhouses.

Isolation of chloroplasts. Intact chloroplasts were prepared by two methods, that of Leech (1964) and a method based on Stocking (1959). Leech's (1964) method employs a discontinuous density gradient of sucrose in phosphate buffer, and that based on Stocking (1959) employs a discontinuous density gradient of carbon tetrachloride and hexane (Scheme 2). In each case the final chloroplast pellet was dispersed in 1.0 ml. of 0.3 M-sucrose in 0.10 M-phosphate buffer, pH 7.4, immediately before use.

Acetone-dried powders. Acetone-dried powders (Loomis, 1959) were prepared in the cold room. Plant-cell cultures of meristematic tissue of Paul's Scarlet Rose (Williams & Goodwin, 1965) were concentrated by centrifugation. The paste-like sediment was added dropwise to cold acetone (-10°) with continuous vigorous stirring. The white powder was filtered off and dried at room temperature before storage in tightly capped jars until use.

Acetone-dried powders of the green and etiolated parts of variegated leaves of *Pelargonium kewensis* and *Hibiscus* sp. were prepared by first vigorously homogenizing small pieces of leaf in acetone at -10° with an Ultra-Turrax homogenizer (Hudes Merchandising Co. Ltd., London, W.1). Subsequent

stages were the same as those described for the plant-tissue cultures.

Protein determinations. The protein content of the buffer extracts of acetone-dried powders was determined by the biuret method of Gornall, Bardawill & David (1949). Crystalline bovine serum albumin was used as a standard. Determination of protein in chloroplast preparations was impracticable because of the small yield and the relatively high proportion of lipid and pigment present.

Detection of enzyme activity. The assay method was based on that used by Markley & Smallman (1961). The substrate was DL-[2-¹⁴C]MVA prepared by dissolving the lactone (The Radiochemical Centre, Amersham, Bucks.) in water, adding a slight excess of KOH and keeping for 30 min. at 37°. The solution (0.1 mc radioactivity) was adjusted to pH 7.4 and made up to 16 ml. with 0.04 M-potassium phosphate buffer, pH 7.4, containing MgSO₄ (0.04 M). The final incubation medium, made up in pointed centrifuge tubes, contained (per tube): KH₂PO₄, 14.0 μmoles; MgSO₄, 4.0 μmoles; sucrose, 120 μmoles; L-cysteine, 6.0 μmoles; ATP, 2.4 μmoles; DL-[2-¹⁴C]MVA, 0.2 μmole (23 100 disintegrations/sec.); protein, 1–2 mg. The medium was adjusted to pH 7.4 before the addition of enzyme (0.1 ml.), and the final volume per incubation tube was 0.4 ml. All incubations were performed at 36° with intermittent shaking. To show whether the chloroplasts were permeable to MVA, some intact preparations were incubated with the assay medium and other preparations were broken by ultrasonic treatment or by suspension of the chloroplasts in assay medium not containing sucrose before incubation.

Investigations to confirm the identity of labelled products were carried out with DL-[1-¹⁴C]MVA as substrate. In this case reactions were carried out in tris-HCl buffer, pH 7.4. The standard incubation mixture used for these studies contained, in a total volume of 1.25 ml.: DL-[1-¹⁴C]MVA, 0.5 μmole; ATP, 6.0 μmoles; MgSO₄, 10.0 μmoles; L-cysteine, 15.0 μmoles; enzyme solution from ultrasonically treated chloroplasts, 0.5 ml. All solutions were made up in 0.05 M-tris-HCl buffer, pH 7.4.

At the end of the incubation the reaction was stopped by immersion of the tubes in a boiling-water bath for 2 min. After centrifuging to remove the precipitated protein, samples of the incubation mixture (usually approx. 0.02 ml.) were transferred to Whatman no. 1 chromatography paper and chromatographed at room temperature in butan-1-ol-formic acid-water (77:10:13, by vol.), isobutyric acid-aq. NH₃ (sp.gr. 0.88)-water (22:1:10, by vol.) or 2-methylpropan-2-ol-formic acid-water (20:5:8, by vol.) (Ohnoki,

Suzue & Tanaka, 1962). The reported *R_F* values of MVA, MVA-5P, MVA-5PP and IPP in these systems have been gathered from several sources for convenience and are summarized in Table 1.

The labelled compounds in the assay medium at the end of incubation were identified from these values.

Radioassay. Radioactive spots were located on dried chromatograms by exposure to Kodak X-ray film for 3–5 days. For quantitative assessment, chromatograms were cut into strips 1.5 in. wide parallel to the length of the paper. The strips were joined and markers of [2-¹⁴C]MVA placed at marked positions between the solvent front on one strip and the origin of the succeeding strip. These aided subsequent alignment of chromatogram strip and recording chart. In a typical experiment the strips were then scanned with a C100 Actigraph automatic chromatogram scanner (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) at a speed of 2 in./min., with a slit width of 1/8 in., an integration time of 10 sec. and a scale setting of 3000 counts/min. The relative radioactivity of each spot was estimated from the area of the peaks on the recording chart.

Finally, selected strips were cut into rectangles 1 1/2 in. × 1 in. and, after being lightly sprayed with N-NaOH, were counted in a Packard Tri-Carb scintillation counter (Packard Instrument Co., La Grange, Ill., U.S.A.) after immersion in scintillation fluid [5 g. of 2,5-diphenyloxazole and 0.3 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l. of toluene] in glass vials.

RESULTS

Preliminary results indicated that buffer-extracted acetone-dried powder of French-bean leaves on incubation with [2-¹⁴C]MVA at pH 6.5 or 7.4 for up to 3 hr. produced one radioactive product, which from its chromatographic behaviour was identified as MVA-5PP. Conversion was greatest at pH 7.4. Acetone-dried powders of cell cultures of Paul's Scarlet Rose, green tissue of the variegated leaves of *Pelargonium kewensis* and *Hibiscus* sp. and white pigmentless leaf tissue of *Hibiscus* sp. gave a similar result. However, extracts of the acetone-dried powder of the white portion of *Pelargonium kewensis* leaves gave a radioactive product that was chromatographically identifiable as MVA-5P.

Intact chloroplasts prepared in a discontinuous sucrose density gradient (Leech, 1964) and incubated for 3 hr. with [2-¹⁴C]MVA exhibited no mevalonate-kinase activity. Activity was, however, found under the same conditions with chloroplasts ruptured by ultrasonic treatment or by osmotic shock. The amount of MVA-5PP formed was slightly less after 15 hr. incubation than after 3 hr. incubation. In one experiment with a weakly active chloroplast preparation both MVA-5P and MVA-5PP were detected. The *R_F* values in three solvents of the metabolites from [1-¹⁴C]MVA and [2-¹⁴C]-MVA and their derived products (see below) are compared with the *R_F* values of MVA-5P and MVA-5PP in these solvents in Table 2. With

Table 1. Reported *R_F* values of MVA, MVA-5P, MVA-5PP and IPP in various solvents

Solvent 1, butan-1-ol-formic acid-water (77:10:13, by vol.); solvent 2, isobutyric acid-aq. NH₃ (sp.gr. 0.88)-water (22:1:10, by vol.); solvent 3, 2-methylpropan-2-ol-formic acid-water (20:5:8, by vol.).

	Solvent 1	Solvent 2	Solvent 3
MVA	0.75	0.65–0.69	0.80–0.85
MVA-5P	0.12–0.18	0.35–0.43	0.53–0.65
MVA-5PP	0–0.03	0.20–0.27	0.29–0.35
IPP	0.30–0.32	—	0.54–0.62

Table 2. R_F values in different solvent systems of the metabolites of MVA and their products after hydrolysis

Experimental details are given in the text. Solvent 1, butan-1-ol-formic acid-water (77:10:13, by vol.); solvent 2, isobutyric acid-aq. NH_3 (sp.gr. 0.88)-water (22:1:10, by vol.); solvent 3, 2-methylpropan-2-ol-formic acid-water (20:5:8, by vol.).

	Solvent 1	Solvent 2	Solvent 3
MVA-5P	0.13-0.17	0.40	0.55
MVA-5PP	0.01-0.03	0.25-0.26	0.35

Fig. 1. Radioautograph of descending chromatogram in butan-1-ol-formic acid-water (77:10:13, by vol.) prepared from the reaction mixture: (a) after incubation of ultrasonically treated 'non-aqueous' chloroplasts with $[2\text{-}^{14}\text{C}]\text{MVA}$ (i); (b) after incubation of intact chloroplasts with $[2\text{-}^{14}\text{C}]\text{MVA}$ for 3 hr. (ii) and 15 hr. (iii); (c) after incubation of ultrasonically treated heat-inactivated chloroplasts with $[2\text{-}^{14}\text{C}]\text{MVA}$ (iv). Experimental details are given in the text. The vertical lines indicate the strips scanned for radioactivity with the Actigraph automatic chromatogram scanner. A, Position occupied by MVA; B, position occupied by MVA-5P; C, position occupied by MVA-5PP.

chloroplasts that were osmotically burst and then incubated with $[2\text{-}^{14}\text{C}]\text{MVA}$, an unidentified product, R_F 0.47 in butan-1-ol-formic acid-water, was observed.

Chloroplasts isolated in 'non-aqueous' media were also examined. As with 'aqueously' isolated chloroplasts intact chloroplasts were found to be inactive in converting $[2\text{-}^{14}\text{C}]\text{MVA}$ into its phosphorylated derivatives even after incubation for

Fig. 2. Radioautograph and scan for radioactivity of the chromatographically separated reaction products obtained by the phosphorylation of MVA by enzymes from chloroplasts isolated in non-aqueous media (a) ultrasonically treated chloroplasts incubated with $[1\text{-}^{14}\text{C}]\text{MVA}$ in tris-HCl buffer, pH 7.4, for 3 hr. at 36° ; (b) ultrasonically treated heat-inactivated chloroplasts incubated with $[1\text{-}^{14}\text{C}]\text{MVA}$ under the same conditions as for (a). Similar traces to (b) were obtained with ultrasonically treated chloroplasts if no ATP was present, or with $[1\text{-}^{14}\text{C}]\text{MVA}$ alone. Enzyme assays were carried out as described in the text and reaction products separated by chromatography in butan-1-ol-formic acid-water (77:10:13, by vol.). Chromatograms were scanned for radioactivity with the Actigraph automatic chromatogram scanner under the conditions described in the Experimental section.

24 hr. However, ultrasonically treated chloroplasts were very active and on radioautography of the chromatogram developed with butan-1-ol-formic acid-water a radioactive spot very near the origin, corresponding to MVA-5PP, was clearly evident in a 3 hr. incubation mixture together with a very slight spot at an R_F characteristic of MVA-5P (Fig. 1).

When sections of this chromatogram were placed in vials containing scintillator fluid and counted in a scintillation counter the counts on areas of the chromatogram corresponding to the positions of radioactive spots were: MVA-5PP, 42350 disintegrations/min.; MVA-5P, 4440 disintegrations/min.; MVA, 187000 disintegrations/min. Only very slight radioactivity was evident with intact chloroplasts even if the chromatogram was substantially overloaded with radioactive material. This radioactivity was so slight that peaks corresponding to MVA-5P and MVA-5PP could not be recorded when strips of the chromatogram were scanned for radioactivity by the method described above. Similarly

the MVA-5P peak is very difficult to detect in the reaction mixture from ultrasonically treated chloroplasts although the MVA-5PP peak is clearly revealed. In the absence of ATP from the incubation medium no conversion of [2- 14 C]MVA into products observable by the methods used occurred.

The approximate extent of conversion of MVA into MVA-5PP in the best 'non-aqueous' chloroplast preparations was 32% (Fig. 2). This represents about 64% of the theoretically possible conversion since only one isomer of DL-MVA is biologically active. The principal metabolite from incubation of [2- 14 C]MVA with ruptured chloroplasts appeared from chromatographic studies to be MVA-5PP. Similar experiments in which [1- 14 C]MVA was the substrate also produced the same metabolite, which was conclusively identified as MVA-5PP by its behaviour when subjected to conditions that might cause hydrolysis (Bloch, Chaykin, Phillips & de Waard, 1959; Williamson & Kekwick, 1965). The reaction mixture after incubation of enzyme solution from ruptured chloroplasts with [1- 14 C]MVA in tris-hydrochloric acid buffer, pH 7.4, was chromatographed in butan-1-ol-formic acid-water. The sample was applied as a band across the width of the chromatography paper, and after chromatography the area of paper containing the metabolite was removed and the metabolite eluted with water into a boiling tube. Elution was followed by scanning the strip for radioactivity at suitable intervals and was continued until no radioactivity remained

Fig. 3. Paper-chromatographic separation and identification of the product obtained on treatment with *n*-HCl at 100° for 7 min. of the metabolite obtained on the phosphorylation of [1- 14 C]MVA by chloroplasts isolated in non-aqueous media: (a) with isobutyric acid-aq. NH_3 (sp.gr. 0.88)-water (20:1:10, by vol.); (b) with 2-methylpropan-2-ol-formic acid-water (20:5:8, by vol.); (c) with butan-1-ol-formic acid-water (77:10:13, by vol.). Subsequent treatment of this hydrolytic product with enzyme solution from chloroplasts in the presence of ATP and MgSO_4 reversed the positions of the two peaks (see also Fig. 4).

on the paper. The resulting eluate was evaporated *in vacuo* over phosphorus pentoxide until the volume was reduced to approx. 1.0 ml. This was divided into three portions (0.25, 0.25 and 0.5 ml.), which were treated with *N*-hydrochloric acid, *N*-sodium hydroxide and calf-intestinal alkaline phosphatase respectively. When the metabolite was heated in *N*-hydrochloric acid at 100° for 7 min. it was converted almost completely into a product whose R_F values in the solvent systems used were characteristic of MVA-5P, and were the same as those values obtained for the minor metabolite formed in some of our investigations (Fig. 3). However, treatment of the metabolite with *N*-sodium hydroxide at 100° for 7 min. produced no detectable change in the R_F of the material on subsequent chromatography. Incubation of enzyme solution from ruptured chloroplasts with the product of acid hydrolysis in tris-hydrochloric acid buffer containing ATP and magnesium sulphate produced a product having the chromatographic characteristics of MVA-5PP (see Fig. 4). When a solution of the metabolite (0.5 ml.) was incubated for 4 hr. with an

Fig. 4. Confirmation of the identity of the principal metabolite obtained when [1- 14 C]MVA was incubated with ultrasonically treated 'non-aqueous' chloroplast preparations. The paper-chromatographic separations indicated are those in butan-1-ol-formic acid-water (77:10:13, by vol.) of: (a) the isolated metabolite; the metabolite after treatment with (b) *N*-HCl at 100° for 7 min.; (c) *N*-NaOH at 100° for 7 min.; (d) alkaline phosphatase at pH 7.6 and 36° for 4 hr.; (e) shows the chromatographic separation obtained when the reaction mixture from (b) was reincubated with ultrasonically treated chloroplasts in the presence of ATP and MgSO_4 . Chromatograms were scanned for radioactivity with the Actigraph automatic chromatogram scanner at a speed of 1.5 in./min., with a slit width of $\frac{1}{8}$ in., an integration time of 2 sec. and a scale setting of 3000 counts/min.

equal volume of 0.1 M-tris-hydrochloric acid buffer, pH 7.6, containing 4mg. of alkaline phosphatase, nearly all the metabolite was converted into a product having the chromatographic characteristics of MVA. The results, summarized in Fig. 4, show conclusively that the metabolites obtained by the action of enzyme solutions from chloroplasts on MVA were MVA-5P and MVA-5PP.

DISCUSSION

Mevalonate kinase catalyses the reaction:

However, with crude enzyme preparations and in the presence of sufficient ATP and Mg^{2+} two further reactions occur:

catalysed by phosphomevalonate kinase and pyrophosphomevalonate decarboxylase (ATP-5-pyrophosphomevalonate carboxy-lyase) respectively. Thus at the end of reaction with the incubation medium used in the present work we might expect to find MVA-5P, MVA-5PP or IPP in varying proportions. In nearly all cases, however, the only detectable product was MVA-5PP, although MVA-5P was observed when a weakly active chloroplast preparation was used; it might have been in evidence more often if less prolonged incubations had been used. These findings were not entirely unexpected; the reaction catalysed by phosphomevalonate kinase has been shown to proceed, in yeast autolysates, far towards formation of MVA-5PP provided that sufficient ATP is present (Bloch *et al.* 1959; Henning, Moslein & Lynen, 1959). Loomis & Battaile (1963) have also commented on the production of MVA-PP as well as MVA-5P by crude enzyme extracts of pumpkin cotyledons.

No IPP was convincingly detected in any of the experiments. Possibly an essential cofactor for the conversion of MVA-5PP into IPP has been leached from the chloroplasts or the equilibrium of the reaction in the systems studied *in vitro* is unfavourable for the formation of IPP. It is also possible that any small amount of IPP formed is rapidly converted into further products not detected by our methods; or, as is known to occur for some enzymes, the pyrophosphomevalonate decarboxylase is inactivated during isolation of chloroplasts in 'non-aqueous' media. As discussed by Leech (1964), the interpretation of biochemical data obtained from isolated chloroplasts in terms of their intracellular function depends on a knowledge of the structural

integrity of the preparations and especially on the absence of other cellular components. Chloroplasts prepared by conventional differential centrifugation techniques would have been valueless for the studies described above because of contamination by other cell organelles, by cytoplasm, and by enzymes leached out of broken organelles. Therefore density-gradient techniques were used. Chloroplasts prepared by the sucrose-density-gradient method have stroma and matrix intact and retain their bounding membranes (Leech, 1964). The final purified chloroplast preparation consists of about 90.5% of intact chloroplasts, 5.0% of chloroplast fragments and 4.5% of mitochondria. Since the preparation is washed several times before examination no extrachloroplastidic mevalonate-activating enzymes nor any released from broken chloroplasts should be still present. This was confirmed by the total inactivity towards MVA of intact chloroplasts prepared by this method. It is worth noting that, although the commonly used method of James & Das (1957), in which glycerol-sucrose density gradients are employed, gives a purer preparation in terms of contamination by other cellular components, it was unsuitable for the present studies because the chloroplasts so prepared are largely devoid of stroma and bounding membranes (Leech, 1964).

The chloroplasts isolated in non-aqueous media have lost the outer (lipid) membrane of the chloroplast and some of the chloroplast pigments (Stocking, 1959). However, the loss of soluble protein is far less than for chloroplasts prepared with aqueous media. The main difficulties associated with the method are the possible inactivation of some enzymes and the contamination through adsorption to the chloroplast of enzymes of cytoplasmic origin. However, even were the latter to occur the exposed enzymes would in all probability be inactivated through prolonged contact with organic solvents. Since the dry chloroplast pellet is suspended in buffer before incubation with $[2\text{-}^{14}\text{C}]\text{MVA}$ it is likely that some enzyme leached from structurally damaged chloroplasts would be present in preparations not treated with ultrasonics. This would explain the very slight activity shown by these preparations (Fig. 1). Our results confirm the reported structural integrity of chloroplasts prepared by this method, and the experiments described above are now used as a routine in this Department in studies of terpenoid biosynthesis as a test of the structural integrity of chloroplast preparations and their enzymic activity.

Our studies showed mevalonate kinase and phosphomevalonate kinase to be present in chloroplasts prepared by both the techniques used. The fact that intact chloroplasts from both methods were inactive in converting MVA into MVA-5P, but became active

on rupture, indicates that the chloroplast membrane is impermeable to MVA. The unknown product obtained from 'aqueously prepared' osmotically ruptured chloroplasts in addition to MVA-5PP is possibly a degradation product of IPP.

The presence of an extrachloroplastidic mevalonate kinase in addition to a chloroplastidic enzyme is difficult to demonstrate unequivocally. One cannot use cell homogenates from which chloroplasts have been spun off as the enzyme may be easily leached from broken or damaged chloroplasts. However, we have indicated the presence of extrachloroplastidic mevalonate-activating enzymes by demonstrating enzymic activity in acetone-dried powders of cultures of meristematic tissue of Paul's Scarlet Rose and in acetone-dried powders of the white pigmentless portions of variegated leaves of *Pelargonium* and *Hibiscus*. These tissues have no chloroplasts. Supporting evidence is the finding of Loomis & Battaile (1963) that mevalonate kinase occurs in the cytoplasm of cotyledons of pumpkin seedlings grown in the dark.

An understanding of the role of mevalonate-activating enzymes in plant cell metabolism depends partly on a knowledge of the intracellular sites in which they occur. The work described demonstrates their presence both inside and outside the chloroplast, and shows that the chloroplast membrane is impermeable to MVA. The findings support the view that a combination of 'compartmentation' of enzymes and specific membrane permeability may be one of the primary ways by which the regulation of terpenoid biosynthesis in the developing seedlings is achieved.

We thank Dr R. M. Leech, Department of Botany, Imperial College, London, and Dr K. J. Treharne of this Department for helpful discussions of various aspects of

this work. This work was supported by a grant from the Scientific Research Council, which we gratefully acknowledge.

REFERENCES

- Bloch, K., Chaykin, S., Phillips, A. H. & de Waard, A. (1959). *J. biol. Chem.* **234**, 2595.
 Goodwin, T. W. (1958a). *Biochem. J.* **68**, 26p.
 Goodwin, T. W. (1958b). *Biochem. J.* **70**, 612.
 Goodwin, T. W. (1965). In *Biosynthetic Mechanisms in Higher Plants*, p. 57. Ed. by Pridham, J. B. & Swain, T. London: Academic Press (Inc.) Ltd.
 Goodwin, T. W. & Mercer, E. I. (1963). *Symp. biochem. Soc.* **24**, 37.
 Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
 Griffiths, W. T., Threlfall, D. R. & Goodwin, T. W. (1964). *Biochem. J.* **90**, 40p.
 Henning, U., Moslein, E. M. & Lynen, F. (1959). *Arch. Biochem. Biophys.* **83**, 259.
 James, W. O. & Das, V. S. R. (1957). *New Phytol.* **56**, 325.
 Leech, R. M. (1964). *Biochim. biophys. Acta*, **79**, 637.
 Loomis, W. D. (1959). *Plant Physiol.* **34**, 541.
 Loomis, W. D. & Battaile, J. (1963). *Biochim. biophys. Acta*, **67**, 54.
 Markley, K. & Smallman, E. (1961). *Biochim. biophys. Acta*, **47**, 327.
 Mercer, E. I. & Goodwin, T. W. (1962). *Biochem. J.* **85**, 13p.
 Mercer, E. I. & Goodwin, T. W. (1963). *Biochem. J.* **88**, 46p.
 Ohnoki, S., Suzue, G. & Tanaka, S. (1962). *J. Biochem., Tokyo*, **52**, 423.
 Rogers, L. J., Shah, S. P. J. & Goodwin, T. W. (1965). *Biochem. J.* **96**, 7p.
 Stocking, C. R. (1959). *Plant Physiol.* **34**, 56.
 Treharne, K. J., Mercer, E. I. & Goodwin, T. W. (1964). *Biochem. J.* **90**, 39p.
 Williams, B. L. & Goodwin, T. W. (1965). *Phytochemistry*, **4**, 81.
 Williamson, I. P. & Kekwick, R. G. O. (1965). *Biochem. J.* **96**, 862.