Mevalonate Kinase in Green Leaves and Etiolated Cotyledons of the French Bean *Phaseolus vulgaris*

By J. C. GRAY and R. G. O. KEKWICK

Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

(Received 4 December 1972)

1. Partially purified preparations of mevalonate kinase were obtained from green leaves and etiolated cotyledons of *Phaseolus vulgaris*. 2. After removal of interfering polyphenols both enzyme preparations behaved identically on gel filtration, ion-exchange chromatography and density-gradient centrifugation. 3. The kinetic parameters of the preparations from the two sources were indistinguishable. The preparation from etiolated cotyledons had a K_m of 4.26×10^{-5} M for *RS*-mevalonate and 1.54×10^{-3} M for ATP. The preparation from green leaves had a K_m of 4.55×10^{-5} M for *RS*-mevalonate and 1.75×10^{-3} M for ATP. The pH optimum of both enzyme preparations was pH 7.0. 4. The effect of inhibitors on the two enzyme preparations was similar, both being inhibited by reagents known to react with thiol groups, and the two preparations had similar inhibitor constants for competitive inhibition by prenyl pyrophosphates. 5. The molecular weight of the enzyme in both preparations was estimated to be 100000; the enzymes from the two preparations had similar mobilities on polyacrylamide-gel electrophoresis.

Interest in the nature and intracellular location of mevalonate kinase (ATP-mevalonate 5-phosphotransferase, EC 2.7.1.36) has been stimulated by the original observations of Goodwin (1958) and Mercer & Goodwin (1962), who found that, in conditions in which $^{14}\text{CO}_2$ was incorporated into β -carotene and phytol by illuminated maize seedlings, no incorporation of $[2-^{14}\text{C}]$ mevalonate occurred. $[2-^{14}\text{C}]$ -Mevalonate was, however, incorporated into sterols in these conditions. Rogers *et al.* (1966a) demonstrated that, although isolated chloroplasts were impermeable to mevalonate, the presence of mevalonate kinase could be demonstrated on disruption. On the basis of these and other experiments (Treharne *et al.*, 1966) it was suggested by Threlfall *et al.* (1967) that there were within the cells of the green leaf two distinct sites of terpenoid biosynthesis, one inside and the other outside the chloroplast. Evidence for the existence of two isoenzymic forms of mevalonate kinase in the green leaf of *Phaseolus vulgaris*, one inside and the other outside the chloroplast, was obtained by Rogers *et al.* (1966b). When the total mevalonate 5-phosphate and mevalonate 5-pyrophosphate formed from mevalonate was assayed it was found that, whereas the optimum activity of the enzyme of etiolated cotyledons was at pH 5.5, that of the chloroplast preparations was at pH 7.5. The green leaves showed optima of activity at both pH 5.5 and pH 7.5 and the etiolated leaves had an activity with an optimum at pH 5.5 which predominated over that at pH 7.5.

The relatively acidic pH optimum found for the preparation from the etiolated cotyledons is in contrast to that found for the mevalonate kinase of

Hevea brasiliensis latex (Williamson & Kekwick, 1965), pig liver (Levy & Popják, 1960) and rabbit liver (Markley & Smallman, 1961), although preparations of mevalonate kinase from plant sources have been described from *Cucurbita pepo* cotyledons (Loomis & Battaile, 1963), *Pinus radiata* seedlings (Valenzuela *et al.*, 1966) and orange juice vesicles (Potty & Bruemmer, 1970) which have acid pH optima.

A further investigation of the nature of the mevalonate kinase of *Phaseolus vulgaris* was therefore of interest, particularly because the presence of a characteristic chloroplast mevalonate kinase might be of interest in relation to the development of terpenoid biosynthesis in the greening plastid. An attempt was therefore made to characterize and to compare mevalonate kinase preparations from the etiolated cotyledons and green leaves of the French bean *Phaseolus vulgaris*. Part of this work is the subject of a preliminary communication (Gray & Kekwick, 1969).

Materials and Methods

Plant material

Seed of *Phaseolus vulgaris* cv. Canadian Wonder was obtained from Sutton and Sons Ltd., Reading, Berks., U.K. Seeds were surface-sterilized with aq. 10% (v/v) sodium hypochlorite before planting in trays of vermiculite, supplied by Vermipeat Ltd., Bitton, Bristol, U.K. Etiolated cotyledons were obtained from plants grown in darkness for 4-5 days at 20°C with regular watering. Cotyledons were harvested under a dim green safe-light. Green leaves were obtained from plants grown under natural

illumination for 10–12 days at 20°C with regular watering.

Leaves of spinach beet, *Beta vulgaris*, were obtained from plants grown outside at the University Botanical Gardens.

Special chemicals

DL-Mevalonic acid lactone was obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. DL-[2-¹⁴C]Mevalonic acid lactone was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The specific radioactivity of [2-¹⁴C]mevalonic acid lactone was adjusted to the required value by the addition of the appropriate amount of the unlabelled substrate, and the lactone ring was opened by incubation with a 2% molar excess of KOH for 30 min at 37°C. ATP, NADP⁺, ribose 5-phosphate and glucose 6-phosphate were obtained from Sigma (London) Ltd. The nucleotides, ADP and ITP, were obtained from Boehringer Corporation (London) Ltd., London W5 2TZ, U.K. CTP, GTP, UTP and dithiothreitol were obtained from Calbiochem Ltd., London W1H 1AS, U.K. The reagents required for the spectrophotometric assay, i.e. NAD⁺, NADH, phosphoenolpyruvate, rabbit muscle lactate dehydrogenase (EC 1.1.1.27, specific activity 360 enzyme units/mg of protein) and rabbit muscle pyruvate kinase (EC 2.7.1.40, specific activity 150 enzyme units/mg of protein) were all obtained from Boehringer Corporation (London) Ltd. Geranyl pyrophosphate, farnesyl pyrophosphate, geranylgeranyl pyrophosphate and phytyl pyrophosphate were prepared as described by Gray & Kekwick (1972). Mevalonate 5-phosphate and isopentenyl pyrophosphate were prepared as described by Skilleter & Kekwick (1967). Salmine sulphate was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Sephadex, DEAE-Sephadex and Blue Dextran 2000 were obtained from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Of the proteins used as markers for determination of molecular weight by gel filtration, bovine serum albumin, bovine haemoglobin, acid-modified horse heart cytochrome *c* and ovalbumin were obtained from Sigma (London) Ltd., horse myoglobin was obtained from British Drug Houses Ltd., and pig heart malate dehydrogenase (EC 1.1.1.37, sp. enzyme activity 1100 units/mg of protein), horse liver alcohol dehydrogenase (EC 1.1.1.1, 2.7 enzyme units/mg of protein) and rabbit muscle lactate dehydrogenase were obtained from Boehringer Corporation (London) Ltd.

Enzyme assays

Mevalonate kinase activity. The two procedures used were essentially similar to those previously described (Williamson & Kekwick, 1965). A radio-

chemical assay was most generally used in this investigation. Standard incubation mixtures contained: DL-[2-¹⁴C]mevalonate (0.5 μmol, sp. radioactivity 0.1 Ci/mol), ATP (5 μmol), [MgCl₂ (5 μmol), potassium phosphate buffer, pH 7.0 (50 μmol of phosphate) and a suitable amount of enzyme protein in a total volume of 1.0 ml. Incubations, which were carried out for 30 min at 30°C unless otherwise stated, were terminated by the addition of 1.0 ml of ethanol; protein was precipitated by heating to 80°C and was removed by centrifugation. The supernatant was freeze-dried and redissolved in 0.2 ml of water; a portion of this solution (50 μl) was then subjected to chromatography on Whatman no. 1 paper in ethanol–aq. NH₃ (sp.gr. 0.88)–water (7:2:1, by vol.). After development the radioactive compounds were detected by radioautography; the radioactivity was assayed and the enzyme activity calculated as previously described (Skilleter & Kekwick, 1971).

The spectrophotometric method could be used only with partially purified preparations and was used to determine *K_m* values. The concentrations of substrates and MgCl₂ were varied and are given in the text. The coupled system for assay of ADP contained KCl (40 μmol), phosphoenolpyruvate (4 μmol), NADH (0.3 μmol), lactate dehydrogenase (50 μg of protein, specific activity 360 units/mg of protein), pyruvate kinase (20 μg of protein, 150 units/mg of protein), and potassium phosphate buffer, pH 7.0 (50 μmol of phosphate), in a total volume, including mevalonate kinase, substrates and MgCl₂, of 1.0 ml. The incubations were carried out at 30°C and the decrease in *E*₃₄₀ was followed in a recording spectrophotometer. Control incubations containing no mevalonate were included in each assay.

Ribulose diphosphate carboxylase (EC 4.1.1.39). The activity of this enzyme was assayed by the radiochemical measurement of ¹⁴CO₂ fixation. Incubations contained Tris–HCl buffer, pH 7.8 (50 μmol), MgCl₂ (5 μmol), NaH¹⁴CO₃ (10 μmol; sp. radioactivity 0.2 μCi/μmol), ribulose diphosphate (0.2 μmol), β-mercaptoethanol (5 μmol), together with a suitable sample of the material for assay in a total volume of 1.0 ml. Ribulose diphosphate required for the assay was synthesized enzymically from ribose 5-phosphate by using ribose phosphate isomerase (EC 5.3.1.6) prepared by the procedure of Hurwitz *et al.* (1956) and phosphoribulokinase (EC 2.7.1.19) prepared by the method of Racker (1957); the product was isolated as the barium salt after chromatography on Dowex 1 (chloride form) and was converted into the sodium salt by the method of Rabin & Trown (1964).

Malate dehydrogenase. This enzyme was assayed spectrophotometrically by following the oxidation of NADH by the decrease in *E*₃₄₀. Incubations contained sodium phosphate buffer, pH 7.0 (100 μmol), sodium oxaloacetate (4 μmol) and NADH (0.3 μmol), to-

gether with the material to be assayed in a total volume of 2.0 ml.

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49). This enzyme was also assayed spectrophotometrically by following the increase in E_{340} due to NADPH. Incubations contained Tris-HCl buffer, pH 7.8 (100 μ mol), $MgCl_2$ (5 μ mol), $NADP^+$ (4 μ mol) and glucose 6-phosphate (4 μ mol) together with a sample of the material to be assayed in a total volume of 2.0 ml.

Units of enzyme activity. All enzyme activities are expressed as units defined as μ mol of substrate converted into product/min under the standard conditions specified.

Other determinations

Protein. The protein content of enzyme preparations was assayed by the biuret method of Gornall *et al.* (1949). The protein content of column eluates was assayed by measuring the E_{280} .

Chlorophyll. The method of Arnon (1949) was used to assay the chlorophyll content of leaf preparations.

Chloroplast preparations

For aqueous preparations from *Phaseolus vulgaris*, 10-day-old dark-grown seedlings were illuminated for 24 h with three 40 W Mazda universal fluorescent white tubes supplemented with six 15 W incandescent light bulbs at a distance of 40 cm. The leaves were then harvested and homogenized in 4 vol. of a solution containing 0.3 M-sucrose, 0.1 M-Tris-HCl buffer, pH 7.6, 2 mM-EDTA and 2 mM-sodium D-iso-ascorbate for 5 s in an MSE top-drive homogenizer. The resulting slurry was poured through twelve layers of surgical gauze, and the filtrate was centrifuged at 2500g for 1 min. After decanting the supernatant the small chloroplast pellet was suspended in a solution containing 0.1 M-Tris-HCl buffer, pH 7.8, 5 mM- $MgCl_2$ and 5 mM-mercaptoethanol.

Beta vulgaris chloroplasts were prepared from green leaves by the procedure of Ramirez *et al.* (1968). The green leaves (60 g fresh wt.) were de-ribbed and torn into 1 cm strips before homogenization for 5 s in an MSE Ato-Mix homogenizer in 200 ml of a solution containing 0.35 M-sucrose, 25 mM-Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer, pH 7.6, 2 mM-EDTA and 2 mM-sodium D-iso-ascorbate. The subsequent stages in the preparation were the same as for the aqueous bean chloroplast preparations.

Non-aqueous preparations of *Phaseolus vulgaris* chloroplasts were prepared by the method of Rogers *et al.* (1966a) from primary leaves of 10-day-old plants. Dark-grown plants were illuminated for 36 h and then placed in the dark for 18 h before use. The leaves were gathered in a room at -20°C , frozen on

solid CO_2 and freeze-dried. The freeze-dried leaves (1 g) were then homogenized in 40 ml of a CCl_4 -hexane mixture (adjusted to sp. gr. 1.35) in a MSE top-drive homogenizer and were subsequently ground in a mortar. The filtrate obtained by straining the macerated leaves through 12 layers of surgical gauze was poured into a centrifuge tube, overlaid with a CCl_4 -hexane mixture (sp. gr. 1.32) and centrifuged at 3000g for 15 min. The green top layer and the debris at the bottom were retained for enzyme assay. Material for enzyme assay was obtained from the upper layer and from a portion of the original homogenate by diluting with an equal volume of hexane and centrifuging at 3000g for 15 min; the green pellets thus obtained were dried under N_2 before suspending in the assay medium.

Gel filtration

The enzyme preparations were subjected to gel filtration at 4°C on Sephadex G-200 during the course of purification, and their molecular weights and diffusion coefficients were estimated from the elution volumes of the partially purified preparations from both Sephadex G-100 and G-200 also determined at 4°C . The procedure for estimating molecular weight was essentially that of Andrews (1965). Sephadex G-100 (lot No. 226) was packed in a column 60 cm $\times$ 2.5 cm and Sephadex G-200 (lot no. 1680) was packed in a column 90 cm $\times$ 2.5 cm. Preparations of mevalonate kinase were dissolved together with the marker enzymes, which were selected from lactate dehydrogenase (mol. wt. 135000; Jaenicke & Knof, 1968), horse liver alcohol dehydrogenase (mol. wt. 83000; Ehrenberg & Dalziel, 1958), bovine serum albumin (mol. wt. 65000; Creeth, 1952), pig heart malate dehydrogenase (mol. wt. 50000; Siegel & England, 1961), ovalbumin (mol. wt. 45000; Kegeles & Gutter, 1951), cytochrome *c* (mol. wt. of tetramer 48000, trimer 36000, dimer 24000; Margoliash & Lustgarten, 1962) and myoglobin (mol. wt. 17000; van den Oord *et al.*, 1969), in 0.08 M-potassium phosphate buffer, pH 7.0, in a total volume of 2 ml. The protein was eluted from the column with the same buffer in 3 ml fractions at 15–20 ml/h. The void volume of the column was obtained by measuring the elution volume of Blue Dextran 2000 and the total volume by measuring the elution volume of 2 M-KCl. The elution volumes of the proteins were estimated to the nearest 1 ml from the elution profiles.

Centrifugation on a sucrose-density gradient

The method of Martin & Ames (1961) was used to determine the sedimentation coefficients and molecular weights of the mevalonate kinase present in the two enzyme preparations. Samples of the enzyme preparations together with three or four marker proteins (as used for gel filtration) were layered on to a

linear gradient of 5–20% (w/v) sucrose in 0.08M-potassium phosphate buffer, pH 7.0, and were centrifuged at 135000g for 18 h in a 3 × 5 ml swing-out rotor in an MSE Superspeed 50 centrifuge. At the conclusion of the run each centrifuge tube was pierced at the bottom and 25 fractions were collected. Each fraction was diluted to 1.0 ml with 0.08M-potassium phosphate buffer, pH 7.0, before assay for mevalonate kinase or marker protein. Molecular weights and sedimentation coefficients were estimated by interpolation on graphs of the number of the peak fraction containing activity or protein plotted against the known molecular weights or sedimentation coefficients of the marker proteins.

Polyacrylamide-gel electrophoresis

Disc electrophoresis was carried out in a polyacrylamide gel by the method of Davis (1964). The resolving gel contained 7% (w/v) acrylamide, the tracking dye was Bromophenol Blue, and a current of 3 mA per gel was applied for about 3 h. Protein was stained with Amido Schwartz [1% (w/v) in aq. 7% (v/v) acetic acid] and assays for mevalonate kinase activity were carried out on 2 mm slices of gel by the radiochemical assay.

Results

Purification of the enzymes

The requirement for purification of the enzyme from etiolated cotyledons was that the phosphatase found to contaminate the crude preparations should be removed to give a preparation suitable for kinetic studies. The problem with the crude preparation from green leaves was not so much the phosphatase, although there was some present, as can be seen in Table 1, but rather the contaminating polyphenols. Although ATPase* activity is used as the measured index of phosphatase activity in the brei supernatants from both green leaves and etiolated cotyledons, these preparations also hydrolysed mevalonate 5-phosphate, mevalonate 5-pyrophosphate and isopen-tenyl pyrophosphate at rates considerably in excess of mevalonate 5-phosphate formation. Initially, purified preparations from green leaves were found to behave as heterogeneous mixtures of mevalonate kinase enzymes on chromatography on DEAE-Sephadex. As described below, it was shown that this apparent heterogeneity could be ascribed to the interaction of the contaminating polyphenols with the

* Abbreviation: ATPase, adenosine triphosphatase.

Table 1. *Purification of mevalonate kinase from etiolated cotyledons and green leaves*

Enzyme activities were measured by the radiochemical procedure.

Purification stage	Total activity of mevalonate kinase (units)	Total protein (mg)	$10^3 \times$ Specific activity of mevalonate kinase (units/mg of protein)	Total activity of ATPase (units)	$10^3 \times$ Specific activity of ATPase (units/mg of protein)
Etiolated cotyledons					
1. Brei supernatant	2.760	2927	0.94	260	89
2. 20–45% satd.- (NH ₄) ₂ SO ₄ precipitate	1.170	337	3.48	41	122
3. Elution from Sephadex G-200	1.390	80	17.3	9.7	121
4. Elution from DEAE-Sephadex	1.360	15	89.0	0.3	20
Green leaves					
1. Brei supernatant	1.300	1330	0.98	95	71.5
2. Salmine sulphate supernatant	1.420	1050	1.34	98.5	94
3. 0–60% satd.- (NH ₄) ₂ SO ₄ precipitate	0.880	750	1.17	47.7	63.5
4. Elution from Sephadex G-200	0.880	160	5.50	7.5	44
5. Elution from DEAE-Sephadex	0.883	41	21.5	0.27	6.6

enzyme, giving a number of molecular species carrying different charges. Care had therefore to be taken to remove polyphenols from the preparation and to prevent the formation of quinones from any polyphenols that were present in the preparation.

Etiolated cotyledons. To minimize the amount of the contaminating phosphatase activity it was essential to start with fresh preparations obtained from macerated cotyledons rather than from the acetone-dried powder previously used (Rogers *et al.*, 1966b).

Cotyledons were macerated in a domestic vegetable grater and the brei was suspended in an equal volume of 0.08M-potassium phosphate buffer, pH7.0. The debris were removed by straining the preparation through surgical gauze and the extract was centrifuged at 105000g for 1.5h. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to give a 20%-satd. solution, resulting in the precipitation of 4.4% of the protein present in the original supernatant; this precipitate, which contained 1% of the enzyme activity, was discarded.

When further $(\text{NH}_4)_2\text{SO}_4$ was added to give a concentration of 45% saturation, 61% of the activity present in the original supernatant was precipitated together with 17% of the protein; this precipitate

contained only 19% of the ATPase activity present in the supernatant of the extract. This precipitate was dissolved in 0.08M-potassium phosphate buffer, pH7.0; when this solution was applied to a Sephadex G-200 column (90cm $\times$ 2.5cm) previously equilibrated with the same buffer, the mevalonate kinase and phosphatase activities were separated, most of the ATPase activity being eluted in the void volume (Fig. 1a). When the fractions containing mevalonate kinase were applied to a DEAE-Sephadex (A-50) column (12cm $\times$ 1.5cm) previously equilibrated with the same buffer the enzyme was eluted by a linear gradient of increasing KCl concentration at concentrations of 0.04 to 0.12M (Fig. 2a). The specific activity of the ATPase present in this final product was about 25% of that of the starting material and 22% of that of the mevalonate kinase present (Table 1). This material was used for the characterization of the enzyme from etiolated cotyledons.

Green leaves. The objects of this purification were to obtain active preparations relatively free of polyphenols and with minimal phosphatase activity. Fresh green leaves were cut into small pieces about 1cm² and were vacuum-infiltrated with 0.08M-potassium phosphate buffer, pH7.0, containing

Fig. 1. Gel filtration on Sephadex G-200 of $(\text{NH}_4)_2\text{SO}_4$ fractions containing mevalonate kinase activity

(a) The 20–45%-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate from etiolated cotyledon preparations; (b) the 0–60%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction from green leaf preparations; ○, protein concentration; ●, mevalonate kinase. For details see the Materials and Methods section.

Fig. 2. Elution of mevalonate kinase preparations from DEAE-Sephadex

(a) Etiolated cotyledon; (b) green leaf; $\circ$, protein concentration; $\bullet$, mevalonate kinase; ----, KCl gradient. For details see the Materials and Methods section.

Fig. 3. Effect of pH on the activity of the two mevalonate kinase preparations

Incubations contained the standard radiochemical assay mixture except for the buffer, which was: pH 4.0–5.5, potassium acetate–phosphate; pH 6.0–7.0, potassium phosphate; pH 7.5–9.0, Tris–potassium phosphate. $\bullet$, Cotyledon enzyme; $\circ$, leaf enzyme.

10 mM-sodium metabisulphite, 1 mM-KCN and 0.1% (w/v) bovine serum albumin. The leaves were then ground in a mortar with sand in 3 vol. of the modified phosphate buffer also containing Dowex 1 (X10) resin (20%, w/v) previously equilibrated with the modified phosphate buffer. The brei thus produced was strained through gauze and the extract centrifuged at 105 000g for 1 h. Inactive protein was then removed by the addition of salmine sulphate (2%, w/v) in 0.08 M-phosphate buffer, pH 7.0, to give a final concentration of 0.1%. The dark-brown precipitate left a supernatant which contained very little polyphenol and which had slightly more mevalonate kinase activity than the original extract. The enzyme activity was precipitated from this supernatant by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 60% saturation. The active precipitate was then dissolved in 0.08 M-potassium phosphate buffer, pH 7.0, and applied to a Sephadex G-200 column (90 cm $\times$ 2.5 cm), and was eluted by the phosphate buffer in a volume similar to that for the enzyme from the cotyledon extract (Fig. 1b). The active material was then applied to a DEAE-Sephadex column (12 cm $\times$ 1.5 cm) equilibrated with 0.08 M-potassium phosphate buffer, pH 7.0. The enzyme was eluted by applying a linear gradient of increasing KCl concentration to 0.5 M; as with the cotyledon extract the enzyme was eluted at KCl concentration of 0.04–0.12 M, as shown in Fig. 2(b).

Fig. 4. Effect of (a) mevalonate and (b) ATP on the activities of the cotyledon and leaf enzyme preparations. Enzyme activities were assayed by the spectrophotometric procedure. ●, Cotyledon enzyme; ○, leaf enzyme.

Table 2. Nucleotide specificity of the mevalonate kinase preparations

Enzyme activities were measured by the radiochemical procedure. Each incubation contained nucleotide (2mM) and enzyme protein (45 μg of protein from cotyledon preparation, 220 μg of protein from leaf preparation).

Nucleotide	Enzyme activity (nmol of mevalonate 5-phosphate formed/min)	
	Etiolated-cotyledon preparation	Green- leaf preparation
ATP	3.14	3.44
CTP	0.17	0.21
GTP	0.29	0.34
UTP	0.54	0.65
ITP	1.44	1.68
ADP	0.58	0.45

Kinetic properties of the two enzyme preparations

pH-dependence of enzyme activity. When the rates of phosphorylation of mevalonate by the two enzymes were compared over the pH range 4.0–9.5 by the radiochemical procedure no differences could be detected. As shown in Fig. 3 both preparations showed maximum activity between pH6.5 and pH7.5. When the enzyme from etiolated cotyledons was assayed over the same pH range by the spectrophotometric procedure a similar pH-activity curve was obtained.

Variation of reaction rate with substrate concentration. Measurement of the rate of reaction catalysed by the two enzyme preparations at constant ATP and different mevalonate concentrations and at constant mevalonate and different ATP concentrations showed a linear relationship between $1/v$ and $1/s$ for

Fig. 5. Effect of Mg²⁺ and Mn²⁺ on the activities of the two enzyme preparations

Enzyme activities were assayed by the radiochemical procedure under standard conditions except for the metal ion concentration which was varied as indicated. ●, Cotyledon enzyme; ○, leaf enzyme; ---, Mg²⁺; —, Mn²⁺.

both preparations (Fig. 4); there was therefore no detectable kinetic evidence of heterogeneity of the two enzyme preparations. Both preparations showed slight inhibition at high mevalonate concentration. The K_m values for each substrate were almost identical for the two preparations. The etiolated-cotyledon preparation had a K_m for *RS*-mevalonate of

4.26×10^{-5} M and that of leaf preparation for the same substrate was 4.55×10^{-5} M. The K_m values for ATP were: etiolated-cotyledon preparation, 1.54×10^{-3} M, green-leaf preparation, 1.75×10^{-3} M. The maximum velocities were attained at 5×10^{-4} M-mevalonate and 6×10^{-3} M-ATP with both the preparations. Both preparations were inhibited by mevalonate 5-phosphate and very slightly by ADP. Inhibition of the leaf enzyme by these substances is shown in Fig. 6.

Nucleotide specificity. The nucleotide specificity of the two enzyme preparations was compared by the radiochemical assay procedure. As shown in Table 2, ATP was by far the most effective phosphate donor for both enzyme preparations, but both preparations could utilize all the nucleotides tested and there was no detectable difference in their specificity patterns. When the activity of the cotyledon enzyme preparations was examined at different concentrations of ITP it was found that the affinity of the enzyme for this substrate was less than that for ATP ($K_m = 2.0$ mM).

Metal ion activation. Both enzyme preparations showed a requirement for bivalent metal ions; either Mg^{2+} or Mn^{2+} was satisfactory. High concentrations of Mn^{2+} were inhibitory, as shown in Fig. 5, the optimum activity being observed at 2 mM- Mn^{2+} . Maximum activity was observed with 9 mM- Mg^{2+} .

Sensitivity to thiol reagents. The two enzyme preparations behaved similarly with respect to this class of reagent. Neither preparation was activated by thiols such as dithiothreitol (1 mM), β -mercaptoethanol (10 mM) or *N*-acetylcysteine (10 mM). Although *p*-chloromercuribenzoate (1 mM) produced almost total inhibition of both preparations, as also did $CuSO_4$ (10 mM), iodoacetamide produced a slight activation (20% at 10 mM). A somewhat unusual stimulation of the activity of both preparations was observed with sodium arsenite (10 mM). It did not seem likely that this effect was due to decreased residual phosphatase activity, since the latter was insensitive to 20 mM-sodium arsenite.

Inhibition by prenyl pyrophosphates. Mevalonate kinase preparations from both animal and plant sources are inhibited by prenyl pyrophosphates (Dorsey & Porter, 1968; Flint, 1970; Gray & Kekwick, 1972). The inhibition of the mevalonate kinase preparation from etiolated cotyledons of the French

bean has been described (Gray & Kekwick, 1972); the inhibitor constants, K_i , for the inhibition of mevalonate kinase preparations from *Phaseolus* green leaves by geranyl pyrophosphate, farnesyl pyrophosphate, geranylgeranyl pyrophosphate and

Fig. 6. Inhibition of leaf mevalonate kinase by intermediates of isoprenoid biosynthesis

Enzyme activity was determined by radiochemical assay under standard conditions with the addition of the following compounds at the concentration indicated: mevalonate 5-phosphate, ▼; ADP, □; isopentenyl pyrophosphate, ■; geranyl pyrophosphate, ●; farnesyl pyrophosphate, ○; geranylgeranyl pyrophosphate, △; phytyl pyrophosphate, ▲.

Table 3. Inhibitor constants for the inhibition of the two mevalonate kinase preparations by prenyl pyrophosphates

Inhibitor constants were obtained as described by Gray & Kekwick (1972).

Enzyme preparation	K_i (μ M)			
	Geranyl pyrophosphate	Farnesyl pyrophosphate	Geranylgeranyl pyrophosphate	Phytyl pyrophosphate
Cotyledon	30.8	7.1	48.6	3.6
Leaf	32.5	7.5	48.0	3.5

phytyl pyrophosphate for the green-leaf preparation did not differ appreciably from those of the cotyledon preparation (Table 3 and Fig. 6). Isopentenylpyrophosphate did not appreciably inhibit mevalonate kinase activity in preparations from etiolated cotyledons or green leaves.

Molecular properties of the two enzyme preparations

Molecular size. An attempt was made to differentiate between the cotyledon and green-leaf enzyme preparations on the basis of their molecular parameters estimated by gel filtration in Sephadex G-100 and G-200, and by centrifugation in a sucrose-density gradient. The partition coefficients, K_D , for the enzyme activity in the two preparations were indistinguishable on both Sephadex G-100 and G-200. Estimates of molecular weights obtained by the procedure of Andrews (1965) gave values of 97000–104000 for both preparations. Estimates of the diffusion coefficients by gel filtration (Laurent & Killander, 1964) gave values of 5.4×10^{-7} – 5.5×10^{-7} cm²/s for both preparations. Sedimentation coefficient estimates obtained by centrifugation in a

Fig. 7. Electrophoresis of the two mevalonate kinase preparations in polyacrylamide gel at pH 8.3

(a) Cotyledon enzyme; (b) leaf enzyme. Samples of purified cotyledon enzyme (50 μ g) and purified leaf enzyme (100 μ g) were applied to the respective gels. Distances migrated are expressed relative to the distance moved by the Bromophenol Blue tracking dye.

Fig. 8. Effect of the presence of polyphenols on the elution of mevalonate kinase from DEAE-Sephadex

(a) Chromatography of leaf enzyme. The enzyme, extracted from leaves by homogenization in 0.08 M-potassium phosphate buffer, pH 7.0, containing 10 mM-sodium metabisulphite, was partially purified by precipitation at 0–60% saturated $(\text{NH}_4)_2\text{SO}_4$ and by gel filtration on Sephadex G-200, before application to the DEAE-Sephadex column. (b) Chromatography of cotyledon enzyme after treatment with a polyphenol preparation. Cotyledon enzyme eluted from Sephadex G-200 was treated at 4°C for 2 h with an ethanol extract of a quantity of leaves which would contain the mevalonate kinase activity present in the cotyledon preparation. The treated enzyme preparation was then dialysed overnight against 0.08 M-potassium phosphate buffer, pH 7.0, before application to the DEAE-Sephadex column. —, E_{280} ; ---, KCl gradient; ●, enzyme activity.

sucrose-density gradient by the procedure of Martin & Ames (1961) gave a value of 5.9S for both preparations. Substitution of these values of the diffusion coefficients and sedimentation coefficients, together with an assumed value of the partial specific volume of $0.73\text{ cm}^3/\text{g}$, in the Svedberg equation gave a value of the molecular weight of the enzyme in both preparations of approx. 100 000.

Charge on the enzymes. As shown in Fig. 2 the enzyme activity of both preparations was eluted at 0.06 M-KCl in $0.08\text{ M-potassium phosphate buffer, pH 7.0}$, suggesting that the enzyme has about the same charge in both preparations. Further attempts to distinguish the enzymes from the two preparations on the basis of charge, by electrophoresis in polyacrylamide gel at pH 8.3 , showed that at this pH the charge on the enzyme in the two preparations was virtually identical and that there was no reason to suppose that there was any difference between the preparations when judged by this criterion (Fig. 7).

Interaction with polyphenols. When mevalonate kinase was prepared from green *Phaseolus* leaves by the method successful with cotyledons great difficulty was experienced in obtaining a final product in which the mevalonate kinase activity was eluted as a single peak from DEAE-Sephadex. Because initial leaf homogenates became brown rapidly, sodium metabisulphite (10 mM) was added to the $0.08\text{ M-phosphate buffer, pH 7.0}$, of the homogenization medium. Despite this initial precaution the mevalonate kinase preparation behaved as a heterogeneous enzyme when finally eluted from DEAE-Sephadex (Fig. 8). The cotyledon preparation, on the other hand, which appeared to contain no polyphenols, consistently yielded a final preparation of mevalonate kinase in which the activity was eluted from DEAE-Sephadex as a single peak. The apparent heterogeneity of the leaf enzyme preparations could, as shown in Fig. 8, be simulated by incubating the apparently enzymically homogeneous cotyledon preparation with a

Fig. 9. Changes in mevalonate kinase activity, soluble protein and chlorophyll content of greening bean leaves

Etiolated bean plants (12 days old) were greened under continuous illumination from three 40W fluorescent strips supplemented with six 15W incandescent bulbs. A control group of beans were kept in the dark. Samples of five leaf pairs were harvested at 4h intervals and homogenized in 3 vol. of $0.08\text{ M-phosphate buffer, pH 7.0}$, containing $10\text{ mM-sodium metabisulphite}$, 1 mM-KCN , 0.1% (w/v) bovine serum albumin and 20% (w/v) Dowex 1 (X10) in a mortar with sand. The homogenate was centrifuged at $105\,000g$ for 60min; mevalonate kinase and protein assays were carried out on the supernatant and the pellet was assayed for chlorophyll. ○, Mevalonate kinase; ●, protein; △, chlorophyll.

Table 4. *Distribution of mevalonate kinase and other enzymes in French bean and spinach-beet leaf homogenates*

For details see the text. The term 'precipitate' refers to the material sedimenting in the layered solvent system for the flotation of chloroplasts.

Enzyme source		Chlorophyll ($\mu\text{g}/\text{mg}$ of protein)	Specific enzyme activities (nmol/min per mg of protein)			
			Ribulose diphosphate carboxylase	Mevalonate kinase	Malate dehydrogenase	Glucose 6-phosphate dehydrogenase
<i>Phaseolus vulgaris</i>						
Aqueous preparation	Homogenate	14.7	81.8	0.31	1540	5.74
	Chloroplasts	23.8	108.8	0.09	393	1.66
Non-aqueous preparation	Homogenate	27.0	82.2	0.57	1560	10.5
	Precipitate	9.6	32.3	1.81	2980	41.7
	Chloroplasts	27.8	104.0	0.39	1230	8.3
<i>Beta vulgaris</i>						
Aqueous preparation	Homogenate	4.8	119.0	0.26	1475	—
	Chloroplasts	14.3	160.0	0.04	286	—

preparation of *Phaseolus* leaf polyphenols before chromatography. The elution patterns shown in Fig. 8 are typical of those obtained in a number of experiments; the relative heights and areas under peaks of enzyme activity varied somewhat with each experiment but the overall pattern was always similar. It was also possible to produce apparently polymorphic forms of mevalonate kinase by treating an apparently enzymically homogeneous preparation of the enzyme from cotyledons with papain. The heterogeneity observed in the product of this treatment on DEAE-Sephadex was slightly different from that observed with polyphenol-contaminated preparations. Because the apparent enzymic heterogeneity of mevalonate kinase preparations from green leaves was obviated when great care was taken (a) to absorb existing polyphenols on Dowex 1 (Lam & Shaw, 1970), (b) to inhibit polyphenol oxidase activity present in the leaves by including KCN (1 mM) in the homogenization medium and (c) to remove quinones by including bovine serum albumin (0.1 % w/v) in the homogenization medium (Pierpoint, 1969), it was concluded that protease activity was not a factor contributing to the observed enzymic heterogeneity of leaf mevalonate kinase.

Mevalonate kinase in greening leaves

Increase in mevalonate kinase activity on greening. Since the nature of mevalonate kinase present in green leaves did not appear to differ materially from that of the cotyledons it was decided to ascertain whether there was an overall increase in the mevalonate kinase of leaves on greening. The results in Fig. 9 show that the mevalonate kinase activity of leaves did increase on greening but this appeared to be part of an overall increase in the protein content because the

specific enzyme activity remained constant during this process.

Locus of mevalonate kinase activity. It was decided to ascertain whether the increase of mevalonate kinase activity observed on greening could be associated with an increase of the activity present in the plastids. An attempt was therefore made to assess the proportion of the total mevalonate kinase activity present in the green leaves which was located in the chloroplasts. Chloroplasts from French-bean leaves were prepared by both aqueous and non-aqueous procedures. The mevalonate kinase activity present in the chloroplasts and in the parent homogenate was assayed and the distribution was compared with those obtained by assaying ribulose diphosphate carboxylase, known to be located in the chloroplasts (Smillie & Fuller, 1959), and glucose 6-phosphate dehydrogenase and malate dehydrogenase, both known to be present in the cytoplasm but absent from the chloroplasts (Smillie, 1963; Tolbert *et al.*, 1969; Rocha & Ting, 1970). The specific activities of the four enzymes are given in Table 4 which shows that the distribution of mevalonate kinase in both the aqueous and non-aqueous chloroplast preparation is similar to that of the two enzymes known to be present in the cytoplasm but not in the chloroplast. All attempts to measure the mevalonate kinase in disrupted chloroplasts failed to show any appreciable activity; results obtained with spinach-beet preparations were similar to those obtained with *Phaseolus*.

Discussion

The comparison of the molecular and kinetic characteristics of mevalonate kinase preparations obtained from etiolated cotyledons and green leaves

has shown that, when care was taken to remove contaminating phosphatase from, and to minimize the effect of polyphenols on, the enzyme, no evidence could be obtained for enzymic heterogeneity of mevalonate kinase in green leaves or for any differences between such preparations and those prepared from etiolated cotyledons.

The kinetic characteristics of both preparations obtained from *Phaseolus vulgaris* were quite similar to those reported for preparations from *Hevea brasiliensis* latex (Williamson & Kekwick, 1965). In particular the pH optimum was between pH 6.5 and 7.5 for both preparations, in contrast to the report of Rogers *et al.* (1966b) that, whereas the enzyme from etiolated cotyledons had a pH optimum of pH 5.5, that from green leaves showed optima at pH 5.5 and 7.5. It is noteworthy that acid pH optima for mevalonate kinase have been reported for crude plant preparations containing a considerable amount of phosphatase (Rogers *et al.*, 1966b; Valenzuela *et al.*, 1966; Potty & Bruemmer, 1970; Loomis & Battaile, 1963). This phosphatase activity could perhaps have had the effect of altering the apparent pH optimum of mevalonate kinase. This is, however, not likely, for in the present work no maximum of mevalonate kinase activity below pH 6.5 was detected in any preparation, even in crude acetone-dried preparations. The pH optimum of the crude enzyme preparations was always 7.0 and the activity at pH 5.5 about half that at pH 7.0. The ATPase activity of crude preparations had optima at pH 5.0 and 7.8.

Not only was it not possible to demonstrate an isoenzymic form of mevalonate kinase characteristic of the chloroplast, but the overall activity of disrupted chloroplasts prepared by both aqueous and non-aqueous methods were very low.

Comparison of the specific activity of mevalonate kinase and of ribulose diphosphate carboxylase, malate dehydrogenase and glucose 6-phosphate dehydrogenase present in disrupted chloroplast preparations with that of the parent homogenate showed that the distribution of mevalonate kinase was very similar to that of malate dehydrogenase and glucose 6-phosphate dehydrogenase, enzymes known to be cytoplasmic (Smillie, 1963; Tolbert *et al.*, 1969; Rocha & Ting, 1970), rather than to the ribulose diphosphate carboxylase, a chloroplast enzyme. It is, however, possible that any mevalonate kinase present may be much more readily lost from the chloroplast than ribulose diphosphate carboxylase on slight damage, since the molecular weight of mevalonate kinase reported here is about 100000 whereas that of ribulose diphosphate carboxylase is about 550000 (Paulsen & Lane, 1966). Cooper & Benedict (1967) failed to demonstrate mevalonate kinase activity in *Euglena* chloroplasts, but it is not clear to what extent their chloroplast preparations had been disrupted.

Circumstantial evidence that synthesis of some

isoprenoids does nevertheless occur within the chloroplast has come from the work of Threlfall *et al.* (1967), who showed that in excised barley shoots $^{14}\text{CO}_2$ was more readily incorporated into some characteristic chloroplast isoprenoids than was $[2-^{14}\text{C}]$ mevalonate and that the converse situation prevailed when incorporation into isoprenoids localized in the cytoplasm was investigated. More direct evidence for synthesis of isoprenoids by chloroplasts was obtained by Charlton *et al.* (1967) who demonstrated the incorporation of $[2-^{14}\text{C}]$ -mevalonate into phytoene by disrupted chloroplasts from *Phaseolus vulgaris* prepared by the non-aqueous procedure.

It seems therefore that, notwithstanding the conclusion that there is no evidence for an isoenzymic form of mevalonate kinase characteristic of the chloroplast and that little mevalonate kinase could be demonstrated in material obtained by disrupting isolated chloroplasts, some isoprenoid biosynthesis does occur in chloroplasts and that some of the enzymes concerned therewith occur within the chloroplast.

References

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Arnon, D. I. (1949) *Plant Physiol.* **24**, 1–15
- Charlton, J. M., Treharne, K. J. & Goodwin, T. W. (1967) *Biochem. J.* **105**, 205–212
- Cooper, C. Z. & Benedict, C. R. (1967) *Plant Physiol.* **42**, 515–519
- Creeth, J. M. (1952) *Biochem. J.* **51**, 10–17
- Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
- Dorsey, J. K. & Porter, J. W. (1968) *J. Biol. Chem.* **243**, 4667–4670
- Ehrenberg, A. & Dalziel, K. (1958) *Acta Chem. Scand.* **12**, 465–469
- Flint, A. P. F. (1970) *Biochem. J.* **120**, 145–150
- Goodwin, T. W. (1958) *Biochem. J.* **70**, 612–617
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Gray, J. C. & Kekwick, R. G. O. (1969) *Biochem. J.* **113**, 37P
- Gray, J. C. & Kekwick, R. G. O. (1972) *Biochim. Biophys. Acta* **279**, 290–296
- Hurwitz, J., Weissbach, A., Horecker, B. L. & Smyrniotis, P. Z. (1956) *J. Biol. Chem.* **218**, 769–783
- Jaenicke, R. & Knof, S. (1968) *Eur. J. Biochem.* **4**, 157–163
- Kegeles, G. & Gutter, F. J. (1951) *J. Amer. Chem. Soc.* **73**, 3770–3777
- Lam, T. H. & Shaw, M. (1970) *Biochem. Biophys. Res. Commun.* **39**, 965–968
- Laurent, T. C. & Killander, J. (1964) *J. Chromatogr.* **14**, 317–330
- Levy, H. R. & Popják, G. (1960) *Biochem. J.* **75**, 417–428
- Loomis, W. D. & Battaile, J. (1963) *Biochim. Biophys. Acta* **67**, 54–63
- Margoliash, E. & Lustgarten, J. (1962) *J. Biol. Chem.* **237**, 3397–3405

- Markley, K. & Smallman, E. (1961) *Biochim. Biophys. Acta* **47**, 327–335
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
- Mercer, E. I. & Goodwin, T. W. (1962) *Biochem. J.* **85**, 13p
- Paulsen, J. M. & Lane, M. D. (1966) *Biochemistry* **5**, 2350–2357
- Pierpoint, W. S. (1969) *Biochem. J.* **112**, 619–629
- Potty, V. H. & Bruemmer, J. H. (1970) *Phytochemistry* **9**, 99–105
- Rabin, B. R. & Trown, P. W. (1964) *Proc. Nat. Acad. Sci. U.S.* **51**, 497–501
- Racker, E. (1957) *Arch. Biochem. Biophys.* **69**, 300–310
- Ramirez, J. M., Del Campo, F. F. & Arnon, D. I. (1968) *Proc. Nat. Acad. Sci. U.S.* **59**, 606–612
- Rocha, V. & Ting, I. P. (1970) *Arch. Biochem. Biophys.* **140**, 398–407
- Rogers, L. J., Shah, S. P. J. & Goodwin, T. W. (1966a) *Biochem. J.* **99**, 381–388
- Rogers, L. J., Shah, S. P. J. & Goodwin, T. W. (1966b) *Biochem. J.* **100**, 14c–17c
- Siegel, L. & Englard, S. (1961) *Biochim. Biophys. Acta* **54**, 67–76
- Skilleter, D. N. & Kekwick, R. G. O. (1967) *Anal. Biochem.* **20**, 171–180
- Skilleter, D. N. & Kekwick, R. G. O. (1971) *Biochem. J.* **124**, 407–417
- Smillie, R. M. (1963) *Can. J. Bot.* **41**, 123–154
- Smillie, R. M. & Fuller, R. C. (1959) *Plant Physiol.* **34**, 651–656
- Threlfall, D. R., Griffiths, W. T. & Goodwin, T. W. (1967) *Biochem. J.* **103**, 831–851
- Tolbert, N. E., Oeser, A., Yamazaki, R. K., Hageman, R. H. & Kisaki, T. (1969) *Plant Physiol.* **44**, 135–147
- Treharne, K. J., Mercer, E. I. & Goodwin, T. W. (1966) *Biochem. J.* **99**, 239–245
- Valenzuela, P., Cori, O. & Yudelevich, A. (1966) *Phytochemistry* **5**, 1005–1011
- van den Oord, A. H. A., Wesdorp, J. J., van Dam, A. F., & Verheij, J. A. (1969) *Eur. J. Biochem.* **10**, 140–145
- Williamson, I. P. & Kekwick, R. G. O. (1965) *Biochem. J.* **96**, 862–871