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PROPERTIES AND PARTIAL PURIFICATION OF MEVALONATE KINASE FROM *AGAVE AMERICANA*

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Key Word Index—*Agave americana*; Amaryllidaceae; mevalonate kinase; mevalonate-5-phosphate.

Abstract—Mevalonate kinase activity was demonstrated in acetone powder extracts from *Agave americana* leaves, flowers and scape. ATP was the most effective phosphate donor. The enzyme had an optimum pH of 7.9 in Tris–HCl buffer. Dialysis decreased the ability to phosphorylate mevalonic acid (MVA). Partially purified mevalonate kinase reached maximum activity in the presence of 2 mM Mn^{2+} or 6–8 mM Mg^{2+}. Higher concentrations of Mn^{2+} were inhibitory, whereas higher concentrations of Mg^{2+} produced only a small decrease in the activity. The amount of mevalonate-5-phosphate (MVAP) formed depended on protein concentration and incubation time. During short incubations, the MVAP formed increased as protein concentration rose, whereas during prolonged incubations (1–6 hr), there was a decrease in the MVAP formed when a certain amount of protein was exceeded. It is suggested that MVAP formed was hydrolysed by a phosphatase present in the extracts. This interfering activity was eliminated when mevalonate kinase is partially purified. The apparent K_m values of the enzyme from leaves were 0.05 mM for MVA and 0.14 mM for ATP. Similar K_m values are obtained with partially purified mevalonate kinase. The enzyme was purified by ammonium sulphate precipitation, Sephadex G-100 filtration and DEAE-Sephadex A-50 fractionation.

INTRODUCTION

Mevalonate kinase (ATP:mevalonate-5-phosphotransferase, E.C. 2.7.1.36) catalyses the phosphorylation of mevalonic acid to mevalonate-5-phosphate, as the first stage in the conversion of MVA into numerous terpenoids in animals, plants and microorganisms. This enzyme is known to be present in yeast [1], in the livers of the rat [2], pig [3] and rabbit [4], in the superovulated rat ovary [5] and in the larva of *Sarcophaga bullata* [6, 7]. In plants, mevalonate kinase has been partially purified from *Cucurbita pepo* seedlings [8], *Hevea brasiliensis* latex [9] and green leaves and etiolated cotyledons of *Phaseolus vulgaris* [10]. Recently, we have reported the purification and some properties of MVA kinase from *Pinus pinaster* seedlings [11].

The genus *Agave* is a source of steroidal sapogenins, which occur in the plant as saponins. Hecogenin, chlorogenin, neotigogenin, kammogenin and 9-dehydrohecogenin have been isolated from *A. americana*. Little information is available on the biosynthesis of these compounds in *Agave*. Only Ehrhardt *et al.* [12] have shown that *A. toumeyana* incorporates acetate-[1-^{14}C] into cycloartenol. In a previous communication (13) we reported the mevalonate phosphorylation to mevalonate-5-phosphate and mevalonate-5-pyrophosphate in *A. americana*.

The present paper describes some properties and partial purification of MVA kinase of acetone powder extracts from *A. americana* leaves, flowers and scape.

RESULTS

Nucleotide and pH dependence of MVA kinase activity

The formation of MVAP from MVA-[2-^{14}C] by acetone powder extracts from *A. americana* leaves was followed by paper chromatography. The efficiency of UTP, ITP and GTP as phosphate donors at two different concentrations was compared with that of ATP. The results given in Table 1 show that ATP was by far the most effective phosphate donor. The enzyme preparations could utilize all the nucleotides tested at a 5 mM concentration, but the MVAP formed was only 20% of that obtained with 1 mM ATP.

MVAP formation by acetone powder extracts from *A. americana* leaves was studied at different pH values by using 0.1 M Tris–HCl buffer. The protein concentration was adjusted to 10–12 mg/ml. As shown in Table 2, the maximal activity was obtained at pH 7.9.

Effect of dialysis and metal ions on MVA kinase activity

The acetone powder extracts from *A. americana* leaves were able to phosphorylate MVA without addition of

Table 1. Nucleotide specificity of *A. americana* mevalonate kinase

Nucleotide	Concentration (mM)	MVAP formed (dpm × 10^{-3}/mg protein)
ATP	1	24.0
	5	24.0
UTP	1	0.0
	5	5.5
ITP	1	2.0
	5	6.0
GTP	1	2.5
	5	4.5

Incubations contained the standard radiochemical assay mixture, nucleotide were added as shown, 12 mg of protein were added. Incubations were carried out for 1 hr.

Table 2. Effect of pH on the activity of *A. americana* mevalonate kinase

pH		MVAP formed
Buffer	Extract	(dpm × 10^{-3}/mg protein)
7.2	6.0	8.2
7.5	7.2	12.5
8.0	7.9	32.3
8.5	8.5	28.4

Incubations contained the standard radiochemical assay mixture and adjusted to the pH shown.

Table 3. Effect of dialysis and metal ions on the activity of *A. americana* mevalonate kinase

	MVAP formed (dpm × 10^{-3}/mg protein)		
	—	Mg^{2+}	Mn^{2+}
Undialysed acetone powder extract	17.4	27.6	11.4
Dialysed acetone powder extract	7.2	23.1	1.8

Incubations contained the standard radiochemical assay mixture, except for the metal ions (10 mM). 5 mg of protein were added in all the cases.

metal ions (Table 3). When the extracts were dialysed, a lower specific activity was found. Supplementation of 10 mM Mg^{2+} strongly increased the phosphorylation of MVA, both in undialysed and dialysed extracts, whereas Mn^{2+} at the same concentration caused inhibition.

Partially purified mevalonate kinase reached its maximum activity in the presence of 2 mM Mn^{2+} or 6–8 mM Mg^{2+} (Fig. 1). High concentrations of Mn^{2+} were clearly inhibitory, whereas high concentrations of Mg^{2+} produced only a small decrease in the MVA kinase activity.

Influence of incubation time and protein concentration on MVAP formation

The MVAP formation by acetone powder extracts from *A. americana* leaves, flowers and scape was investigated in reaction carried out for 30 min to 6 hr. Protein

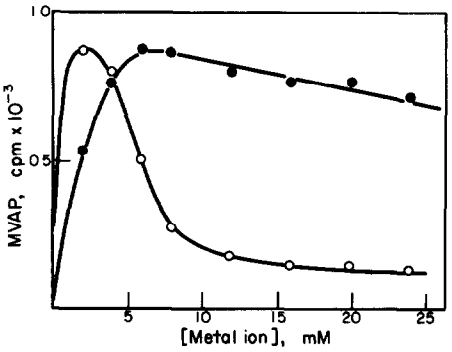

Fig. 1. Effect of Mg^{2+} and Mn^{2+} on the activity of partially-purified mevalonate kinase from *A. americana* leaves. Incubations contained the standard radiochemical assay mixture, except for the metal ions. Enzyme protein was partially purified by ammonium sulphate precipitation and Sephadex G-100 gel filtration. (●), Mg^{2+}; (○), Mn^{2+}.

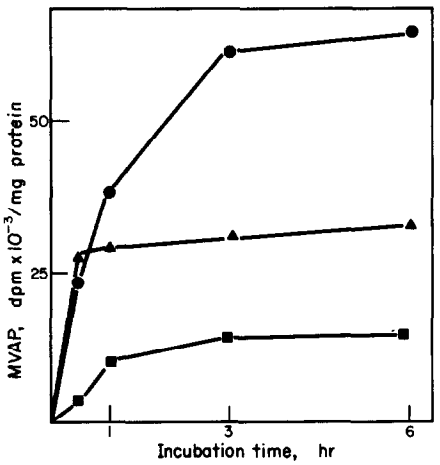

Fig. 2. MVAP formation by acetone powder extracts from *A. americana* leaves, flowers and scape at different incubation times. Incubations contained the standard radiochemical assay mixture. Protein concentrations were 18 mg/ml with leaf extract (■), 8 mg/ml with flowers extract (▲) and 4 mg/ml with scape extract (●).

concentration in the incubations was 18 mg/ml with leaf extract, 8 mg/ml with flower extract and 4 mg/ml with scape extract. As shown in Fig. 2, the scape extract showed the maximum specific activity, being the less concentrated. The variations in the protein concentration could also explain the different slope of the curves.

To elucidate this influence, different amounts of acetone powder extracts were incubated with equal amounts of substrate and cofactors for 3 hr. The MVAP formed decreased when a definite amount of protein was exceeded in the three extracts assayed (Fig. 3). A concomitant increase of the remaining MVA was also observed, suggesting that the MVAP formed was hydrolysed by a phosphatase present in the extracts. In order to investigate this interference, reactions were carried out for 15 min to 6 hr with different amounts of acetone powder extracts from leaves. In short-time incubations, there was no decrease in the MVAP formed at increasing protein concentrations, whereas with

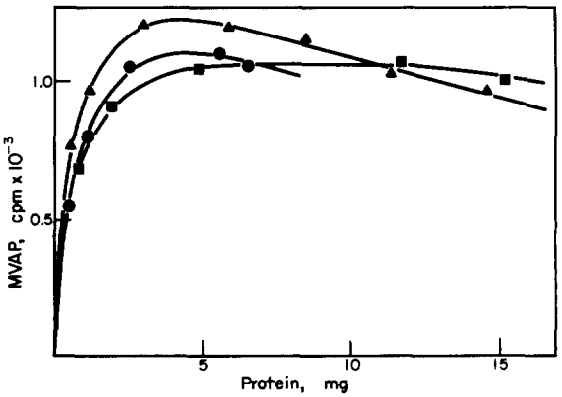

Fig. 3. MVAP formation by different amounts of acetone powder extracts from *A. americana* leaves, flowers and scape. Incubations contained the standard radiochemical assay mixture. Incubation time was 3 hr. (■) leaf extract; (▲) flower extract; (●) scape extract.

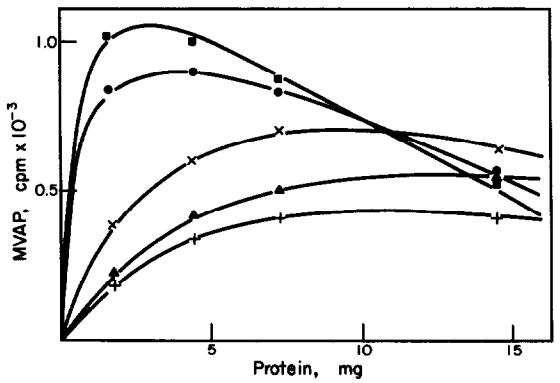

Fig. 4. Influence of protein concentration on MVAP formation by acetone powder extracts from *A. americana* leaves. Incubations contained the standard radiochemical assay mixture. Incubation times were: (+) 15 min; (▲) 30 min; (×) 1 hr; (●) 3 hr; (■) 6 hr.

prolonged incubation times (1–6 hr), there was a decrease in the MVAP formed when a definite amount of protein was exceeded (Fig. 4). In general, similar results were obtained in the other extracts (flowers or scape acetone powder). The inner leaves (unexposed to light) showed profiles similar to those of green leaves.

Further experiments have been carried out with supplementation of 1 ml of boiled extract (15 mg protein/ml) to incubations with low protein concentration. No decrease of MVAP formed was found, even when incubation time was prolonged for 6 hr. This interfering thermolabile activity was also eliminated when the MVA kinase was partially purified.

Kinetic properties of mevalonate kinase

The rate of MVA phosphorylation by acetone powder extracts from *A. americana* leaves was studied at MVA concentrations varying from 0.04 mM to 0.5 mM in the presence of 8 mM ATP. Similar experiments were carried out under conditions in which the ATP concentration varied between 0.01 mM to 4 mM in the presence of 0.057 mM MVA. The reciprocal plots of $1/V$ against $1/S$ provided the following apparent K_m values: for MVA 0.05 mM and for ATP 0.14 mM. Similar K_m values were obtained working with partially purified mevalonate kinase. In experiments carried out with acetone powder extracts from *A. americana* flowers and scape, similar K_m values were also obtained. A slight inhibition at high mevalonate and ATP concentrations was observed in all the cases.

Partial purification of mevalonate kinase

Table 4 shows the results of partial purification of mevalonate kinase of acetone powder extracts from *A. americana* leaves. These extracts were subjected to ammonium sulphate fractionation. The fraction obtained at 30% saturation showed practically no activity. About 85–90% of the enzyme precipitated between 30–45% ammonium sulphate saturation with a 2-fold increase in specific activity.

Further purification was carried out by Sephadex G-100 gel filtration. Two fractions with mevalonate kinase activity were eluted, with a 23-fold and 37-fold increase in specific activity over the original acetone powder extracts. However both fractions differed in their stability, the second losing its activity rapidly at 4° even in the presence of 10 mM β-mercaptoethanol.

The first active fraction from Sephadex G-100 column was applied to a DEAE-Sephadex A-50 column. On stepwise elution with the Tris–HCl buffer containing increasing concentration of KCl the enzyme was present in the fraction eluted at 0.2 M KCl with a 33-fold increase in specific activity over the original acetone powder extracts. Nevertheless, when electrophoresis on polyacrylamide gel was used as a test for purity, a main protein band was observed together with a more diffused band.

In parallel experiments, the first active fraction from Sephadex G-100 column was applied to a DEAE-cellulose column and eluted with Tris–HCl buffer containing increasing concentrations of KCl. A fraction eluted at 0.2 M KCl contained the mevalonate kinase but no significant increase in the specific activity was obtained by this procedure.

DISCUSSION

In this paper, we have studied some characteristics of mevalonate kinase from *A. americana*. Regarding nucleotide requirements, the mevalonate kinase from *A. americana* is more specific for ATP. Thus, the *Hevea* enzyme can utilize ATP and ITP with equal effectivity at similar concentrations [9], and shows a little activity with UTP. The pig liver enzyme can utilize only ATP and ITP [3], whereas the *Sarcophaga bullata* larval enzyme is able to use UTP and ITP at low concentrations, although it shows a marked preference for ATP [6]. Mevalonate kinase from yeast autolysate can also utilize GTP, UTP or CTP in the place of ATP [1]. Working with the orange enzyme Potty and Bruemmer [14] have reported that neither CTP nor GTP was effective in replacing ATP as a cofactor. In *Pinus pinaster* we have also shown that mevalonate kinase can only utilize ATP as a phosphate donor, GTP, UTP and ITP being inactive [11].

Maximum phosphorylation of MVA occurred at pH 7.9 in the presence of acetone powder extracts from *A. americana* leaves. In a previous communication [13]

Table 4. Purification of mevalonate kinase from *A. americana* leaves

Purification step	Protein (mg/ml)	Activity (dpm × 10^{-3})	Sp. act. (dpm × 10^{-3}/mg)	Purification
Acetone powder extract	13.70	216	15.8	1
Ammonium sulphate (30–45%)	10.00	316	31.6	2
Sephadex G-100 (1st fraction)	0.30	109	363.3	23
DEAE-Sephadex A-50	0.15	78	520.0	33
Sephadex G-100 (2nd fraction)	0.15	87	580.0	37

we reported 7.3–7.5 as optimum pH, but at higher pH values an increased specific activity was found. This result differs from that obtained by other workers with other plant sources. Valenzuela *et al.* [15], working with the cell-free extracts from *Pinus radiata* seedlings, reported the maximum activity at pH 6. With the orange enzyme, the MVA phosphorylation was maximal at pH 6.3 [14]. However, the mevalonate kinase from animal sources [3–5, 7] has an optimum pH of 7.3–7.5, similar to that reported for *Hevea* latex enzyme [9].

Some differences have been observed in the pH-activity profiles obtained for mevalonate kinase from plant preparations. In French-bean seedlings, Rogers *et al.* [16] demonstrated that the mevalonate kinase occurs outside as well as inside the chloroplast by finding that the optimum pH for activity of the chloroplastidic and extrachloroplastidic enzymes was significantly different: pH 7.5 for the chloroplastidic enzyme and 5.5 for the extrachloroplastidic enzyme. Homogenates of green and etiolated leaves showed two peaks at pH 5.5 and 7.5, while in the chloroplasts only the latter could be detected. Loomis and Battaille [8] reported that the optimum pH of mevalonate kinase in the cytoplasm of etiolated cotyledons of pumpkin seedlings was at pH 5.5. Recently, we reported [17] the separation by Sephadex G-100 of two fractions with mevalonate kinase activity from *Pinus pinaster* seedlings and *A. americana* leaves, both fractions being active at pH 7.9.

When acetone powder extracts were dialysed enzyme activity decreased, being increased by 10 mM Mg^{2+} supplementation. On the other hand, metal ion requirements of partially purified mevalonate kinase are similar to those from other animal and plant sources. As with the hog liver enzyme [18], at low concentration Mn^{2+} is a better activator than Mg^{2+}. Maximum enzyme activity was reached at 2 mM; at higher concentration Mn^{2+} was inhibitory. Similar results were observed with *Hevea* latex [9], *Phaseolus* cotyledons and leaves [10] and *Pinus radiata* seedlings [15]. However, at higher concentrations, Mg^{2+} was the best activator, but a slight inhibition was found at 10–25 mM. A most pronounced decrease with Mg^{2+} has been reported for the *Hevea* latex enzyme [9]. It is accepted that Mg·ATP is the substrate of mevalonate kinase; therefore, the ratio ATP/Mg^{2+} may be more important than the absolute concentrations. With *A. americana* enzyme, the optimum values seem to be 1 for the ATP/Mg^{2+} and 4 for the ATP/Mn^{2+}.

Working with extracts of different protein concentrations from *A. americana* leaves, flowers and scape, we have obtained quite different values for the mevalonate kinase specific activities. With the more concentrated extracts, lower specific activities were found.

The amount of MVAP formed is dependent on both protein concentration and incubation time. The concomitant increase of remaining MVA observed when MVAP decreased suggests hydrolysis of MVAP. The interference of phosphatases with assays of mevalonate activating enzymes in crude plant extracts has been reported by other workers [14, 19]. Mitchell and Downing [20] reported the separation of phosphatases from mevalonate activating enzymes in a cell-free homogenate of *Nepeta cataria*. Hill and Rogers [21] have investigated the phosphatase activity in French-bean preparations over the pH range 4–9. The acid phosphatase activity is some thousand-fold greater than that of mevalonate kinase in extracts of higher plants. In our case, this activity has been found at pH 7.9. The presence of this phosphatase activity would affect the mevalonate kinase assay, through removal of ATP necessary for phosphorylation of MVA and through degradation of the phosphorylated products of mevalonate metabolism [21]. Thus, the presence of isopentenyl phosphate among the products of MVA metabolism by orange preparations [22] seems to be due to the enzymatic dephosphorylation of isopentenyl pyrophosphate by the phosphatase present in these preparations. Similar observations were also made [23] in *Hevea brasiliensis* latex.

Cell-free extracts from *Pinus radiata* seedlings contain a phosphatase which splits ATP and phosphomonoesters at rates 40–100 times the rate of phosphorylation of MVA [15]. This phosphatase competes with the kinase for ATP. In acetone powder and cell-free extracts from *Pinus pinaster*, we have also found a similar phosphatase activity [11]. This interfering activity was not present in experiments carried out with supplementation of boiled extract applied to incubations with low protein concentration and when the mevalonate kinase was partially purified.

The kinetic characteristics of mevalonate kinase from *A. americana* were quite similar to those reported for preparations from *Pinus pinaster* [11]. The K_m for MVA resembles that of the pig liver enzyme [3], whereas the K_m for ATP is similar to those of the superovulated rat ovary [5] and hog liver [18] and lower than those of *Hevea* latex [9] and *Sarcophaga bullata* larvae [7].

Our results on the purification of the mevalonate kinase from *A. americana* leaves agree with those reported from other plant sources [10]. Precipitation with 30–45% ammonium sulphate saturation doubled the specific activity. We had obtained a similar result with the enzyme from *Pinus pinaster* [11] whereas Valenzuela *et al.* [15] reported that no increase in specific activity was obtained by this procedure with the *Pinus radiata* enzyme.

When the precipitate obtained with ammonium sulphate was applied to a Sephadex G-100 column, two fractions with mevalonate kinase activity were eluted. The second fraction showed a higher specific activity than the first, but lost its activity even at 4° in the presence of 10 mM β-mercaptoethanol [13].

Although DEAE-cellulose chromatography has been successfully used as a purification procedure for hog liver mevalonate kinase [18], the partially purified enzyme from *A. americana* leaves for Sephadex G-100 (1st fraction) could not be successfully eluted from DEAE-cellulose column. Similar results were reported for the *Hevea* mevalonate kinase [9]. However, when the same active fraction from Sephadex G-100 was applied to a DEAE-Sephadex A-50 column, most of the mevalonate kinase was eluted in the 0.2 M KCl fraction. This procedure produced an increase in the specific activity 33 times that of the original extract, although we have obtained indications of other proteins in this preparation by electrophoresis on polyacrylamide gel.

The partial purification and characterization of the mevalonate kinase from *A. americana* leaves is a significant step towards understanding one of the first enzymes in the isoprenoid pathway in this source. Demonstration that the greater part of this enzyme appears in the chloroplastidic fraction [24] and the

isolation by Sephadex G-100 filtration of two active fractions with different stabilities seems to suggest the presence of two isoenzymes, both being active at the same pH value. Hill and Rogers [21] attempted to separate molecular species of mevalonate kinase from cotyledons of *Phaseolus vulgaris* by isoelectric focusing. Several peaks were obtained by this procedure. Further investigations will be carried out on the molecular parameters and the compartmentation of these isoenzymes, in order to elucidate the role of the mevalonate kinase in the regulation of the isoprenoid biosynthesis.

EXPERIMENTAL

Materials. Locally-grown *A. americana* plants were used. MVA-[2-^{14}C] was supplied as the lactone by the Radiochemical Centre, Amersham, England. The K salt was prepared by treating the lactone at 37° for 30 min with an excess of KOH soln. The final pH of the soln was *ca* 10.

Enzyme preparations. The Me_2CO powders were prepared as described previously [13]. Prior to use, the powder was extracted for 10 min with a volume of 0.1 M Tris–HCl buffer pH 7.9, containing 10 mM β-mercaptoethanol equivalent to 10× the weight of the powder and centrifuged at 10000 *g* for 15 min at 4 . Dialysis was carried out in 'Visking' tubes against 1 mM Tris–HCl buffer pH 7.9, containing 0.1 mM β-mercaptoethanol, changing the buffer 3 × (0.5, 1 and 2 hr). Protein was determined by the method of Lowry *et al.* [25]. Crystalline bovine serum albumin was used as a standard. Protein concentration of the column eluates was determined by absorption at 280 nm.

Purification procedures. The protein content of the 10000 *g* supernatants was fractionated with solid $(NH_4)_2SO_4$. The fraction obtained between 30–45% saturation was dissolved in 0.1 M Tris–HCl buffer, pH 7.9, containing β-mercaptoethanol and filtered upward through a Sephadex G-100 column (4.5 × 45 cm), previously equilibrated with the same buffer. Protein was eluted at a flow rate of 40 ml/hr. Fractions of 10 ml collected. The active fractions eluted from the Sephadex G-100 column were applied to a DEAE-Sephadex A-50 column (1.5 × 20 cm) equilibrated with 0.1 M Tris–HCl buffer, pH 7.9, containing 10 mM β-mercaptoethanol and 0.1 M KCl; they were eluted with the same buffer using continuous gradient of 0.1–0.5 M KCl and a flow rate of 60 ml/hr. Fractions of 10 ml were collected. In parallel experiments, the active fractions eluted from Sephadex G-100 were applied to a DEAE-cellulose column (1.5 × 20 cm) equilibrated with 0.1 M Tris–HCl buffer, pH 7.9, containing 10 mM β-mercaptoethanol and 0.1 M KCl. Protein was eluted with the same buffer at a continuous gradient of 0.1–0.5 M KCl and a flow rate of 60 ml/hr. Fractions of 10 ml were collected.

Analytical methods. Disc electrophoresis in 6% polyacrylamide gels was performed in glass tubes (7.5 × 0.5 cm) according to the discontinuous method of Davis [26] and Ornstein [27], with 377 mM Tris titrated to pH 8.9 with HCl, used in conjunction with tank buffer soln of 38 mM glycine soln titrated to pH 8.2 using conc Tris soln. The gel used was 'Cianogum 41' (95% acrylamide and 5% bisacrylamide). The gel soln (25 ml) was prepared in the Tris–HCl buffer, pH 8.9, containing 1.425 g acrylamide and 0.78 g bisacrylamide. 60 µl of dimethylaminopropionitrile were adding by stirring and the soln filtered. Polymerization was initiated by adding 0.8 ml 7% (w/v) freshly prepared ammonium persulphate soln. Polymerization was complete within 10 min. The protein soln was thickened by adding 10% sucrose and made visible with a trace of bromophenol blue. Electrophoresis was carried out for 15 min at 1 mA per tube and 1 hr at 5 mA per tube. After electrophoresis, the gels were detached and stained for protein using Amido Black 10B (0.2 g of dye dissolved in 1000 ml of 7% aq. HOAc). After 30 min, the gels were destained by repeated washing in 7% aq. HOAc.

Enzyme assays. Unless otherwise stated, enzymatic reactions were carried out by incubating the extracts at 37° for 30 min. The reaction system contained 24 µmol ATP, 12 µmol $MgCl_2$, 145 nmol MVA-[2-^{14}C], 300 µmol Tris–HCl buffer, pH 7.9, 30 µmol β-mercaptoethanol and enzyme preparation in a total vol. of 3 ml. Reactions were stopped by heating the tubes at 90° for 2 min. Precipitated protein was centrifuged off at 2000 *g* for 5 min. Aliquots (25 µl) of supernatants from the reaction mixture were applied to Whatman No. 1 paper strips and developed in several solvents [11]. Radioactive spots on the dried strips were detected in a Nuclear-Chicago Actigraph III system.

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