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MEVALONATE-ACTIVATING ENZYMES IN THE ORANGE

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Abstract—Orange juice vesicles were examined for enzymes associated with the activation of mevalonic acid (MVA). An ammonium sulfate fractionated preparation from the vesicles converted MVA to mevalonic acid-5-phosphate and mevalonic acid-5-pyrophosphate. Inclusion of insoluble polyvinyl pyrrolidone and ascorbate in the extraction medium protected the phosphorylating enzymes during their preparation. The reaction was specific for adenosine triphosphate and was stimulated by thiol compounds. Activation of MVA is probably the first step in the biosynthetic pathway to citrus terpenoids.

INTRODUCTION

MEVALONIC acid (MVA) has been implicated as the precursor of isoprenoid compounds in liver,[1] yeast,[2–4] and plants.[5–14] MVA was incorporated into plant terpenes by *in vitro* systems,[5–7] and formation of a number of phosphorylated intermediates from MVA has been demonstrated with cell-free systems from plants.[8–14] Loomis suggested that MVA is synthesized within the plant cell to explain the poor *in vivo* incorporation of MVA into certain plant terpenes.[5] However, MVA synthesis from acetyl CoA could not be demonstrated in orange fruit extracts. This paper reports the enzymatic phosphorylation of MVA by adenosine triphosphate (ATP) with extracts from orange juice vesicles. These reactions could be the first steps in the biosynthesis of terpenes from MVA in the orange.

RESULTS AND DISCUSSION

Phosphorylation of MVA-2-^{14}C

Extracts from orange juice vesicles phosphorylated MVA and ATP. Paper chromatographic analyses of the reaction mixture revealed that two products were formed. They were

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identified as mevalonic-5-phosphate (MVAP) and mevalonic-5-pyrophosphate (MVAPP) from their R_fs in three solvent systems (Table 1). MVA is converted to MVAP by MVA kinase (ATP-mevalonate-5-phosphotransferase, EC 2.7.1.36). MVAP kinase (ATP-5-phosphomevalonate phosphotransferase, EC 2.7.4.2) phosphorylates MVAP to MVAPP. In a typical experiment 120 μg of enzyme protein was incubated with 2×10^5 cpm MVA-2-^{14}C and $5{\cdot}85 \times 10^4$ cpm was recovered in MVAP, $4{\cdot}69 \times 10^3$ cpm in MVAPP and $1{\cdot}32 \times 10^5$ cpm in MVA fractions. No other product was detected. Rogers, Shah and Goodwin,[16] and Loomis and Battaile[9] were also unable to detect any other products beyond MVAPP.

TABLE 1. R_f VALUES OF MVA METABOLITES ON PAPER CHROMATOGRAPHY

Sample	R_f of spots in solvents*		
	1	2	3
Control (no enzyme)	0·78	0·78	0·74
Control (heated enzyme)	0·79	0·79	0·77
Complete reaction system†	0·78	0·78	0·74
	0·17	0·40	0·18
	0·03		
MVA‡	0·75	0·80–0·85	0·68–0·72
MVAP‡	0·15–0·18	0·53–0·61	0·18–0·20
MVAPP‡	0·03–0·05	0·29–0·35	

* The solvents were: 1. *n*-butanol–formic acid–water (77:10:13)[1,22]; 2. *tert*-butanol–formic acid–water (40:10:16)[2]; 3. *n*-Propanol–ammonia–water (60:20:20)[25]

† The reaction system, in a total volume of 0·2 ml, contained 20 μmoles of phosphate buffer, pH 6·5, 5 μmoles of mercaptoethanol, 2 μmoles of ATP, 2 μmoles of $MgCl_2$, 20 m μmoles of MVA-2-^{14}C ($\sim$100 m μc) and about 124 μg enzyme protein. The mixture was incubated at 37° for 3 hr, heated to coagulate proteins and centrifuged. Aliquots of supernatant were spotted on paper strips and subjected to chromatographic separation in three solvent systems.

‡ The R_f values of MVA, MVAP, MVAPP are those reported in literature, cited for the solvent systems, (1)[1,22], (2)[2], and (3)[25].

Enzyme Extraction

Inclusion of Polyclar AT (insoluble polyvinyl pyrrolidone) and sodium ascorbic in the extraction medium resulted in preparations with highest specific activities (Table 2). Dowex-1(Cl^-), nylon powder and supercel were not as effective as Polyclar AT. Loomis and Battaile[17] also observed that Polyclar AT and ascorbate were essential for successful extraction of alkaline phosphatase and MVA kinase from peppermint leaves. Polyclar AT removes bulk of the phenolic materials of the extract and ascorbate reduces any quinones formed from residual phenols which otherwise would react with the enzyme protein. Treatment of the precipitated enzyme fraction with Dowex resin increased the specific activity of the preparation. The Dowex anion exchanger might be removing enzyme inhibitors of anionic nature from these preparations. Water-clear enzyme extracts were obtained by Polyclar AT and Dowex treatment.

[16] L. J. ROGERS, S. P. J. SHAH and T. W. GOODWIN, *Biochem. J.* **99**, 381 (1966).
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TABLE 2. EXTRACTION OF MVA KINASE ACTIVITY FROM ORANGE JUICE VESICLES*

Additions†		m μmoles of MVA phosphorylated per mg proteins in 3 hr
1. *To extraction medium*	(g dry wt. equivalent/ 100 g of vesicles)	
None	—	29·2
Polyclar AT	20	66·4
Dowex-1(Cl^-)	20	51·4
Nylon Powder	20	48·2
Supercel	20	39·0
2. *To ammonium sulfate fraction*‡	(% dry wt. equivalent/V)	
None	—	68·7
Dowex-1(Cl^-)	50	92·8
Polyclar AT	50	71·2
Supercel	50	66·9
Nylon Powder	50	65·9

* Extracts were prepared from juice vesicles with various additions to the extraction medium and to the ammonium sulfate fraction after dialysis. MVA kinase was assayed with MVA-2-^{14}C and estimated from difference in radioactivity of unreactive MVA-2-^{14}C between heat inactivated and test systems.

† Added after pre-swelling in water. The extraction medium also contained 1% sodium ascorbate and Tris to pH 6·9.

‡ $(NH_4)_2SO_4$ fraction was obtained from Polyclar AT-treated juice vesicle extracts.

pH and ATP Dependence of MVA Kinases

Maximum phosphorylation of MVA occurred in 3 hr, after which there was a decrease in the amount of MVA phosphates formed (Fig. 1). This decline was probably due to phosphatase present in the extract. After 3 hr the phosphatase activity exceeded the kinase activity. Figure 1 also shows that MVA phosphorylation was maximal at pH 6·5. At higher pH values lesser amounts of MVA phosphates were detected in the reaction system. Rogers *et al.*,[18] reported pH 5·5 and 7·5 for two MVA kinase isoenzymes from bean seedlings. A pH maximal of 6·0 was reported for MVA kinase from pine seedlings[19] and pH of 7·3 and 7·5 for the kinase from rat liver[20] and rubber latex.[21]

The reaction between MVA and ATP was dependent both on enzyme as well as ATP concentrations. The data in Figure 2 show that the formation of MVA phosphates is directly proportional to enzyme concentration. Optimal ATP concentration for the reaction was 7·5 mM. Higher ATP levels probably complexed Mg^{++} to limit phosphorylation.[9]

Specificity for ATP

It was reported by Tchen[22] that MVA kinase from yeast autolysate could effectively use uridine triphosphate (UTP), cytidine triphosphate (CTP) or guanosine triphosphate (GTP) in place of ATP. Table 3 shows that neither CTP nor GTP was effective in replacing ATP as a cofactor.

[18] L. J. ROGERS, S. P. J. SHAH and T. W. GOODWIN, *Biochem. J.* **100**, 14C (1966).
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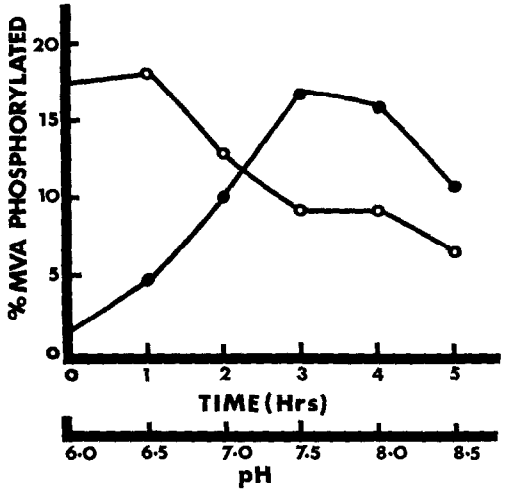

FIG. 1. DEPENDENCE OF MVA KINASE ACTIVITY ON TIME ● AND pH ○.

In the time experiment the reaction system contained 20 μmoles of phosphate buffer, pH 6·5, 5 μmoles of mercaptoethanol, 2 μmoles of ATP, 2 μmoles of $MgCl_2$, 20 m μmoles of MVA-2-^{14}C and 75 μg enzyme protein in total volume of 0·2 ml. Phosphate served as the buffer for all tests in the pH experiment.

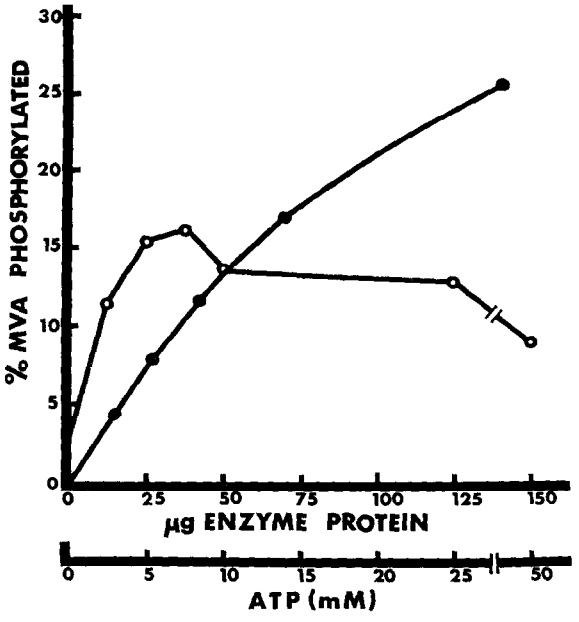

FIG. 2. DEPENDENCE OF MVA KINASE ACTIVITY ON ENZYME (●) AND ATP CONCENTRATIONS (○).

The reaction system for both experiments contained 20 μmoles of phosphate buffer pH 6·5, 5 μmoles of mercaptoethanol, 2 μmoles of $MgCl_2$,20 m μmoles of MVA-2-^{14}C and the indicated amounts of ATP and enzyme protein in total volume of 0·2 ml. In the enzyme concentration experiment 2 μmoles of ATP was used and in the ATP concentration experiment 68 μg enzyme protein was used.

TABLE 3. EFFECT OF VARIOUS NUCLEOSIDE TRIPHOSPHATES ON MVA KINASE ACTIVITY

Cofactor	Concentration $M \times 10^{-3}$	Percent MVA phosphorylated
None	—	2·56
ATP	10	15·10
	20	13·24
GTP	10	3·95
	20	4·02
UTP	10	4·10
	20	4·42

Cofactor was added to reaction system containing 20 μmoles of phosphate buffer, pH 6·5, 62 μg enzyme protein, 5 μmoles of mercaptoethanol, 2 μmoles of $MgCl_2$ and 20 mμmoles of MVA-2-^{14}C in total volume of 0·2 ml and assayed for MVA kinase activity.

MVA kinase from rubber latex was also specific for ATP.[21]

Activation and Inhibition of MVA Kinases

The MVA kinase from orange required reducing agents for maximum enzyme activity, as evidenced by the increase in activity observed in presence of reduced glutathione, mercaptoethanol and dithiothreitol (Table 4). Evidently they protect the active sites of the enzymes resulting in an increased yield of MVA phosphates as compared to controls. The inhibition by iodoacetamide which is known to react with thiol groups suggests that a thiol group in the kinase may participate in the active site.

TABLE 4. ACTIVATION AND INHIBITION OF MVA KINASE ACTIVITY

Addition	Concentration $M \times 10^{-3}$	Percent MVA phosphorylated
None	—	14·0
Reduced glutathione	25	29·7
Mercaptoethanol	25	26·4
Dithiothreitol	25	21·2
Nitrogen	—	17·1
Iodoacetamide	25	7·5
Potassium fluoride	25	27·4

Additions were made to reaction system containing 114 μg enzyme protein, 20 μmoles phosphate buffer, pH 6·5, 2 μmoles of ATP, 2 μmoles of $MgCl_2$ and 20 mμmoles of MVA-2-^{14}C in total volume of 0·2 ml and assayed for MVA kinase activity.

The increase in MVA phosphates in presence of potassium fluoride is probably due to inhibition of phosphatase activity thus preventing the breakdown of the reaction products.

Gosselin[23] reported an increase in MVA incorporation into hydrocarbons by rat liver microsomes in presence of added sodium fluoride.

These results are the first demonstration that the necessary enzymes to activate MVA are contained in the juice vesicles of orange fruit. For terpene synthesis the phosphorylated derivatives of MVA have to be transformed into other intermediates. This requires the mediation of a variety of enzymes. It will be necessary to examine orange juice vesicles for these enzymes to see whether the conventional biosynthetic pathway of terpenes prevails in these fruits.

EXPERIMENTAL

Materials

Valencia orange fruits with Brix/acid ratio of 6/1 and above, were obtained from local groves.

Polyclar AT was a product of General Aniline and Film Corporation, New York, New York. It was hydrated before use. Dowex-1 (Cl^-) was obtained from J. T. Baker and Company, Philipsburg, New Jersey. DL-MVA-2-^{14}C was obtained as its *N,N'*-dibenzylethylenediamine (DBED) salt from New England Nuclear Corporation, Boston, Massachusetts. An aqueous solution of this salt was treated with an excess of $NaHCO_3$ and the free DBED was removed by ether extraction. ATP, GTP, UTP, crystalline ammonium sulfate and tris (hydroxymethyl) amino-methane were purchased from Sigma Chemical Company, St. Louis, Missouri. All other chemicals used were of highest purity available in the market.

Enzyme Preparations

Juice vesicles from the orange fruit were separated from other tissues, as described previously[15] and stored at −96° for use as needed.

Weighed amounts of frozen vesicles were comminuted in a Waring blendor for 10–15 sec and then added to the extraction medium slowly with stirring. The extraction medium contained previously swelled Polyclar AT (20 g dry wt. equivalent/100 g of juice vesicles), sodium ascorbate (1 g/100 g of juice vesicles) and tris (calculated from the acidity of the fruit used). The pH of the resulting slurry was adjusted to pH 6·9 either with 1 M tris buffer, pH 10·5 or with 10% ascorbic acid. After straining through a four-layered cheesecloth, the filtrate was spun at 10,000 *g* for 30 min (Beta-Fuge, Model A-2, Lourdes Instrument Corporation, Old Bethpage, New York). $(NH_4)_2SO_4$ 65 g/100 ml of supernatant, was added to obtain 90% saturation. After 30 min equilibration at 4°, the precipitate was collected, suspended in 0·1 M tris buffer, pH 7·0 and dialyzed against distilled water at 4° for 2 hr. The dialyzed preparation, appearing pale yellow in color, was treated with pre-swelled Dowex-1(Cl^-) resin (50% dry wt. equivalent/V) and filtered through glasswool. The clear filtrate containing 1–2 mg protein was used as enzyme source.

Protein Estimation

Protein levels in the enzyme preparations were determined by a modification of Lowry's procedure that corrected for phenol interference.[24]

Enzyme Assays

MVA kinase and MVAP kinase were detected by using MVA-2-^{14}C as substrate and analyzing the reaction mixture for the presence of MVAP and MVAPP. Enzyme extract containing about 100 μg protein was incubated with 20 μmoles of phosphate buffer, pH 6·5, 5 μmoles of mercaptoethanol, 2 μmoles of $MgCl_2$, 2 μmoles of ATP and 20 m μmoles of MVA-2-^{14}C (~100 m μc) in a total volume of 0·2 ml. The reaction mixtures taken in pointed centrifuge tubes were incubated at 37° for 3 hr. Controls containing heat-inactivated enzymes were employed in each experiment. At the termination of incubation, the enzyme proteins were inactivated by immersing the tubes in a boiling water bath for 3 min and the coagulated proteins were sedimented by centrifugation. The supernatants were analyzed by paper chromatography.

Paper Chromatographic Separation of MVA Phosphates

Aliquots of supernatants from the reaction mixture were spotted on Whatman no. 1 paper strips (2 × 20 cm) and the paper strips were developed by descending chromatography in three separate solvent systems in Thomas–Kolb chromatography jars. (Arthur H. Thomas Company, Philadelphia, Pa.) The solvent systems

[23] L. GOSSELIN, *Biochim. J.* **89**, 23P (1963).
[24] V. H. POTTY, *Anal. Biochem.* **29**, 535 (1969).

used were *n*-butanol–formic acid–water (77:10:13),[1,22] *tert*-butanol–formic acid–water (40:10:16)[2] and *n*-propanol–ammonia–water (60:20:20)[25]. Radioactive spots on the dried strips were detected by cutting into 0·5 cm wide pieces and counting them by liquid scintillation. In some experiments two dimensional chromatography of the reaction mixture was carried out and the spots detected by exposure to Kodak X-ray film for a week. Repeated analysis of the reaction mixtures by paper chromatography showed that more than 95 per cent of the radioactivity added to the system could be recovered in MVA, MVAP and MVAPP fractions. So, for quantitative assessment of the reaction, the strips were developed in *n*-butanol–formic acid-water system and the unreacted MVA-2-^{14}C estimated by cutting out the corresponding area and counting by liquid scintillation. The amount of MVA phosphorylated was computed from the difference in radioactivity between control and test systems.

Radioassay

Radioactivities in paper strips were estimated in a Packard Tri-Carb Scintillation Spectrometer (Packard Instrument Co., LaGrange, Illinois, Model 4322) after immersing in the scintillation fluid in glass vials. The scintillation fluid contained per litre of toluene, 5 g of 2,5-diphenyloxazole and 0·1 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl]benzene.

[25] D. H. SHAH, W. W. CLELAND and J. W. PORTER, *J. Biol. Chem.* **240**, 1946 (1965).