BBA 12130

BIOSYNTHESIS OF TERPENES

III. MEVALONIC KINASE FROM HIGHER PLANTS

W. D. LOOMIS AND J. BATTAILE*

Science Research Institute and Department of Chemistry, Oregon State University, Corvallis, Oreg. (U.S.A.)

(Received May 14th, 1962)

SUMMARY

Mevalonic kinase activity has been demonstrated in extracts from several plant species. Pumpkin *(Cucurbita pepo)* seedlings had the highest activity of the various plant materials tested, and they were therefore used as a source of the enzyme for further investigations. Mevalonic kinase from pumpkin seedlings was partially purified, and its properties were investigated. Maximum activity is obtained with ATP as the phosphate donor, though several other nucleotides can also be utilized. A divalent metal ion is required: Mn^{2+} is the most effective, but several others, including Mg^{2+}, can replace it. The optimum pH for the enzyme is about 5.7. The enzyme remains in the supernatant solution after fractionation of the cell components by centrifugation, suggesting that it is associated with the soluble cytoplasm. The product of the enzymic reaction contains by analysis one mole of phosphate per mole of mevalonate, and gives R_F values in paper chromatography which agree with published values for 5-phosphomevalonic acid. With crude enzyme extracts this product is partly converted into a second derivative, which is tentatively identified, from R_F values, as 5-pyrophosphomevalonic acid.

INTRODUCTION

Extensive investigations of cholesterol biosynthesis and less-extensive work on other isoprenoid compounds indicate that the biological "isoprene unit" arises from acetyl coenzyme A *via* mevalonic acid (3-methyl-3,5-dihydroxyvaleric acid). Recent investigations have implicated several phosphorylated derivatives of mevalonic acid as intermediates in the biosynthesis of cholesterol and squalene in animals and yeast. These include (listed in the sequence of formation from mevalonate): 5-phosphomevalonic acid[1,2], 5-pyrophosphomevalonic acid[2], Δ^3-isopentenyl pyrophosphate[2], γ,γ-dimethylallyl pyrophosphate[3,4], geranyl pyrophosphate[3], and farnesyl pyrophosphate[3]. Other isoprenoid compounds, including several plant products, appear to be synthesized from the same precursors. For example, one or more of the cholesterol precursors have been reported to be incorporated into rubber[5-7], plant sterols[8],

* Present address: Science-Mathematics Division, Southern Oregon College, Ashland, Oregon (U.S.A.).

carotenoids[9–14], monoterpenes[15,16], phytoene[17], and lycopersene[18]. Mevalonic kinase, the enzyme responsible for the initial phosphorylation of mevalonic acid to form 5-phosphomevalonic acid, has been partially purified from yeast[1] and liver[19,20] and its properties investigated.

The ability of higher plants to utilize mevalonate, and of crude cell-free preparations to utilize certain phosphorylated derivatives of mevalonate[7,17], suggested that the same enzymic pathway for metabolism of mevalonic acid might be found in plants. We wish to report the finding of mevalonic kinase in higher plants and to describe some of the properties of the enzyme.

MATERIALS AND METHODS

dl-[2-^{14}C]Mevalonic acid was obtained in the form of the *N,N'*-dibenzylethylenediamine salt from Tracerlab, Inc., Waltham, Mass. (U.S.A.). Unlabeled mevalonic acid, also as the dibenzylethylenediamine salt, was obtained through the courtesy of Dr. K. FOLKERS of the Merck Sharpe and Dohme Research Laboratories, Rahway, N.J. (U.S.A.). These were converted into the potassium salt by adjusting an aqueous solution to a pH of about 11 with KOH, extracting the free amine with ether, removing traces of ether under vacuum at room temperature, and then neutralizing the solution with HCl.

ATP and ADP, both as sodium salts, were obtained from Sigma Chemical Company, St. Louis, Mo. (U.S.A.). CTP, GTP, ITP, and UTP, as sodium salts, were obtained from Pabst Laboratories, Milwaukee, Wis. (U.S.A.).

The buffer used in most experiments was a mixture of maleic acid and Tris, 0.4 *M* with respect to each component, adjusted to the desired pH with KOH.

Plant tissues were dried with cold acetone by standard procedures described previously[21], and the resulting "acetone powders" were stored in tightly-capped jars in a freezer until needed. The preparation used for most of the investigations was made as follows: Pumpkin (*Cucurbita pepo*, var. Sugar Pumpkin) seedlings grown in the dark were harvested at an age of 10 days, and the cotyledons were acetone dried. 5 g of the dry powder (corresponding to 28.2 g fresh wt.) were suspended in 50 ml of ice-cold 1% $KHCO_3$ solution for 15 min and then squeezed through fine cotton cloth. The residue was reextracted several times with 1% $KHCO_3$ to yield a final volume of about 100 ml. This milky suspension was dialyzed for 16 h against ice-cold deionized water and then centrifuged for 20 min at 25 000 × *g*. The supernatant solution was fractionated by adjusting it to pH 5.6 with a small amount of 1 *N* acetic acid. Centrifugation for 15 min at 25 000 × *g* yielded a copious white precipitate and a clear, almost colorless, supernatant solution. This supernatant solution was adjusted to pH 7 with KOH, which caused it to turn somewhat yellow. Most of the yellow color could be removed by treatment with charcoal. 52 ml of the extract plus 2.6 ml of 1 *M* potassium phosphate buffer (pH 6.5) were stirred with 520 mg of Norit A charcoal and filtered immediately. The extract was again dialyzed overnight against ice-cold deionized water and filtered again through a fine sintered-glass filter. All operations described above were carried out in the cold. Centrifugation was with a Servall high-speed refrigerated centrifuge. The final solution contained 1.5 mg of protein per ml and accounted for 5% of the protein present in the acetone powder or 12% of that in the crude extract.

The method of assaying mevalonic kinase activity was similar to that used by TCHEN[1]. Labeled mevalonate, enzyme, and the other components of the reaction mixture were pipeted into 12-ml conical centrifuge tubes chilled in dry ice. The total volume was in most cases 0.1 ml in each tube. The reaction was started by placing the tubes in a water bath and mixing the components thoroughly as soon as they melted. The reaction was allowed to proceed in the water bath and was stopped by placing the tubes in boiling water for 1 min. If the protein precipitate was large it was centrifuged down. The supernatant liquid was removed with a capillary dropper, the precipitate was washed once or twice by the same process with one or two drops of water, and the several supernatant solutions were combined. The solutions were concentrated under a stream of air at room temperature, and chromatographed on Whatman No. 1 filter paper. The solvent used was either *n*-butanol–90% formic acid water (77 : 10 : 13, v/v)[1] or *tert.* butanol–90% formic acid water (40 : 10 : 16, v/v)[2], and ascending development was used.

The position of radioactive components was determined by radioautography with Kodak no-screen medical X-ray film. Radioactivity was counted directly on the paper, using for the earlier experiments a Tracerlab TGC-14 ultra-thin window counter and later a Tracerlab LSC-20 liquid scintillation counter[22]. In using the scintillation counter, small shell vials, 18 × 52 mm, were fitted inside the regular screw-cap vials. Radioactive areas of the chromatograms were cut into sections 15.5 × 40 mm, which fit snugly into these inner vials, and the inner vials then filled with a solution containing 3 g of terphenyl and 30 mg 2,2′-paraphenylene-bis-5-phenyloxazole (POPOP) in a liter of toluene. This method has been shown to give about 50% counting efficiency if the radioactivity remains on the paper. The phosphate derivatives of mevalonic acid are not eluted by toluene. Mevalonic acid itself is partially eluted, resulting in variable counting efficiency; it was kept on the paper by spraying the spots very lightly with alcoholic NaOH. Quenching was checked by counting the samples with and without "overpulse reject" and determining that the ratio between the two counts was constant. It could then be assumed that quenching was constant. From the counts, the percentage conversion of the starting material and thence the molar amount of product were calculated.

To characterize the reaction product, 2 μmoles of labeled mevalonate (2.5 μC) plus 18 μmoles of unlabeled mevalonate were added to enzyme extract, buffer, manganous sulfate, and ATP, in a volume of 2.0 ml, and allowed to react for 2 h. The product was isolated by successive paper chromatography first with the *n*-butanol–formic acid–H_2O system described above and then with methanol–28% NH_4OH–H_2O (60 : 10 : 30, v/v)[1]. Remaining traces of adenine nucleotides were removed by treatment with Norit A charcoal (checked by lack of absorption at 260 mμ, using a Beckman Model-DU spectrophotometer). Mevalonate was determined by radioactivity measurements; a portion of the product was digested with concentrated sulfuric acid and used for phosphate determination by the method of MARTIN AND DOTY as modified by ERNSTER *et al.*[23].

Cell fractions of pumpkin seedlings were obtained by differential centrifugation of buffered sucrose homogenates. 13 g of cotyledons were taken from seedlings about one week old which had been grown in the dark. These were ground carefully in a cold mortar with sand and 20 ml of 0.4 *M* sucrose–0.1 *M* potassium phosphate (pH 7.4). Large debris was removed by squeezing through cotton cloth, and the

residue was washed once with the sucrose buffer solution. The suspension was centrifuged for 5 min at about 60 × *g* and the resulting precipitate discarded. The supernatant liquid was centrifuged for 15 min at a maximum relative centrifugal force of 25 600 × *g* in a Servall refrigerated centrifuge. The precipitate was removed, and the remaining supernatant solution was centrifuged again, this time for 90 min at a maximum of 105 000 × *g* in a Spinco preparative ultra-centrifuge. Each precipitate was resuspended in sucrose–buffer solution to the volume of the original extract. A fatty layer which rose to the surface at 25 600 × *g* was discarded. The fractions were dialyzed overnight against cold deionized water and stored frozen for several days before they were assayed. Before assay, water was added as needed so that all fractions were of equivalent concentration with respect to the fresh tissue. The final concentration corresponded to about 220 mg fresh tissue per ml or about 11 mg fresh tissue per reaction tube.

Protein determinations were made by a microkjeldahl method[21] or by the ultraviolet-absorption method of Warburg and Christian[23].

EXPERIMENTAL RESULTS

The enzymic nature of the mevalonic kinase activity obtained from pumpkin seedlings is evidenced by its heat-lability. Heating the extracts for 5 min at 100° completely destroys the kinase activity.

The identification of the reaction product as 5-phosphomevalonic acid is based on several observations. Both mevalonic acid and ATP are required for its formation. R_F values in the three different chromatography solvents which were used agree with those reported in the literature[1,2] for 5-phosphomevalonic acid. Analysis of the isolated product showed a phosphorus to mevalonate ratio of 1 : 1. The phosphate is not energy rich, *i.e.*, it was not hydrolyzed in 7 min at 100° in 1 *N* HCl.

In experiments with crude extracts of pumpkin seedlings, a second derivative appeared, which judging from its R_F values in two solvent systems and from the kinetics of its formation, may be 5-pyrophosphomevalonic acid. Phosphomevalonate, isolated chromatographically from enzyme reaction mixtures and used as a substrate for the crude extracts, was converted in the presence of Mn^{2+} and ATP to the second derivative.

The results of the enzyme purification procedure are shown in Table I. It will be noted that the total measurable mevalonic kinase activity actually increased during the purification. This was presumably due to removal of some inhibitory agent, possibly ATPase. The presence of slight ATPase activity in the final preparation was shown by omitting mevalonic acid from the reaction mixture and measuring the release of inorganic phosphate. Starting with 2 μmoles of ATP, 1 μmole of phosphate was released in the first hour; after 3 h a total of 2 μmoles of inorganic phosphate had been formed. Since the enzyme preparation also contained adenylic kinase, there was presumably still some ATP present even after 3 h. The presence of adenylic kinase was shown spectrophotometrically by coupling it with added hexokinase and glucose-6-phosphate dehydrogenase[26]. The ability of ADP to act as a phosphate donor in this assay system indicates that the pumpkin extract contained adenylic kinase and was thus able to form ATP from ADP.

Several nucleoside triphosphates, as well as ADP, can act as phosphate donors

TABLE I

PURIFICATION OF MEVALONIC KINASE

The reaction mixture contained: enzyme extract, 50 μl; buffer, Tris plus maleate, 3 μmoles of each (pH 7.8); $MnSO_4$, 0.5 μmole; ATP, 2 μmoles; mevalonic acid, 205 mμmoles (250 mμC). Total volume, 0.1 ml. Temperature, 35°. Reaction time, 105 or 120 min. The activities are from two different experiments and have been converted to comparable percentage figures. The activity of the dialyzed crude extract (which formed 15 mμmoles of 5-phospho-mevalonic acid in 105 min) is defined as 100% activity. Protein figures also are based on the dialyzed crude extract as 100%. Actually this extract contained only 41% of the protein originally present in the acetone powder.

Fraction	*Protein (% of original)*	*Kinase activity (% of original)*
Dialyzed crude extract	100	100
pH-5.6 supernatant	23	326
pH-5.6 precipitate	—	100
pH-5.6 supernatant, treated with charcoal	12	290

for the pumpkin mevalonic kinase, though the highest activity was obtained with ATP. Fig. 1 compares the effectiveness of several levels of ADP and ATP. It can be seen clearly that there is no activity in the absence of a nucleotide, and that the activity with ATP is several times greater than that with ADP. The ADP activity is presumably due to adenylic kinase. Simultaneous addition of ADP and ATP at sub-optimal concentration (each 0.01 *M*) resulted in phosphorylation approximately equal to the sum of that obtained with the two when added individually. However, 0.02 *M* ADP when added to 0.02 *M* ATP (optimal for each alone) caused about 70% inhibition of the ATP activity. This may be compared to 55% inhibition by

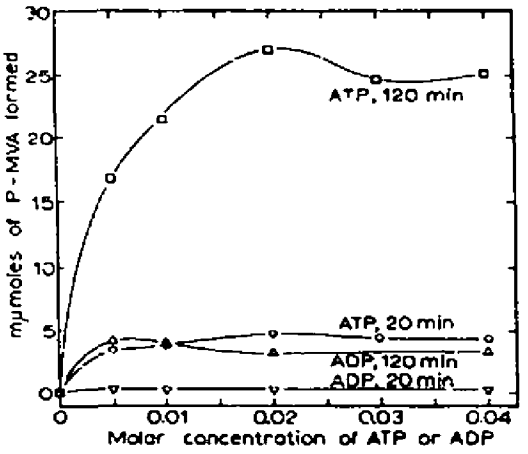

Fig. 1. ATP and ADP as phosphate donors. The reaction mixture contained: enzyme extract, 30 μl (45 μg protein); buffer, Tris plus maleate, 16 μmoles of each (pH 6.2); $MnSO_4$, 0.5 μmole; ATP or ADP, as indicated; mevalonic acid, 102 mμmoles (125 mμC). Total volume 0.1 ml. Reaction times, 20 min and 120 min as indicated. Temperature 37°. P-MVA, 5-phospho-mevalonic acid.

0.02 M sodium pyrophosphate when added to 0.02 M ATP. Either ATP or Mn^{2+} added in excess of the optimum amount is also inhibitory. It seems likely that all of these inhibitory effects are due to formation of inactive manganous pyrophosphate complexes.

In Table II, ATP is compared with other nucleoside triphosphates as the

TABLE II

NUCLEOTIDE SPECIFICITY OF MEVALONIC KINASE

The reaction mixture contained: enzyme extract, 60 µl (90 µg protein); buffer, Tris plus maleate, 17 µmoles of each (pH 6.2); $MnSO_4$, 0.4 µmole; nucleoside triphosphate, as indicated; mevalonic acid, 172 mµmoles (210 mµC). Total volume, 0.11 ml. Reaction time, 105 min. Temperature, 36°.

Nucleotide	5-Phospho-mevalonic acid formed (mµmoles)				
	ATP	ITP	GTP	CTP	UTP
2.0 µmoles (0.02 M)	35.2	20.0	12.9	13.5	5.5
0.2 µmole (0.002 M)	19.0	19.0	12.8	10.0	6.6

phosphate donor for mevalonic kinase. At 0.02 M, the concentration previously shown to be optimal for ATP, ATP is seen to be superior to the other donors. Failure to observe a similar superiority of ATP at lower concentration may be due to destruction by ATPase. Added ADP in catalytic amounts (0.02 µmole of ADP added to 2.0 µmoles of CTP or ITP) did not stimulate phosphorylation by CTP or ITP. This suggests that CTP and ITP are able actually to replace ATP as the phosphate donor, and do not serve merely as sources of energy-rich phosphate for ATP synthesis.

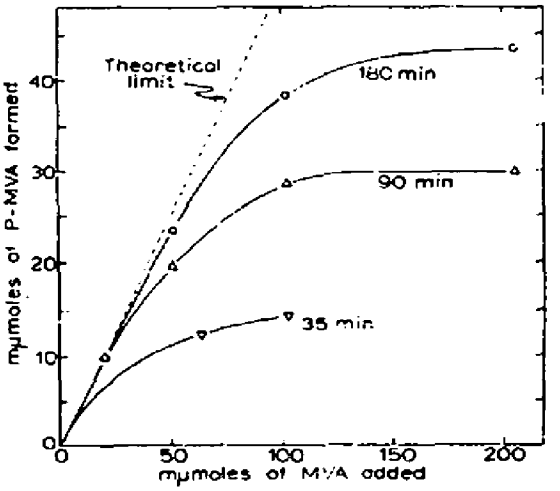

Fig. 2. Effect of mevalonic acid concentration on mevalonic kinase. The reaction mixture contained: enzyme extract, 50 µl (75 µg protein); buffer, Tris plus maleate, 3 µmoles of each (pH 7.8); $MnSO_4$, 0.5 µmole; ATP, 2 µmoles; mevalonic acid, as indicated (specific activity, 1.22 µC/µmole). Total volume, 0.1 ml. Reaction time, as indicated. Temperature, 36°. The line marked "theoretical limit" represents phosphorylation of 50% of the racemic mevalonic acid. MVA, mevalonic acid; P-MVA, 5-phospho-mevalonic acid.

Fig. 2, showing the effectiveness of phosphorylation as a function of mevalonate concentration and time, demonstrates that conditions can be found under which 50% of the added mevalonic acid is phosphorylated. Since synthetic mevalonic acid is a racemic mixture, this corresponds to complete phosphorylation of one of the isomers. Several investigators have reported that only the (+) isomer of mevalonic acid is biologically active[27].

As is generally the case in enzymic reactions involving ATP, the plant mevalonic kinase requires a divalent metal ion. This requirement, and the ability of several metals to fill the requirement, are illustrated in Fig. 3. Manganese is clearly the most effective, the activity with manganous ion being nearly three times that obtained with any of the other metal ions which were tested.

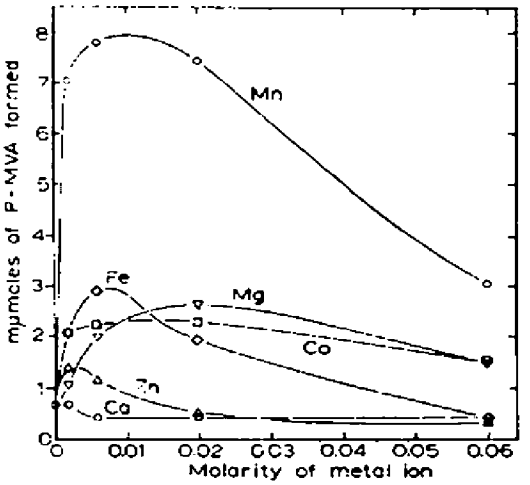

Fig. 3. Metal requirement of mevalonic kinase. The reaction mixture contained: enzyme extract, 50 μl (35 μg protein, purified but not treated with charcoal); buffer, Tris plus maleate, 3 μmoles; of each (pH 7.8); ATP, 2 μmoles; mevalonic acid 156 mμmoles (190 mμC); glutathione, 0.5 μmole. $CaCl_2$, $CoCl_2$, $FeSO_4$, $MgSO_4$, $MnSO_4$, or $ZnSO_4$, as indicated. Total volume, 0.1 ml. Reaction time, 60 min. Temperature, 36°. P-MVA, 5-phospho-mevalonic acid.

The effect of pH on mevalonic kinase is shown in Fig. 4. In this experiment the volume of reaction mixture was increased to 0.2 ml and round-bottom test tubes were used to allow immersion of miniature glass and calomel electrodes. pH measurements were made near the beginning and again near the end of the reaction period. In one tube there was a difference of 0.3 pH unit between the two measurements; all others showed 0.2 unit or less. The figure shows the average of the two pH readings. Actual measurement of the pH was found to be essential. Even though a high concentration of Tris maleate buffer was used, the measured pH of the mixture was lower than the pH of the added buffer, particularly at the higher pH's. Buffer of pH 6.6, for example, gave a measured pH of 6.1 in the mixture, while pH 9.0 buffer gave pH 7.9 in the mixture.

The distribution of mevalonic kinase activity in cell fractions is shown in Table III. The bulk of the activity is seen to remain in the supernatant solution even after centrifugation for 90 min at 105 000 × g. This fraction corresponds approxi-

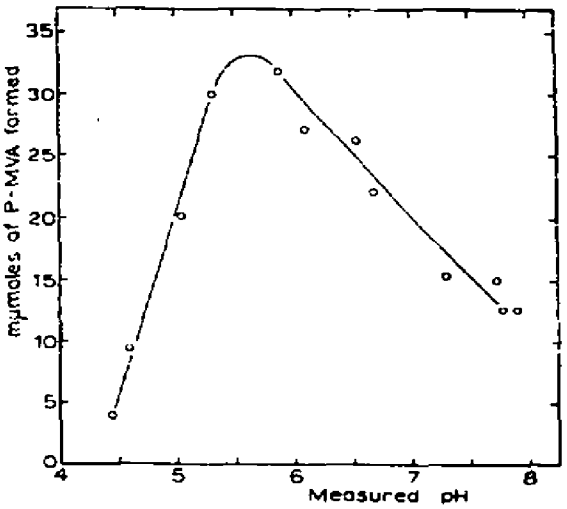

Fig. 4. The effect of pH on mevalonic kinase activity. The reaction mixture contained: enzyme extract, 60 μl (90 μg protein); buffer, Tris plus maleate, 40 μmoles of each; $MnSO_4$, 1 μmole; ATP, 4 μmoles; mevalonic acid, 205 mμmoles (250 mμC). Total volume, 0.2 ml. Reaction time, 75 min. Temperature, 37°. P-MVA, 5-phospho-mevalonic acid.

mately to the soluble cytoplasm or hyaloplasm. Thus it can be concluded that the plant mevalonic kinase is not particulate but is associated with the soluble cytoplasm.

Mevalonic kinase from pumpkin seedlings is a very stable enzyme. Extracts stored for a year or longer in the freezer are still highly active. The addition of compounds such as thioethanolamine or glutathione to protect sulfhydryl groups seems to be neither helpful nor harmful. Glutathione was observed in some experiments to be somewhat inhibitory and in others to be somewhat stimulatory. The effects undoubtedly were due, at least in part, to changes in pH, as changing the buffer

TABLE III

DISTRIBUTION OF MEVALONIC KINASE IN CELL FRACTIONS

The reaction mixture contained: enzyme, 50 μl of the indicated fraction, corresponding to about 11 mg of fresh tissue; buffer, Tris plus maleate, 12 μmoles of each (pH 6.2); $MnSO_4$, 0.5 μmole; ATP, 2 μmoles; mevalonic acid, 102 mμmoles (125 mμC). Total volume, 0.1 ml. Reaction time, 80 min. Temperature, 36°. Values shown are the average of duplicates.

Fraction	*5-Phospho-mevalonic acid formed (mμmoles)*
Homogenate, cleared at 60 × *g*	32.4
Supernatant, 25 600 × *g*	31.6
Precipitate, 25 600 × *g*	1.9
Supernatant, 105 500 × *g*	32.4
Precipitate, 105 500 × *g*	1.5

concentration modified the glutathione effects. When the glutathione was carefully neutralized beforehand and the reaction mixture heavily buffered, 0.015 M glutathione caused only a slight stimulation, of doubtful significance. Relatively high concentrations of iodoacetamide, 0.01–0.02 M, caused inhibition of 20–40%. *p*-Chloromercuribenzoate at 10^{-4} M inhibited by 89%, while the same concentration of mercuric chloride inhibited by 98%. Apparently the inhibition by Hg^{2+} is not due to competition with Mn^{2+}, as increasing the concentration of Mn^{2+} by 5-fold, from $0.5 \cdot 10^{-3}$ M up to $2.5 \cdot 10^{-3}$ M, had no effect on the inhibition by 10^{-4} M $HgCl_2$.

Although pumpkin seedlings were the best source of mevalonic kinase among the plant materials tested, activity was found in extracts prepared from acetone powders of several other species. It was demonstrated without difficulty in extracts from Douglas fir *(Pseudotsuga menziesii)* needles and spinach *(Spinacia oleracea)* leaves. Several assays were made on mint species, in connection with investigations of monoterpene biosynthesis[28]. However, mint extracts browned very rapidly, and it was difficult to find any enzyme activity. Under conditions used with the pumpkin enzyme, peppermint *(Mentha piperita)* leaves were inactive and pennyroyal *(Mentha pulegium)* leaves were slightly active. Considerably better activity was obtained from pennyroyal when extracts were prepared in the presence of sodium cyanide and ascorbic acid to prevent oxidation.

DISCUSSION

The mevalonic kinase from pumpkin seedlings is seen, on comparison, to resemble yeast mevalonic kinase[1] more closely than liver mevalonic kinase[19,20]. Both the yeast enzyme and the plant enzyme can use any of several nucleoside triphosphates as the phosphate donor, while the liver enzymes are reported to be active only with ATP or ITP.

Considerably more than the stoichiometric amount of ATP must be added to obtain maximum activity with any of the mevalonic kinases. A higher concentration of ATP was required with the yeast and pumpkin enzymes than with the liver enzymes, but it is impossible to say, without further investigation, whether the observed differences represent fundamental differences in the affinity of the enzymes for ATP, or only differences in the experimental conditions. For example, there are clearly interactions between ATP and divalent metal ions, and a relative excess of either is inhibitory. In general it can be stated for all of the mevalonic kinases that optimum activities are obtained when the ratio $(ATP)/(Mn^{2+})$ is from 2 to 4, or the ratio $(ATP)/(Mg^{2+})$ is between 1 and 2. These concentration ratios may be more important than the absolute concentrations.

Mevalonic kinase as obtained from liver is very sensitive to oxidation. The pig-liver enzyme is inhibited by oxygen, and this inhibition can be overcome by cysteine or glutathione[19]. Overnight dialysis of the rabbit-liver enzyme[20] resulted in considerable loss of activity unless both cysteine and phosphate buffer were present. Neither the yeast enzyme nor the pumpkin enzyme is sensitive to oxygen, but both are strongly inhibited by *p*-chloromercuribenzoate, as are the liver enzymes. With the plant enzyme, mercuric chloride inhibits even more strongly than *p*-chloromercuribenzoate.

The optimum pH for the plant mevalonic kinase is lower than has been reported

for either yeast or liver mevalonic kinases. We found that even with high concentrations of buffer the true pH of the reaction mixture could only be obtained by measurement and was considerably lower than the buffer pH. This is undoubtedly due to the high buffering capacity of the ATP, mevalonic acid and protein present. In the reports of liver and yeast mevalonic kinases it is not clear whether the pH values were measured. If the final pH was assumed to be the same as the buffer pH, our results suggest that the values thus obtained might easily be in error by as much as 0.5 to 1 pH unit. Thus the difference between the plant enzyme and the liver enzymes with respect to pH optimum might be less than the reported data indicate.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the National Science Foundation (NSF G-9878) and the Graduate School of Oregon State University.

REFERENCES

1 T. T. TCHEN, *J. Biol. Chem.*, 233 (1958) 1100.
2 K. BLOCH, S. CHAYKIN, A. H. PHILIPS AND A. DE WAARD, *J. Biol. Chem.*, 234 (1959) 2595.
3 F. LYNEN, B. W. AGRANOFF, H. EGGERER, U. HENNING AND E. M. MÖSLEIN, *Angew. Chem.*, 71 (1959) 657.
4 B. W. AGRANOFF, H. EGGERER, U. HENNING AND F. LYNEN, *J. Biol. Chem.*, 235 (1960) 326.
5 R. B. PARK AND J. BONNER, *J. Biol. Chem.*, 233 (1958) 340.
6 R. G. O. KEKWICK, B. L. ARCHER, D. BARNARD, G. M. C. HIGGINS, G. P. MCSWEENEY AND C. G. MOORE, *Nature*, 184 (1959) 268.
7 U. HENNING, E. M. MÖSLEIN, B. ARREGUIN AND F. LYNEN, *Biochem. Z.*, 333 (1961) 534.
8 T. W. GOODWIN, *Biochem. J.*, 70 (1958) 612.
9 G. B. BARLOW AND A. D. PATRICK, *Nature*, 182 (1958) 662.
10 G. D. BRAITHWAITE AND T. W. GOODWIN, *Biochem. J.*, 76 (1960) 5.
11 G. D. BRAITHWAITE AND T. W. GOODWIN, *Biochem. J.*, 76 (1960) 194.
12 E. A. SHNEOUR AND I. ZABIN, *J. Biol. Chem.*, 234 (1959) 770.
13 A. E. PURCELL, G. A. THOMPSON, JR. AND J. BONNER, *J. Biol. Chem.*, 234 (1959) 1081.
14 D. G. ANDERSON, D. W. NORGARD AND J. W. PORTER, *Arch. Biochem. Biophys.*, 88 (1960) 68.
15 R. G. STANLEY, *Nature*, 182 (1958) 738.
16 A. J. BIRCH, D. BOULTER, R. I. FRYER, P. J. THOMSON AND J. L. WILLIS, *Tetrahedron Letters*, No. 3 (1959) 1.
17 D. G. ANDERSON AND J. W. PORTER, *Federation Proc.*, 20 (1961) 350.
18 E. C. GROB, K. KIRSCHNER AND F. LYNEN, *Chimia*, 13 (1960) 308.
19 H. R. LEVY AND G. POPJÁK, *Biochem. J.*, 75 (1960) 417.
20 K. MARKLEY AND E. SMALLMAN, *Biochim. Biophys. Acta*, 47 (1961) 327.
21 W. D. LOOMIS, *Plant Physiol.*, 34 (1959) 541.
22 C. H. WANG AND D. E. JONES, *Biochem. Biophys. Research Commun.*, 1 (1959) 203.
23 L. ERNSTER, R. ZETTERSTRÖM AND O. LINDBERG, *Acta Chem. Scand.*, 4 (1950) 942.
24 A. STEYERMARK, *Quantitative organic microanalysis*, Blakiston, New York, 1951, p. 134.
25 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, Inc., New York, 1957 p. 451.
26 P. K. STUMPF, in W. D. MCELROY AND B. GLASS, *Phosphorus Metabolism*, Vol. II, Johns Hopkins Press, Baltimore, Md., 1952, p. 38.
27 A. F. WAGNER AND K. FOLKERS, *Advances in Enzymol.*, 23 (1961) 471.
28 J. BATTAILE AND W. D. LOOMIS, *Biochim. Biophys. Acta*, 51 (1961) 545.