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METABOLISM OF MEVALONIC ACID TO PHOSPHORYLATED INTERMEDIATES IN A CELL-FREE EXTRACT FROM *NEPETA CATARIA* LEAVES*

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Key Word Index—*Nepeta cataria*, Labiatae, mevalonic acid phosphates, isopentenyl pyrophosphate

Abstract—A cell-free extract has been prepared from leaves of *Nepeta cataria* plants which converts mevalonic acid (MVA) to mevalonic acid phosphate (MVAP), mevalonic acid pyrophosphate (MVAPP) and isopentenyl-pyrophosphate (IPP) These enzymes are found in the 30000*g* supernatant The activities are maximal at pH 7 and the formation of mevalonic acid pyrophosphate and isopentenyl-pyrophosphate reaches a maximal level after an incubation time of 180 min whereas the level of mevalonic acid phosphate begins to decrease after 90 min.

INTRODUCTION

THE METHYLCYCLOPENTANE monoterpenoid, nepetalactone, is formed from mevalonic acid-[2-^{14}C] in flowering *Nepeta cataria* plants;[1] however, the per cent incorporation is very low and there is limited randomization of carbon-14 in carbon atoms 3 and 8 and carbon atoms 6 and 9. These findings do not support the mechanism of isomerization of isopentenylpyrophosphate proposed by Agranoff *et al.*[2] and established by Shah *et al.*[3] There are, to our knowledge, no previous reports on the stepwise metabolism of mevalonic acid *in vitro* that produce phosphorylated intermediates and methylcyclopentane monoterpenoids. The requirement for the enzymes which control the first steps of the biosynthesis of monoterpenoids has not been established Studies were initiated in this laboratory to elucidate this pathway, by cell-free extracts in order to clarify the discrepancy that occurs *in vivo* with limited randomization of the isoprenoid methyl carbon atoms. This paper reports the progress with a cell-free system from leaves of *N. cataria* plants, which shows mevalonic acid kinase, mevalonic phosphate kinase and mevalonic acid pyrophosphate decarboxylase activities.

RESULTS AND DISCUSSION

Phosphorylation of mevalonic acid

Incubation of cell-free extracts from *Nepeta cataria* leaves with [2-^{14}C]mevalonic acid, Mg^{2+}, Mn^{2+}, ATP, NADP and dithiothreitol resulted in the production of phosphory-

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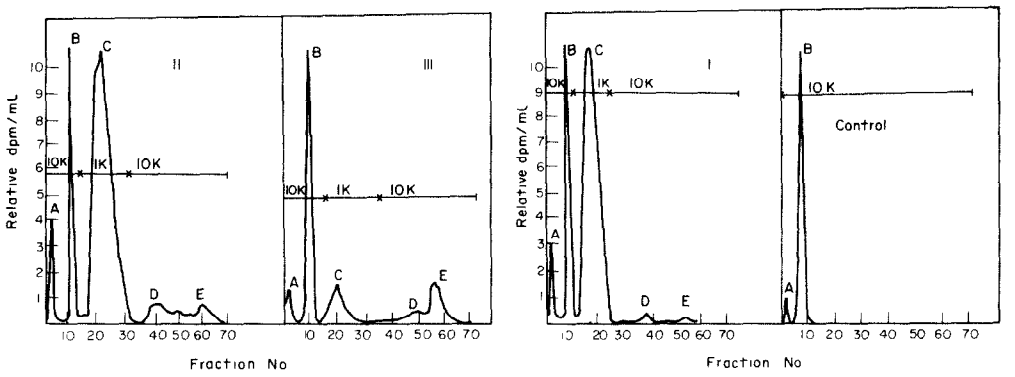

FIG 1 ION EXCHANGE COLUMN CHROMATOGRAPHY (0.5 × 5 cm) OF [2-^{14}C]MEVALONIC ACID INCUBATION WITH CELL FREE EXTRACTS (SEE EXPERIMENTAL) AT VARIOUS TIMES OF INCUBATION I 15 MIN, II, 60 MIN, III 180 MIN
Arrows indicate when solvent changes occurred

lated products. The intermediates were identified by elution from a Dowex-1-formate column and in five paper chromatographic systems The 3000 *g* pellet contained only mevalonic kinase activity whereas the 30000 *g* supernatant produced phosphorylated compounds to isopentenyl pyrophosphate Therefore, the 30000 *g* supernatant was used in all subsequent experiments

Figure 1 shows the results of removing samples from a 30000 *g* supernatant incubation with [^{14}C]mevalonic acid at 15, 60 and 180 min and chromatographing these aliquots through a Dowex-1-formate column as described by Suzuc.[4] At 15 min a large amount of radioactivity resides in peak C which was identified by Suzue[4] as mevalonic acid phosphate (Peaks A, B, D and E have been identified as mevalonolactone, mevalonic acid, mevalonic acid pyrophosphate and isopentenyl pyrophosphate respectively) Our control experiments showed only mevalonolactone and mevalonic acid.

Assuming that one half of the total radioactivity in DL-[^{14}C]MVA is utilized in the biological reaction the percent of available radioactivity that is utilized in the conversion of mevalonic acid, mevalonic acid phosphate, mevalonic acid pyrophosphate and isopentenyl pyrophosphate at various time intervals was calculated At 30 min there is a buildup of mevalonic acid phosphate and a sizeable decrease in mevalonic acid whereas at 180 min mevalonic acid phosphate decreases and mevalonic acid pyrophosphate as well as isopentenyl pyrophosphate increases Beyond 180 min no increase in mevalonic acid pyrophosphate or isopentenyl pyrophosphate was found

Each of the peaks eluted from a Dowex-1-formate column following a 180 min incubation was chromatographed (on paper) in five solvents The peaks B–E were identified as mevalonic acid, mevalonic acid phosphate, mevalonic acid pyrophosphate and isopentenyl pyrophosphate respectively by comparison with published data [3 13] We observed greater

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production of phosphorylated compounds in the presence of dithiothreitol with an absolute requirement for ATP. The results obtained with ATP and dithiothreitol are, in general, in good agreement with those found by Garcia and Major.[14]

The production of phosphorylated intermediates indicates that the cell extract contains kinase enzymes which catalyze the phosphorylation of mevalonic acid to mevalonic acid phosphate, mevalonic acid pyrophosphate and decarboxylation to isopentenyl pyrophosphate. We did not conclusively identify dimethylallyl pyrophosphate; however, since we used dithiolthreitol it is conceivable that the isopentenyl pyrophosphate isomerase would be active. On the other hand, if the isomerase is not present then the production of the unequal labeling in the methyl carbon atoms of nepetalactone (atoms 3 and 8 and atoms 6 and 9) could be explained.[1] These results demonstrate the presence of the mevalonic acid phosphorylation system in a cell-free extract of *N. cataria* and that 95% of the available mevalonic acid is converted to phosphorylated intermediates after 180 min of incubation.

EXPERIMENTAL

Materials and methods DL-Mevalonic acid-[2-^{14}C] was purchased from New England Nuclear, Boston, Massachusetts as the dibenzoylethlenediamine salt with a specific activity of 6·33 mc/mm Enzymes and cofactors were purchased from Sigma Chemical, St Louis, Missouri Poly Clar AT was a gift from GAF Corporation, New York. Protein was determined by the method of Lowry *et al*[15]

Extraction and chromatography A 20·5 g sample of fresh leaves of *Nepeta cataria* was ground with 2·5 g of Polyclar AT and sand in the presence of 20 ml of grinding soln consisting of 10 mM KCl, 5 mM $MgCl_2$, 500 mM sucrose, 1 mM EDTA, 5 mM Dithiothreitol and 0·1% Triton X100 in 500 mM phosphate buffer pH 7·5. The homogenate was squeezed through six layers of cheese cloth. The filtrate (18 ml) was centrifuged at 120 *g* for 20 min and the pellet was discarded The supernatant was then centrifuged at 3000 *g* for 20 min, the pellet discarded and the supernatant centrifuged at 30000 *g* for 20 min The incubation medium contained 4·0 ml incubation soln (3·4 mM $MgCl_2$, 3·4 mM $MnCl_2$, 20·7 mM KF, 3·4 mM dithiothreitol, 20·7 mM ATP, and 6·9 mM $NADP^+$ in 20 mM phosphate buffer pH 7·2), 0·4 ml of ^{14}C-MVA (1 μC), 5 ml of 30 K supernatant (17·5 mg protein/ml) and 0·6 ml of 20 mM phosphate buffer pH 7·2 At 15, 30, 60, 90, 120, 150 and 180 min 1·0 ml aliquots were removed and the enzymic reaction stopped by immersing the solution in a boiling H_2O bath for 4 min The protein was removed by centrifugation at 500 *g* and the pellet was washed with 2 ml of H_2O. The pooled rinse and supernatant were lyophilized The lyophilized samples were dissolved in a 1·0 ml of H_2O and applied to a 0·5 × 5 cm Dowex-1-formate column as described by Suzue *et al*[4] This column was eluted stepwise, at 4°C, with (A) 25 ml of H_2O, (B) 25 ml of 2 N formic acid, (C) 50 ml of 4 N formic acid, (D) 50 ml of 4 N formic acid + 0·4 M ammonium formated and finally (E) 50 ml of 4 N formic acid + 0·8 M ammonium formate Three ml fractions were collected and 50 μl was removed for scintillation counting The fraction containing peaks A–E obtained from the Dowex-1-formate column were collected and treated batchwise with Dowex-50 (H^+) to remove the ammonium ions before lyophilization The lyophilized samples were chromatographed on paper by descent for identification of the phosphorylated inter mediates. Solvents were *n*-PrOH–NH_4OH–H_2O (3·1·1), *n*-PrOH–NH_4OH–H_2O (6·3:1), *n*–BuOH–HCO_2H–H_2O (77·10·13), *t*–BuOH–HCO_2H–H_2O (20.5·8) and *i*-butyric acid–NH_4OH–H_2O (66:3·30)

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