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CELLULAR DISTRIBUTION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE AND MEVALONATE KINASE IN LEAVES OF *NEPETA CATARIA*

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Key Word Index—*Nepeta cataria*, Labiatae, HMG-CoA reductase, mevalonate kinase, sucrose gradient, ion exchange chromatography assay

Abstract—In *Nepeta cataria* leaf tissue there are two separate activities of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and mevalonate (MVA) kinase respectively as determined by the use of a 20–45% discontinuous sucrose density gradient Cell-free extracts of leaf and callus tissue were prepared and HMG-CoA reductase and MVA kinase activities were compared to activities in extracts from porcine livers and yeast autolysates Callus tissue from *N cataria* has only one peak of HMG-CoA reductase and MVA kinase activity located at the top of the sucrose density gradient Isolated chloroplast from *N cataria* leaves have one peak of HMG-CoA reductase and MVA kinase activity, located near the bottom of a sucrose density gradient MVA kinase activities in porcine livers and yeast autolysate also showed only one activity profile, located at the top of the sucrose gradient Partial purification of the leaf extract through the use of differential centrifugation, 30–70% ammonium sulfate precipitation and Bio-Gel P-100 column chromatography shows that MVA kinase, 5-phosphomevalonate (MVAP) kinase and 5-pyrophosphomevalonate (MVAPP) decarboxylase activities remain in the same fractions The extra-chloroplastidic HMG-CoA reductase activity may be separated from MVA kinase activity by differential centrifugation These results suggest the presence of two HMG-CoA reductase and MVA kinase enzymes in *N cataria* leaf tissue—one located in the chloroplast and a second being extra-chloroplastidic

INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A reductase [mevalonate NADP oxidoreductase (acylating CoA) EC 1 1 1 34, HMG-CoA reductase] has been extensively studied in relation to cholesterol biosynthesis and is the major point of feedback control by cholesterol and/or its derivatives [1, 2]

HMG-CoA reductase in rat intestinal crypt cells is located in the microsomal and mitochondrial fractions [3] In *Saccharomyces ceraviseae* [4] and *Tetrahymena pyriformis* [5], HMG-CoA reductase is also present in mitochondria and microsomes On the other hand, Suzuki and Uritani [6] found HMG-CoA reductase in sweet potato roots only in the mitochondria, however, in diseased tissue (infected with *Ceratocystis fimbriala*), HMG-CoA reductase was found in both mitochondrial and microsomal fractions

The localization or compartmentation of enzymes involved in the formation of isopentenyl diphosphate within the plant cell is not unequivocal [7–9] Mevalonate kinase is located in the cytosol and chloroplasts of leaf tissue [10–12] Mevalonate kinase activity in leaf tissue [13] and leaf callus tissue [14] of *Nepeta cataria* has been reported Herein, we report the intracellular distribution of HMG-CoA reductase and mevalonate kinase in *Nepeta cataria* leaf tissue, *N cataria* callus tissue, porcine liver and yeast autolysates A preliminary report has been published [15]

RESULTS

HMG-CoA reductase assay

HMG-CoA reductase activity was determined by the use of Dowex-1-formate ion exchange chromatography to separate the products, mevalonolactone and mevalonate, from the substrate HMG-CoA and its decomposition product 3-hydroxy-3-methyl glutarate (HMG) Rather than using extraction techniques [12] to isolate mevalonolactone we analysed the reaction mixture and all of the products The reaction mixture was placed on a 0 5 × 5 cm Dowex-1-formate (100–200 mesh) column and eluted with a 150 ml gradient consisting of three chambers containing 50 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid respectively, followed by step wise elution with 50 ml each of (a) 4 N formic acid, (b) 4 N formic acid + 0 4 N ammonium formate and, (c) 4 N formic acid and + 0 8 N ammonium formate MVA was eluted first (fractions 3–6) followed by HMG (8–12) and HMG-CoA (57–62) Figure 1 shows the elution profile of a ^{14}C-mevalonate and HMG-CoA solution contained in

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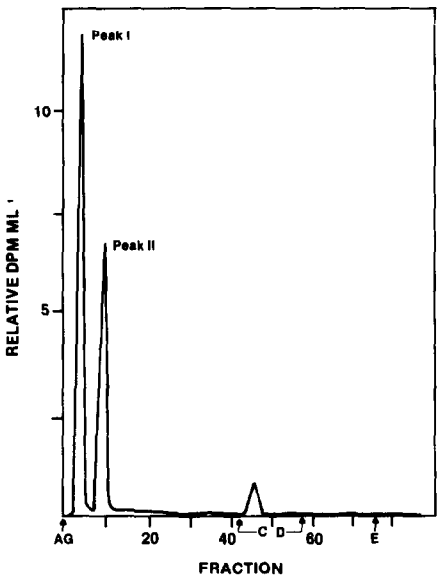

Fig 1 Dowex-1-formate elution pattern of mevalonate and HMG-CoA after incubation at 50 for 3 hr in 100 mM NaOH AG, linear gradient of 0–2 N formic acid using three chambers containing 50 ml H_2O, 50 ml 1N HCOOH and 50 ml 2N HCOOH, C, 50 ml 4N HCOOH, D, 50 ml 4N HCOOH in 0 4 M $HCOONH_4$ and E, 50 ml 4N HCOOH in 0 8 M $HCOONH_4$ Peak I is MVA, Peak II, HMG

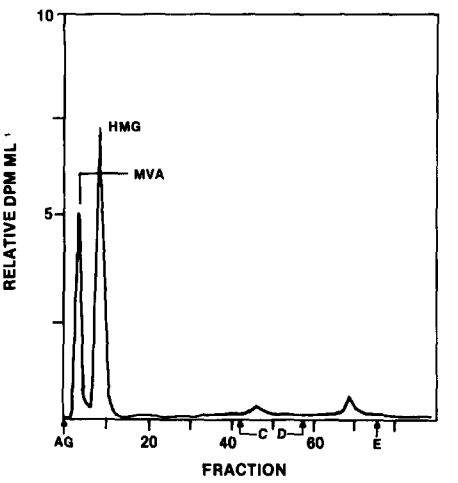

Fig 2 Dowex-1-formate elution profile HMG-CoA reductase assay of 30–70% ammonium sulfate precipitate from leaf tissue (see Experimental for details) Peak II, HMG

100 mM sodium hydroxide and incubated at 50° for 3 hr Mass spectral analysis [13] showed peak I was identical to mevalonic acid, that peak II was 3-hydroxy-3-methylglutarate (HMG), chromatography on paper confirmed these findings

Figure 2 shows a Dowex-1-formate column gradient profile of a reaction mixture used to determine HMG-CoA reductase activity in a 30–70% ammonium sulfate precipitate of a leaf extract This procedure was used for the determination of HMG-CoA reductase activity When the 30–70% ammonium sulfate precipitate is placed on a 20–45% discontinuous sucrose density gradient, two peaks of HMG-CoA reductase activity are observed (Fig 3A) When isolated chloroplasts from *N cataria* leaves are homogenized and placed on a 20–45% discontinuous sucrose density gradient (Fig 3B) only one peak of HMG-CoA reductase activity is observed

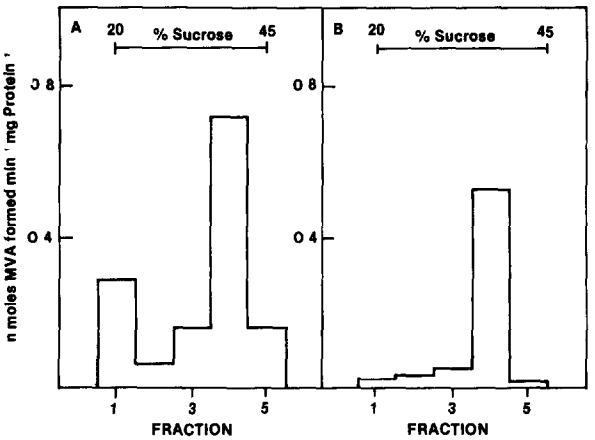

Fig 3 HMG-CoA reductase activity from 20 to 45% sucrose gradient centrifugation of A, 30–70% ammonium sulfate precipitate from leaf tissue, B, homogenized chloroplasts

MVA kinase

The steps used in the partial fraction of mevalonate kinase are shown in Table 1 When the 30–70% ammonium sulfate fraction was centrifuged in a 20–45% discontinuous sucrose gradient (Fig 4A), two peaks of mevalonate kinase activity are observed Results of a Dowex-1-formate column elution profile of an assay of the 30–70% ammonium sulfate precipitated protein are shown in Fig 4C The products indicate the presence of MVA kinase, MVAP kinase and MVAPP decarboxylase activities

Callus tissue from *N cataria* was homogenized and the 30–70% ammonium sulfate precipitate was applied to a 20–45% discontinuous sucrose density gradient Figure 4B shows mevalonate kinase activity distribution in the sucrose gradient Mevalonate kinase activity is located only at the top of the gradient *N cataria* callus tissue do not have chloroplasts Again, the Dowex-1-formate elution profile of the mevalonate kinase assay of fraction one from the sucrose density gradient of callus tissue extract shows the presence of MVA kinase, MVAP kinase and MVAPP decarboxylase (Fig 4D)

Whole chloroplast from *Nepeta cataria* leaves were isolated and homogenized When centrifuged in a 20–30% discontinuous sucrose density gradient, mevalonate kinase activity was found in fraction 3 (Fig 5A), which is identical to the peak of activity observed in whole leaf preparations (Fig 4A) Fraction 3 contains both MVA kinase and MVAP kinase activities as determined by the product identification with a Dowex-1-formate ion exchange column (Fig 5B)

In addition to analysing whole leaf and callus tissue of

Table 1 Partial purification of mevalonate kinase from *N cataria* leaf tissue

Steps	MVA kinase activity (picomol/hr/mg protein)
Part A Fractionation	
1 Crude homogenate	24
2 3000 *g* supernatant	28
3 30000 *g* supernatant	35
4 30–75% ammonium sulfate precipitation	51
5 Sucrose density gradient	
Fraction 1	194
Fraction 3	103
6 Bio-Gel P-100 chromatography of sucrose gradient Fraction 10	1068
Part B Intracellular location of MVA kinase	
1 Crude homogenate	23 5
2 30000 *g* supernatant	31 5
3 150000 *g* pellet (2 hr)	1 0
4 150000 *g* pellet (16 hr)	139 5

N cataria we investigated MVA kinase activities in yeast and hog liver As with *N cataria* callus tissue, only one peak of MVA kinase activity was observed in the sucrose gradient of yeast and hog liver and it was at the top of the gradient

DISCUSSION

We developed a chromatographic method for the separation and direct determination of mevalonate, HMG and HMG-CoA in reaction mixtures Use of this procedure allows us to analyse the reaction mixture directly without using an extraction procedure [16] HMG-CoA reductase and mevalonate kinase in *N cataria* leaf tissue are located both inside and outside the chloroplasts Since chloroplasts are interdependent, it is conceivable that there must be a mechanism for the biosynthesis of carotenoids and the phytyl side chain of the chlorophyll molecule

The location of mevalonate kinase in chloroplast has been convincingly demonstrated by others [10–12, 17], however, our density gradient separation is the first demonstration of two enzymes with different physical properties other than pH optimum The pH optimum distinction has been questioned [12, 17] We did not observe two activities in the density gradient preparations from *N cataria* callus tissue, hog liver or yeast autolysate Our results are in contrast to the suggestions of Kreuz and Kleinig [9] who stated that MVA kinase, MVAP kinase and MVAPP decarboxylase are soluble cytoplasmic enzymes and that they do not occur within the plastids The authors further suggested that isopentenyl diphosphate is synthesized in the cytosol and transported to the plastids for polyprenoid synthesis Their conclusions were based on the observation that ^{14}C-mevalonate was not incorporated into plastid polyprenoids when either pure chloroplasts or chromoplasts were assayed Since they did not determine if MVAP or MVAPP was produced, it is conceivable that the incorporation of ^{14}C-IPP into plastidal polyprenoids would lead to the conclusion that MVA kinase, MVAP kinase and MVAPP decarboxylase are not presented in chloroplasts We did not observe IPP in our chloroplast assay for MVA kinase activity Since MVAPP decarboxylase may be more labile than prenyl transferases and may be a rate controlling enzyme [18] it is conceivable that mevalonate would not be incorporated into polyprenoids in plants!

Table 2 Comparison of HMG-CoA reductase and MVP kinase activities

Source	HMG-CoA reductase (nmol/hr/mg protein)		MVA kinase (nmol/hr/mg protein)	
	Cytosolic	Plastidic	Cytosolic	Plastidic
N cataria leaf	18 0	31 8	12 9	5 4
N cataria callus	1 8	—	8 9	—
Spinach leaf	5 7	9 6	5 3	3 8
Yeast	11 3	—	15 6	—
Porcine liver	26 2	—	43 0	—

Co-purification of MVA kinase, MVAP kinase and MVAPP decarboxylase through the Bio-Gel P-100 column chromatography (Fig 6) step suggests that these enzymes are either physically similar and of high MW or associated in a complex A high MW multi-enzyme complex is consistent with the ideas expressed by Banthorpe *et al* [19] and Francis [20] that 'the enzymes involved in monoterpene biosynthesis may be associated in a multi-enzyme complex with high degree of spatial organization for efficient substrate circulation' Another interpretation is that the enzymes have the same MWs

From our differential centrifugation experiments we might conclude that the extra-chloroplastidic HMG-CoA reductase and MVA kinase are membrane bound, but HMG-CoA reductase is more tightly bound HMG-CoA reductase is present in the 100 K *g* pellet and MVA kinase only sediments after 16 hr at 150 K *g* centrifugation (Fig 7) Another interesting observation in our partial purification of MVA kinase is the fact that MVAP kinase and MVAP decarboxylase were present This observation suggests a cellular association Further fractionation is needed to determine the extent of this association Since a spectrophotometric assay for mevalonate kinase would not demonstrate the presence of

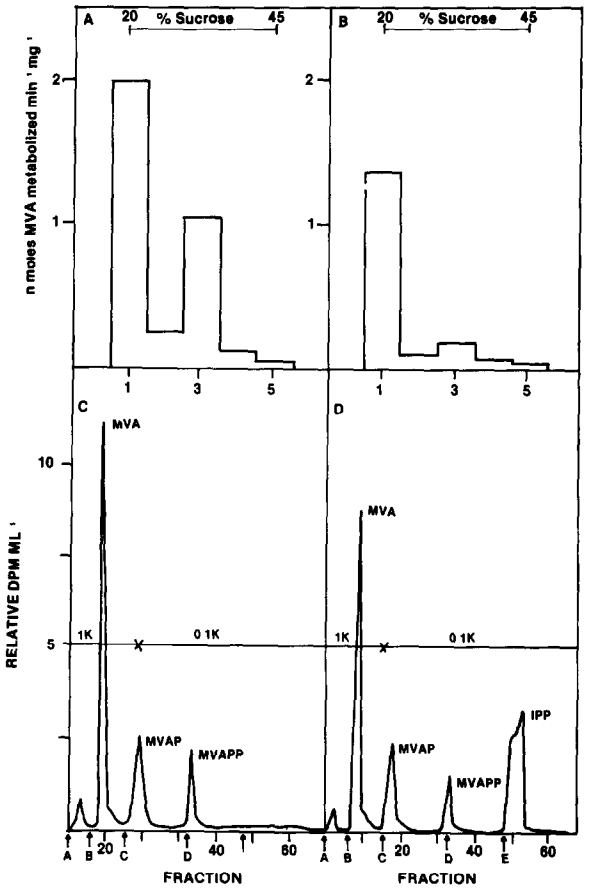

Fig 4 A, Mevalonate activity profile from a 20–45% sucrose density gradient of the 30–70% ammonium sulfate pellet from leaf tissue extracts B, Mevalonate kinase activity profile from a 20–45% sucrose gradient of the 30–70% ammonium sulfate precipitate from callus tissue extract C, Dowex-1-formate elution profile mevalonate kinase assay for 30–70% ammonium sulfate pellet from leaf tissue extracts D, Dowex-1-formate elution pattern mevalonate kinase assay of fraction one from a 20–45% sucrose gradient of the 30–70% ammonium sulfate precipitate from *N cataria* callus tissue (A) 25 ml of H_2O, (B) 25 ml of 2 N HCOOH, (C) 50 ml of 4 N HCOOH, (D) 50 ml of 4 N HCOOH, 0 4 M $HCOONH_4$, (E) 50 ml of 4 N HCOOH, 0 8 M $HCOONH_4$

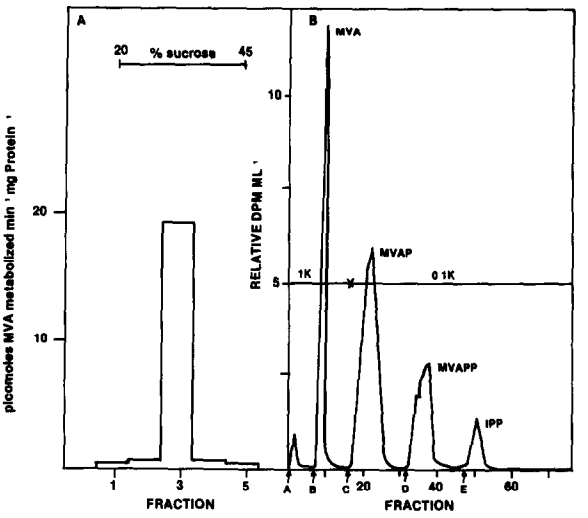

Fig 5 A, Mevalonate kinase activity profile from a 20–45% sucrose density gradient of the homogenized chloroplast preparation (from *N cataria* leaf tissue) B, Dowex-1-formate elution pattern mevalonate kinase assay of fraction 3 of the 20–45% sucrose gradient of homogenized chloroplasts from leaf tissue Elution profile following mevalonate kinase assay from fraction 3 of the 20–45% sucrose gradient of homogenized chloroplasts under standard assay conditions Elution of Dowex-1 column (A) 25 ml H_2O, (B) 25 ml 2N HCOOH, (C) 50 ml of 4N HCOOH, (D) 50 ml of 4 N HCOOH + 0 4 M $HCOONH_4$, (E) 50 ml of 4 N HCOOH + 0 8 M $HCOONH_4$

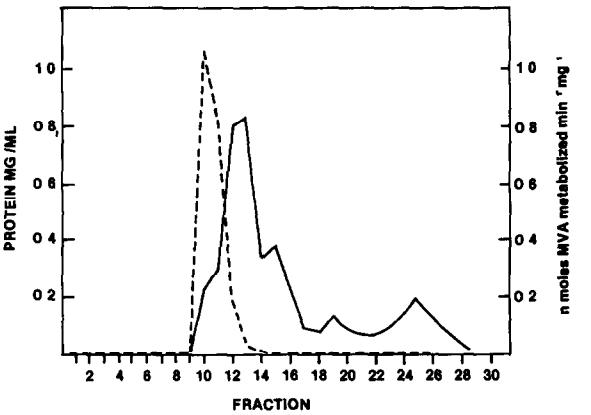

Fig 6 Mevalonate kinase activity profile (– – –) of a Bio-Gel P-100 column chromatography of fraction 1 from the 20–45% sucrose density gradient of the 30–70% ammonium sulfate precipitate of *N cataria* leaf tissue extract (see Table 1)

MVAP kinase or MVAPP decarboxylase, we used ion exchange chromatography to identify all of the products from incubating MVA and ATP with the enzyme preparations

Thus, HMG-CoA reductase is present in leaf tissue, it remains to be determined if the plant enzyme is a major regulation site in terpenoid synthesis as it is in cholesterol synthesis [1] Also, the reaffirmation of two distinct mevalonate kinase enzymes in plant tissue is clearly demonstrated

EXPERIMENTAL

Materials and methods DL-Mevalonic acid (2-^{14}C) (6 33 mCi/nmol), DL-mevalonic acid (5-H(N)) (5 Ci/nmol) and DL-3-hydroxy-3-methylglutaryl CoA (3-^{14}C) (51 9 m Ci/nmol) were purchased from New England Nuclear, Boston, Massachusetts All other chemicals and cofactors were purchased from Sigma Chemicals, St Louis, Missouri PolyClar AT (insoluble polyvinylpyrollidone) was a gift from GAF Corporation, New York, New York

Preparation of cell-free extracts Cell-free extracts of leaf tissue were prepared by grinding the material in a mortar with sand, acid washed Polyclar AT and homogenization buffer which contained 500 mM Tris pH 7 5, 500 mM $MgCl_2$, 1 mM EDTA,

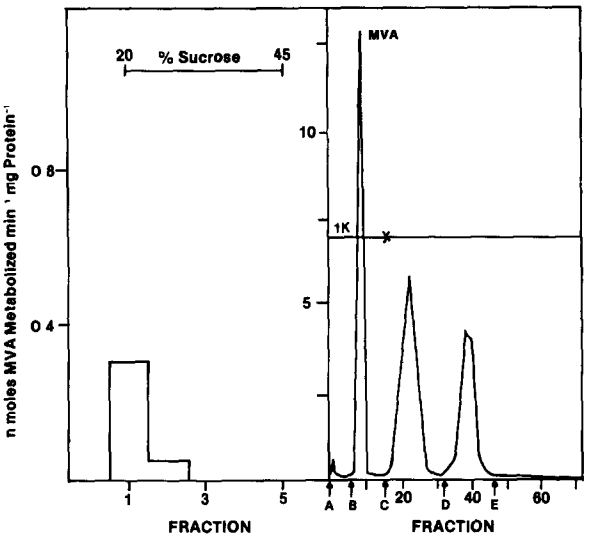

Fig 7 A, Mevalonate kinase activity profile from 20 to 45% sucrose gradient of the 16 hr 150 K pellet from leaf tissue B, Dowex-1-formate elution pattern mevalonate kinase assay of fraction 1 from 20 to 45% sucrose gradient centrifugation of 16 hr 150 K pellet from leaf tissue Elution of Dowex-1 column (A) 25 ml of H_2O, (B) 25 ml of 2N HCOOH, (C) 50 ml of 4N HCOOH (D) 50 ml of 4N HCOOH + 0 4M $HCOONH_4$, (E) 50 ml of 4N HCOOH + 0 8M $HCOONH_4$

and 0 5 mM dithiothrietol, until a smooth homogeneous paste was obtained A 1 1 (w/v) ratio of tissue to homogenization buffer and a 1 10 (w/v) ratio of Polyclar AT to tissue was used in all cell-free preparations

The smooth paste was pressed through six layers of cheese cloth and the exudate was subjected to two centrifugations—first a 3000 *g* (3 K) for 20 min and the pellet discarded followed by a 30 000 *g* (30 K) for 20 min The 30 K supernatant was classified as the cell-free extract All procedures were carried out at 4° using precooled equipment and buffers The 3 K and 30 K pellets showed no MVA kinase activity

Chloroplast isolation Chloroplasts were isolated by the method of Jensen and Bassham [21] with one modification The leaf tissue was homogenized × 5 for 5 sec periods The mildly homogenized leaf tissue was pressed through six layers of cheese cloth and the exudate was centrifuged at 2000 *g* for 50 sec The pellet contained whole chloroplasts For most experiments, the whole chloroplasts were homogenized before centrifuging through a sucrose density gradient

Sucrose gradient A discontinuous sucrose gradient of 20–45% was used in all experiments The sucrose solns were made up in buffer containing 100 mM Tris pH 7 5, 10 M $MgCl_2$, 1 mM EDTA and 0 5 mM dithiothreitol The concn of sucrose was checked with a refractometer The gradient consisted of 5 3 ml of each of the following sucrose concns 20%, 25%, 30%, 34% and 38% layered on 3 2 ml of 45% sucrose soln The sample, layered on the gradient, was centrifuged at 22 000 rpm in a SW 25 1 Spinco Rotor With the use of a density gradient fractionator, samples of either 3 or 6 ml were collected and dialysed against buffer containing 100 mM Tris pH 7 5, 10 mM $MgCl_2$, and 0 5 mM DTT

Protein Protein concns were determined by a modification of the Lowry procedure as described by Hartree [22] using bovine serum albumin as standard

HMG-CoA reductase assay The reaction mixture in 1 0 ml contained 1 mM EDTA, 1 mM dithiothreitol, 10 mM glucose-6-phosphate, 1 mM disodium NADP, 2 units of glucose-6-phosphate dehydrogenase, and various amounts of 3-^{14}C-HMG-CoA and protein as indicated Incubations were at 37° for the time specified in each expt, the reaction was terminated by immersion in boiling water for 3–5 min and the precipitated protein was removed by centrifugation The supernatant was applied to a Dowex-1-formate column and eluted with (a) 150 ml gradient consisting of three chambers (containing 50 ml H_2O, 50 ml 1 0 N and 50 ml 2N HCOOH respectively) followed by a step wise elution with (b) 50 ml 4 N HCOOH, (c) 50 ml of 4 N HCOOH with 0 4 N $HCOONH_4$, (d) 50 ml of 4 N HCOOH with and 0 8 N $HCOONH_4$

Mevalonate kinase assay MVA kinase activity was determined by incubating at 30° 200 mg protein, 10 mM ATP, 10 mM $MgCl_2$, 10 mM potassium phosphate buffer (pH 7 4) and 5 mM 3-glycerol phosphate in a total vol of 400 ml The reaction was initiated by adding 1 milliCurie of (2-C) MVA acid and terminated after 1 hr by heating to 100°

Ion-exchange chromatography of MVA metabolites on 0 5 × 5 cm Dowex-1-formate columns as described by Suzue [23] was used Stepwise elution was achieved with (1) 25 ml H_2O, (2) 25 ml 2N HCOOH, followed by the stepwise elution series above (b–d)

Chromatography Descending PC for the identification of MVA, HMG and HMG-CoA was done on Whatman#1 paper with PrOH–conc NH_4OH (7 3)

Detection and measurement of radioactivity Radioactivity on chromatograms was detected either by autoradiography or by cutting chromatograms into half-in strips and counting each in toluene–EtOH by scintillation counting Radioactive elution profiles from ion exchange chromatography columns were obtained by counting an aliquot of each fraction in toluene–EtOH Instagel by scintillation counting

Mass spectrometry The Prototype LKB 9000 combination gas chromatograph mass spectrometer was used [24] Samples were introduced through the direct-inlet probe

Partial purification of HMG-CoA reductase and mevalonate kinase The cell-free extract was brought to 30% saturation (160 g/l) with $(NH_4)_2SO_4$ sulfate and centrifuged at 3 000 *g* for 20 min, and then the pellet was discarded The 30% supernatant was brought to 70% saturation by the addition of an additional 260 g/l and centrifuged at 3 000 *g* for 20 min The 70% pellet was suspended in a minimum vol of buffer (100 mM Tris pH 7 5, 10 mM $MgCl_2$, 1 0 mM EDTA and 0 5 mM dithiothreitol) and dialysed against the same buffer without EDTA The dialysed solution (1 ml, 22–31 mg of protein) was placed on a 30 ml 20–45% discontinuous sucrose gradient Six fractions (5 ml) were collected The fraction containing the kinase and/or reductase activities were again dialysed against the buffer The dialysed fraction was concd by using a Diaflow ultrafilter apparatus and the concd fraction was applied to a Bio-Gel P-100 column and eluted with the same buffer Fractions (2 ml) were collected and analysed for enzyme activity as previously discussed

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