THE OCCURRENCE AND SOME PROPERTIES OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE AND MEVALONATE KINASE IN *NEPETA CATARIA* LEAF TISSUE

By

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NOMENCLATURE

MVA	-	Mevalonic Acid
MVAP	-	5-Phosphomevalonic Acid
MVAPP	-	5-Pyrophosphomevalonic Acid
HMG	-	β-Hydroxy-β-Methyl Glutaric Acid
HMG-CoA	-	β-Hydroxy-β-Methyl Glutaryl Coenzyme A
ATP	-	Adenosine Triphosphate
IPP	-	Isopentenylphrophosphate
NAD	-	Nicotinamide Adenine Dinucleotide
NADP	-	Nicotinamide Adenine Dinucleotide Phosphate
DTT	-	Dithiothreitol
MES	-	2-(N-Morpholino)ethane Sulfonic Acid
HEPES	-	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
EDTA	-	Ethylene Diamine Tetraacetic Acid

CHAPTER I

INTRODUCTION

The biosynthesis of monoterpenes in higher plants and cholesterol in mammalian and avian tissues have been the subject of a large number of investigations. These investigations have involved a variety of precursors including CO_2, acetate, mevalonate and geranylpyrophosphate. In all studies involving mammalian cholesterol synthesis the major control enzyme was found to be microsomal HMG-CoA reductase.

Monoterpene biosynthesis in higher plants is believed to occur at two sites. The sites are believed to be: 1) the chloroplastic site which is involved in the production of chloroplasts pigments, and 2) an extra-chloroplastic site for the biosynthesis of essential oils.

It is also postulated that the HMG-CoA reductase may be associated with other enzymes of monoterpene biosynthesis. Since HMG-CoA reductase control in plant tissue has not been studied, the following objectives were undertaken: 1) to establish that there is HMG-CoA reductase activity in N. cataria, 2) to determine the intracellular location, 3) to observe the properties of the enzymes involved in mevalonate metabolism and their relationship to HMG-CoA reductase, 4) to study the enzymes involved in mevalonate metabolism in cell-free extracts from a variety of sources for comparison with those from leaf tissue, and 5) to determine if HMG-CoA reductase and the mevalonate activating enzymes might be associated in some way.

CHAPTER II

LITERATURE REVIEW

A. Cholesterol and Terpenoid Biosynthesis

The first evidence that intact animals are capable of the de novo synthesis of cholesterol was provided by Schoenheimer and Breusch in 1933. This finding was later confirmed in 1937 with the observation that deuterated water was rapidly incorporated into cholesterol in rats (1). In 1942 Block and Rittenberg showed that the two carbon compound, acetate, represented the major carbon source for cholesterol biosynthesis in animal tissues (2). Several studies, both in vitro and in vivo, have demonstrated that the major site of cholesterol synthesis in higher animals is in the liver. Srere, Popjak and others have shown that every tissue in the body is probably capable of synthesizing cholesterol from labelled acetate (1).

The work of Block, Lynen, Cornforth, and Popjak led to the elucidation of the biosynthetic pathway of cholesterol from acetate (3).

The first steps of terpenoid synthesis in plants appears to be the same as those of cholesterol in animals (4, 5). The first step of the pathway is considered to be the conversion of β-hydroxy-β-methylglutaryl coenzyme A to mevalonate. Mevalonate is phosphorylated to 5-phosphomevalonate, 5-phosphomevalonate is phosphorylated to 5-pyrophosphomevalonate, and 5-pyrophosphomevalonate is decarboxylated

to isopentenyl pyrophosphate. An isomerization, with loss of 4-S^{3}H from mevalonate (2-R^{3}H of isopentenyl pyrophosphate), is observed in the formation of dimethylallyl pyrophosphate. A simple head to tail condensation of isopentenyl pyrophosphate and dimethylallyl pyrophosphate results in the formation of the simple acyclic monoterpene pyrophosphate.

Various reports of the chemical synthesis and stability of the intermediates of cholesterol and terpenoid synthesis are available and the interested reader is referred to these sources for further information (2, 6, 7, 8, 9, 10).

B. Regulation of Cholesterol and Terpenoid Biosynthesis

There is abundant evidence that the rate of cholesterol biosynthesis in animals is regulated principally by three physiological variables: the amount of cholesterol in the diet; the caloric intake of the animal; and the functional integrity of the enterohepatic circulation of bile acids.

Gould and Popjak (11) and later Bucher et al., (12) were the first to show that cholesterol feeding causes a more marked inhibition in the conversion of acetate-^{14}C to cholesterol than was observed when labelled mevalonate was the sterol precursor. These results implied that regulation occurred before mevalonate formation. This phenomenon was studied further by Siperstein and Guest (13), who demonstrated that liver slices from animals on a high cholesterol diet could incorporate acetate-^{14}C into carbon dioxide, fatty acids and ketone bodies even though cholesterol synthesis was almost completely suppressed.

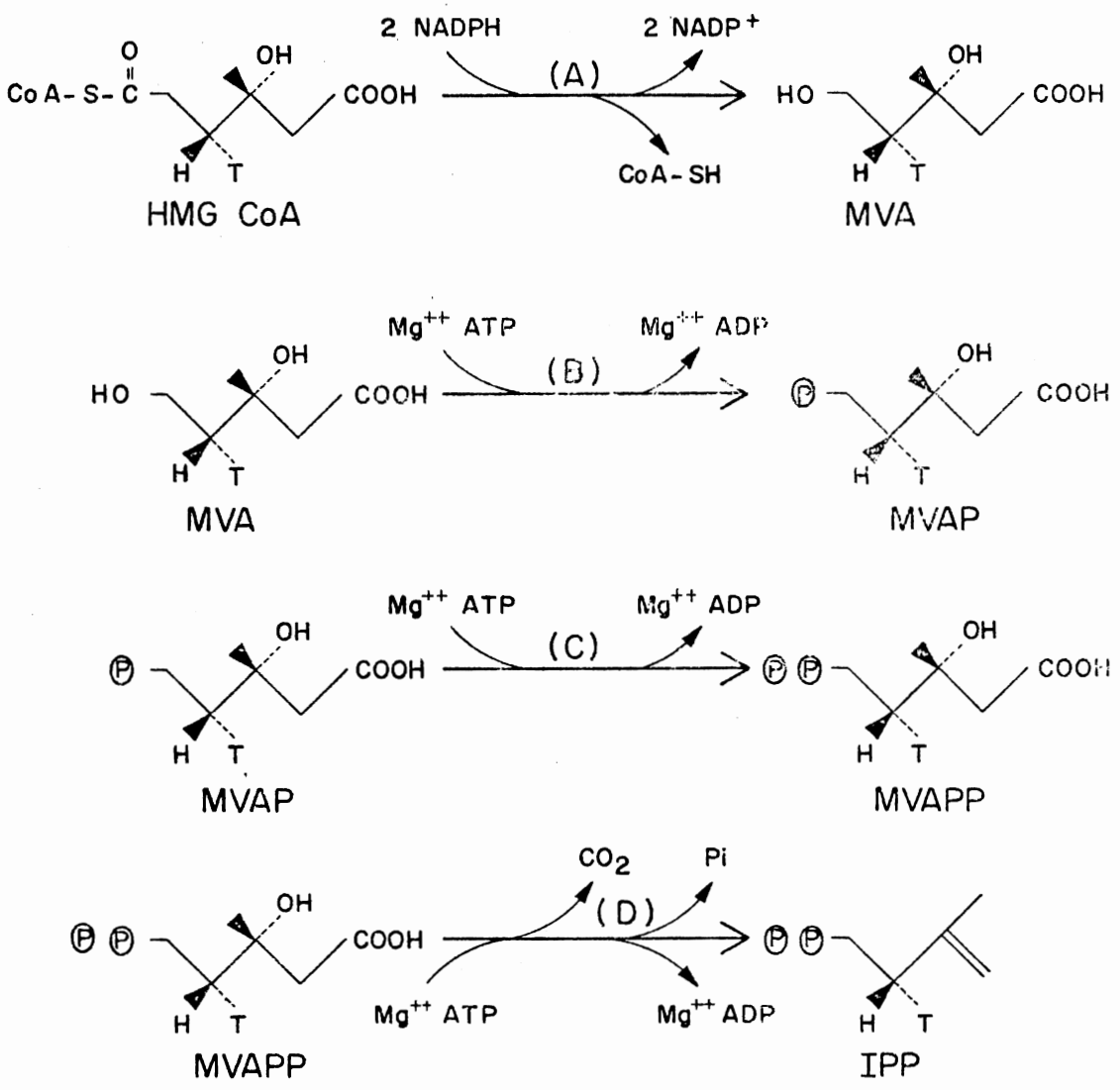
Siperstein and Fagen (14) developed a gas-liquid chromatographic procedure for measurement of the key intermediates in cholesterol biosynthesis. Their studies showed that the incorporation of acetate-^{14}C into mevalonate was inhibited markedly by cholesterol feeding, whereas incorporation of this precursor into β-hydroxy-β-methylglutarate was virtually unaffected (15).

Siperstein and Guest (13) demonstrated that rats kept on a prolonged cholesterol diet diminished the enzyme activity at other steps in cholesterol synthesis in liver tissue. Thus, whereas the rate-limiting step was still the reduction of β-hydroxy-β-methylglutaryl-CoA, marked reduction of enzymatic activity distal to mevalonate was clearly demonstrable. This depression of enzymatic activity is almost certainly secondary to primary feedback inhibition of β-hydroxy-β-methylglutary-CoA reductase during cholesterol feeding and probably has no direct role as a control mechanism of overall sterol synthesis by liver in the intact animal (Figure 1. A).

It has also been shown that high plasma levels of endogenous cholesterol in the form of very low density lipoprotein derived from the liver do not cause an inhibition of hepatic cholesterol synthesis (16). Conversely, hepatic cholesterogenesis is very promptly inhibited when chylomicra are infused into the systemic circulation and greatly stimulated when chylomicra are removed by drainage of lymph cholesterol through a thoracic duct fistula (17). These data suggest that exogenous cholesterol absorbed through the intestine, but not endogenous cholesterol is responsible for the activation of the cholesterol feedback system. This also strongly implies that it is not cholesterol itself, but rather a lipoprotein form of cholesterol, which probably

Figure 1. Enzymes of Cholesterol and Terpenoid Biosynthesis

A. HMG-CoA Reductase
B. Mevalonate Kinase
C. 5-Phosphomevalonate Kinase
D. 5-Pyrophosphomevalonate Decarboxylase


2 NADPH
2 NADP+
(A)
CoA-SH
HMG CoA
MVA
Mg++ ATP
Mg++ ADP
(B)
MVA
MVAP
Mg++ ATP
Mg++ ADP
(C)
MVAP
MVAPP
CO2
Pi
(D)
Mg++ ATP
Mg++ ADP
MVAPP
IPP

represents the actual feedback inhibitor of mevalonate synthesis and hence of cholesterol production. The question whether the cholesterol feedback system operates by direct end-product inhibition or by regulation of enzyme synthesis by means of enzyme repression remains to be answered. Suggestive evidence that this control mechanism involves end-product inhibition is provided by the finding that a significant depression of cholesterol synthesis is observed within 2.5 hours after the intravenous injection of cholesterol containing chylomicra (16), whereas the turnover of HMG-CoA reductase in the liver appears to be much slower (1). A number of attempts to demonstrate an inhibition of either cholesterol or of mevalonate synthesis by the addition of cholesterol in vitro, in its free or esterfied forms (1, 3, 18), have been unsuccessful.

While almost all tissues have the ability to synthesize cholesterol, only the liver demonstrates significant feedback inhibition of cholesterogenesis (19). In vitro studies have clearly shown that, next to liver, the intestine has the greatest cholesterogenic capacity of any body tissue. Normally, liver is the major source of plasma cholesterol, however, the intestine does make some contribution to the circulating cholesterol pool. Since the intestine shows little decrease in cholesterol synthesis in response to exogenous cholesterol, when a high cholesterol diet is fed, the intestine, rather than the liver, becomes the major source of endogenous plasma cholesterol.

The lack of response to exogenous cholesterol in the intestine led investigators to look for another type of metabolic control. Dietschy and Wilson (3) and Siperstein (1) reported in review articles that the external diversion of bile flow leads to a rapid increase in

the rate of cholesterogenesis in the small intestine and the liver. Biliary diversion for 48 hours enhances cholesterol synthesis in the liver two to three times and in the small bowel by as much as 10 times.

Bile contains a number of substances, including bile acids, cholesterol and phospholipids, that undergo an enterohepatic circulation. Evidence from Siperstein (20) indicates that bile acid is the inhibitory component.

Starvation, as well as chronic undernutrition, cause a definite depression in hepatic cholesterol synthesis. This effect is not the result of the absence of a specific dietary requirement since refeeding with any source of calories, fat, carbohydrate, or protein, will promptly restore cholesterogenesis to normal (1).

The existence of the diurnal rhythm in HMG-CoA reductase was first reported for mouse liver (21) and shortly thereafter for rat liver (22, 23), rat intestine (24) and certain rat and mouse hepatomas (25). Rhythmic variations in reductase activity in other species, particularly in humans, or in other tissues has not yet been established.

The only other enzyme in the cholesterol metabolic pathway that is known to have a diurnal rhythm is cholesterol 7-β-hydroxylase. This enzyme catalyzes the first reaction in the catabolism of cholesterol to bile acids (26).

Hormones have also been implicated both in the maintenance of diurnal rhythm in reductase synthesis and in the overall regulation of cholesterol synthesis (27). Hormones believed to be involved in this regulatory process include insulin, glucagon, thyroid hormone and hydrocortisone. Evidence indicates that insulin and thyroid hormone may participate in the chain of events that initiate and maintain the

increased rate of reductase synthesis that characterizes the rising portion of the rhythm (28, 29, 30), while hydrocortisone and glucagon, possibly via cAMP, may be involved in the chain of events that terminate reductase synthesis.

Other hormones that stimulate reductase activity and cholesterogenesis are epinephrine and norepinephrine (31). The steroid sex hormones, estrogens, progestins and androgens, can suppress reductase activity (32). However, it is not clear whether these hormones are acting as hormones or as cholesterol analogs.

Rates of cholesterol synthesis and HMG-CoA reductase activities are extremely variable, but frequently are elevated in primary and transplantable hepatomas. Partial or complete loss of regulation of cholesterol synthesis and of reductase activity by dietary cholesterol appears to be the only consistent feature of all hepatomas studied (1, 27, 32). This defect may be associated with the onset of malignancy, since similar losses of regulation occur in livers of all hepatocarcinogen-treated rats (27, 33, 34).

The nature of the apparent defect in the regulation by dietary cholesterol is not clear. Other aspects of the regulation of cholesterogenesis, such as the diurnal rhythm and suppression by fasting, are not lost (25, 27, 35). A consistent finding in all malignant tissue is reduced accumulation of cholesterol as cholesteryl esters after cholesterol feeding (17, 26, 37). This change may in some way be responsible for the abnormal regulation of reductase activity and cholesterol synthesis in these tissues.

Review articles by Siperstein (1), Dietschy and Wilson (3) and Rodwell *et al.*, (27) cover the literature on the regulation of

cholesterol synthesis and HMG-CoA reductase very thoroughly. The reader is referred to the above reviews for further information on cholesterogenesis other than those covered in this review.

The regulation of terpenoid synthesis in plants has not been studied. The control enzyme in mammalian system, HMG-CoA reductase, only recently has been reported in higher plants (38). No work has been done on the regulation of this enzyme in higher plants. Thus this part of the review was to present as concisely as possible the status of HMG-CoA reductase as a biological control point.

C. Enzymes of Cholesterol and Terpenoid Biosynthesis

1. HMG-CoA Reductase

HMG-CoA reductase (Mevalonate:NADP oxidoreductase [acylating CoA], EC 1.1.1.34) catalyzes the reduction by NADPH of -β-hydroxy-β-methyl-glutaryl-coenzyme A to mevalonate. The enzyme has been partially isolated from a variety of sources including mammalian and avian liver, yeast and plant (38, 39). It has been detected in the soluble and microsomal fractions and is believed to be the major site of control of cholesterol biosynthesis (39).

The intracellular location of cholesterol feedback control has likewise been the subject of several studies which has been reviewed by Siperstein (1). Both microsomes and cytosol are required for the overall conversion of acetate to cholesterol; however, if one uses labelled -β-hydroxy-β-methylglutatyl-CoA as substrate, the reduction of this compound can be observed in the hepatic microsome (1). Other

studies indicated that over 90% of mevalonate production occurs in the microsomes, the remaining activity being in the cytosol (15). The microsomal enzyme is the major target of feedback inhibition of mevalonate synthesis. The residual mevalonate synthesis of the cytosol is relatively unaffected by short term cholesterol feeding. The subcellular localization of -β-hydroxy-β-methylglutaryl CoA reductase, and hence for cholesterol feedback control, can be carried further by subfractionating the microsome into its ribosomes, endoplasmic reticulum and soluble interior. Assay of these fractions indicates that the reductase enzyme is localized exclusively in the lipoprotein that makes up the endoplasmic reticulum or membrane of the microsome (15). The entire lipid component of this enzyme is apparently not necessary for activity since Linn (18) has reported that an acetone powder of whole liver microsomes will reduce -β-hydroxy-β-methylglutaryl CoA to mevalonate.

In contrast to hepatic tissue, yeast HMG-CoA reductase is located in the mitochondria instead of the endoplasmic reticulum (40, 41).

HMG-CoA reductase has only recently been reported in higher plants. Suzuki et al. (38), presented the first evidence for the occurrence of HMG-CoA reductase in sweet potato roots. It was found to be in both the mitochondrial and microsomal fractions. The Km for HMG-CoA was reported between 6.5 μM to 21 μM.

It has been established that HMG-CoA reductase is tightly bound to microsomal membranes in hepatic cells (42). Shefer et al. (24) noted that the enzyme was located in both the mitochondrial and microsomal fractions of rat intestinal crypt cells. However, the enzyme is associated with the mitochondrial fraction in Saccharomyces cerevisiae

(43) and occurs in a soluble form in Tetrahymena pyriformis (44). The difference in the subcellular distribution of HMG-CoA reductase among various living organisms may provide information of physiological and evolutionary significance. For further information concerning the properties of HMG-CoA reductase the reader is referred to a detailed review by Rodwell, McNamara and Shapiro (27).

2. Mevalonate Kinase

The enzyme system that converts mevalonate to isopentenyl pyrophosphate is usually found mainly in the 100,000 x g for one hour of supernatant of most tissue homogenates (45, 46). In higher plants some of the enzymes involved in the synthesis of isopentenyl pyrophosphate have been found in chloroplasts (47, 38).

Mevalonate kinase (ATP:mevalonate 5-phosphotransferase, EC 2.7.1.36) catalyzes the phosphorylation of mevalonate at the five position as shown in Figure 1. The first reported isolation and purification of mevalonate kinase was in 1958 by Tchen (48) from a yeast autolysate. Mevalonate kinase from a large number of sources have been studied. These include pumpkin seedlings (49), rubber plant latex (50), bean seedlings (51, 52, 53, 54), orange fruit (55), pine seedlings (56), N. cataria (57), hog liver (58, 59), chicken and rat liver (60, 61). The phosphorylation of mevalonate by hog liver mevalonate kinase has been shown to be an ordered sequential reaction in which mevalonate adds first followed by MgATP, then 5-phosphomevalonate is released first followed by ADP (58). ATP is the nucleotide that best supports phosphorylation of mevalonate; however, in different systems other nucleotides can also serve as phosphate donors (48, 49).

Inhibition of enzymatic activity by high levels of ATP has been reported (48, 55). Divalent cations are required for activity with Mg^{++} and Mn^{++} giving the best activity although other divalent cations will also support low levels of phosphorylation (48, 49, 50). An active -SH group is suggested in mevalonate kinase by its sensitivity to sulfhydryl binding agents (48, 49, 50, 53, 54, 55) and the requirement for sulfhydryls in isolation buffers (57). The pH optimum of mevalonate kinase varies with source, with a minimum pH optimum of 5.5 to a maximum of 9.0 (49, 50).

It has been suggested that there are two pools of mevalonate kinase in plants. The chloroplastidic pool with a pH optimum of 7.5 and the extra-chloroplastic pool with a pH optimum of 5.5 (51).

Mevalonate kinase can be inhibited by a number of products including geranyl pyrophosphate (54, 58, 61, 62), farnesyl pyrophosphate (54, 60, 61, 62) and higher prenylpyrophosphates (54, 62). ADP and 5-phosphomevalonate have also been shown to inhibit the enzyme (54, 58). The molecular weight of mevalonate kinase from a number of sources has been estimated to be 100,000 daltons (60).

3. 5-Phosphomevalonate Kinase

5-Phosphomevalonate kinase (ATP:5-phosphomevalonate phosphotransferase, EC 2.7.4.2) catalyzes the second phosphorylation by ATP at the 5 position of 5-phosphomevalonate. The enzyme has been partially purified from a number of sources including rubber latex (63), pine (64), yeast (65), and hog liver (59). ATP is the only nucleotide to support phosphorylation (63, 65). A divalent cation is required with Mg^{++}, Fe^{++} and Zn^{++} activating best, and Mn^{++} and Ca^{++} supporting low

levels of phosphorylation (63, 65). The pH optimum varies with source and ranges from pH 5.5 to 7.4. Sulfhydryl reagents tend to increase the activity of 5-phosphomevalonate kinase from pine (64) and rubber latex (63).

4. 5-Pyrophosphomevalonate Decarboxylase

5-Pyrophosphomevalonate decarboxylase (ATP:5-pyrophosphomevalonate carboxy-lyase, EC 4.1.1.33) is the enzyme catalyzing the synthesis of active isoprene units. This enzyme is the most difficult of all the mevalonate activating enzymes to be isolated from hog liver (59). Better yields have been reported from yeast autolysates (65, 66) and the decarboxylase, free of kinase and phosphatase activities, has been purified from rubber latex (63). 5-Pyrophosphomevalonate decarboxylase from yeast has a pH optimum from 5 to 10 (65). In rubber latex a broad peak of activity with an optimum at pH 6.4 is observed (63). Mg^{++}, Mn^{++} and Co^{++} are the cations that best activate the decarboxylase (63). Sulfhydryl binding reagents have been shown to have no effect on decarboxylase activity from some systems (63); iodoacetamide has been shown to decrease isopentenyl pyrophosphate levels in other preparations of the decarboxylase from rubber latex (50). ATP concentrations of 10^{-4} molar are optimum for activity and concentrations higher than 10^{-2} molar are inhibitory (63, 65). ADP and isopentenyl pyrophosphate inhibit decarboxylase activity in the rubber latex system (63).

CHAPTER III

MATERIALS AND METHODS

A. Materials

1. Plants

N. cataria plants were grown in a vermiculite-soil mixture in the Oklahoma State University horticulture greenhouse or in a Percival controlled environmental growth chamber under 16 hour light periods with mixed Grow-Lux-incandescent illumination. Light period temperature was held at 32°C and dark period temperatures were lowered to 21°C. Plants were either propagated vegetatively or grown from seeds.

2. Plant Cell Culture

N. cataria cell cultures were initiated and maintained according to the procedure described by Downing and Mitchell (57).

3. Radioactive Compounds

Mevalonate-2-^{14}C, β-hydroxymethyl-β-methylglutaryl CoA-(HMG-CoA)-3-^{14}C and Instagel were purchased from New England Nuclear, Boston, Massachusetts. The labelled mevalonate was obtained as the dibenzoyl-ethylenediamine salt with a specific activity of 6.3-7.1 microcuries per micromole of acid. The HMG-CoA was obtained in an aqueous solution pH 8.0 with a specific activity of 26.2-26.4 microcuries per micromole.

4. Chemicals and Reagents

Solvents and reagents were of analytical grade or of the highest quality available unless otherwise specified.

Bio-Gels were purchased from Bio-Rad Laboratories, Richmond, California. Dowex ion exchange resins were purchased from Sigma Chemical Company, St. Louis, Missouri. Diaflow ultrafilter apparatus and membranes were purchased from Amicon Corporation, Lexington, Massachusetts.

Whatman number one chromatography paper was purchased from Sargent-Welch in large lots, such that all sheets would be of the same production number, so as to obtain papers with the most nearly uniform flow rate possible.

Polyclar AT (polyvinyl pyrolidone washed in acid) was the generous gift of the GAF Corporation, New York, New York.

Thin layer supplies were purchased from Brinkman Instruments, Westbury, New York.

5. Enzymes, Substrates and Cofactors

Rabbit muscle pyruvate kinase, beef heart lactate dehydrogenase and yeast glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Company, St. Louis, Missouri.

ATP and ADP in the sodium salt form, phosphoenolpyruvate as the trisodium or tricyclohexylamine salt, NAD, NADH and $NADP^+$ as the sodium salts, glucose-6-phosphate as the disodium salt, β-glycerol phosphate, R,S-mevalonate as the lactone and (R,S) HMG-CoA were purchased from Sigma Chemical Company, St. Louis, Missouri.

B. Methods

1. Preparation of Cell-Free Extracts

Cell-free extracts of leaf and callus tissue were prepared by grinding the material with sand, Polyclar AT and homogenization buffer, 500 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothrietol, 0.1 mM potassium mevalonate, 500 mM Tris pH 7.5, in a mortar until a smooth homogenous 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 obtained on grinding of the tissue was pressed through six layers of cheese cloth and the exudate was subjected to a series of centrifugations as described in Figure 2. All procedures were carried out at 4°C using precooled mortar and buffers.

2. Preparation of Microsomes

A homogenate paste was obtained from leaf tissue in a similar manner as described in the preparation of cell free extracts. The homogenate was then subjected to a series of centrifugations as described in Figure 3. The 3,000 x g pellet contained whole chloroplasts and cells. The 30,000 x g pellet contained mitochondria. The 150,000 x g 2 hour pellet contained broken chloroplasts and ribosomes. The 150,000 x g 16 hour pellet contained the microsomal membranes.

3. Chloroplast Isolation

Chloroplasts were isolated by the method of R. G. Jensen and J. A. Bassham (67) with one modification, in that the leaf tissue was

Figure 2. Preparation of Cell Free Extract

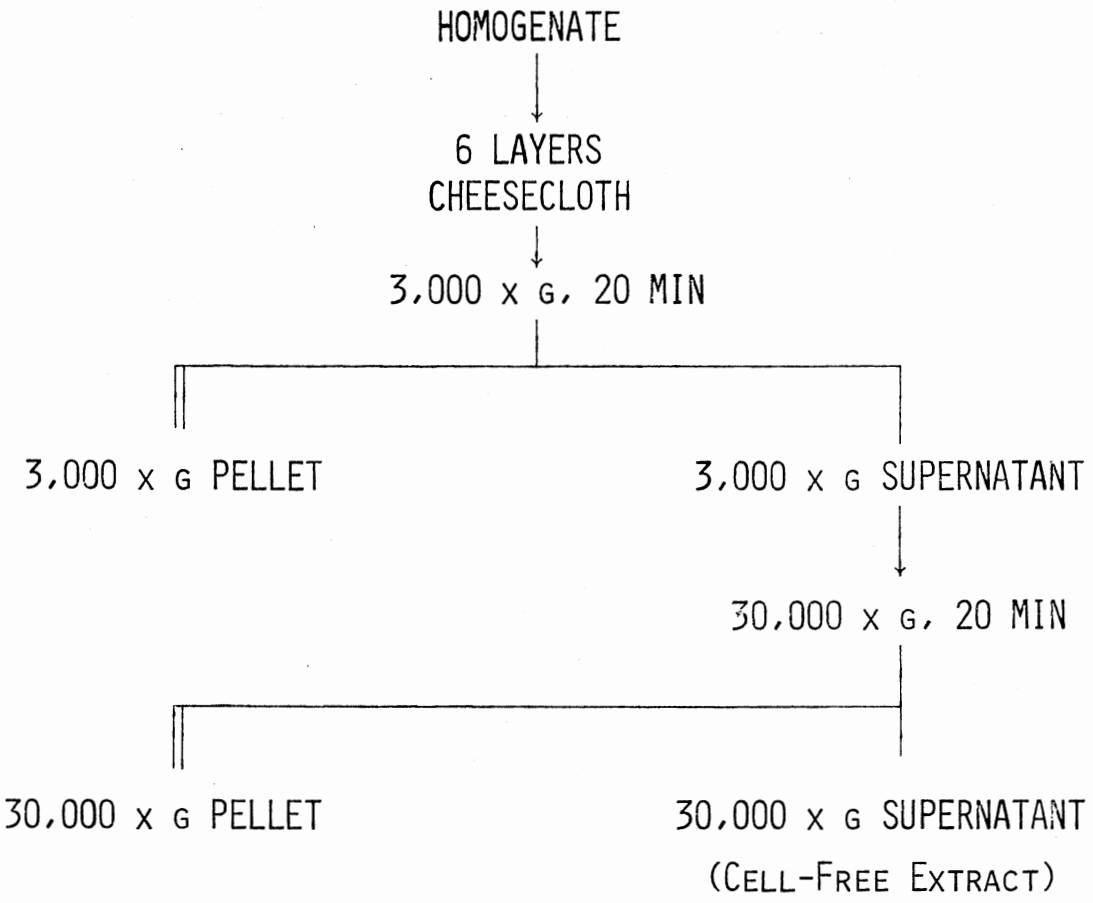
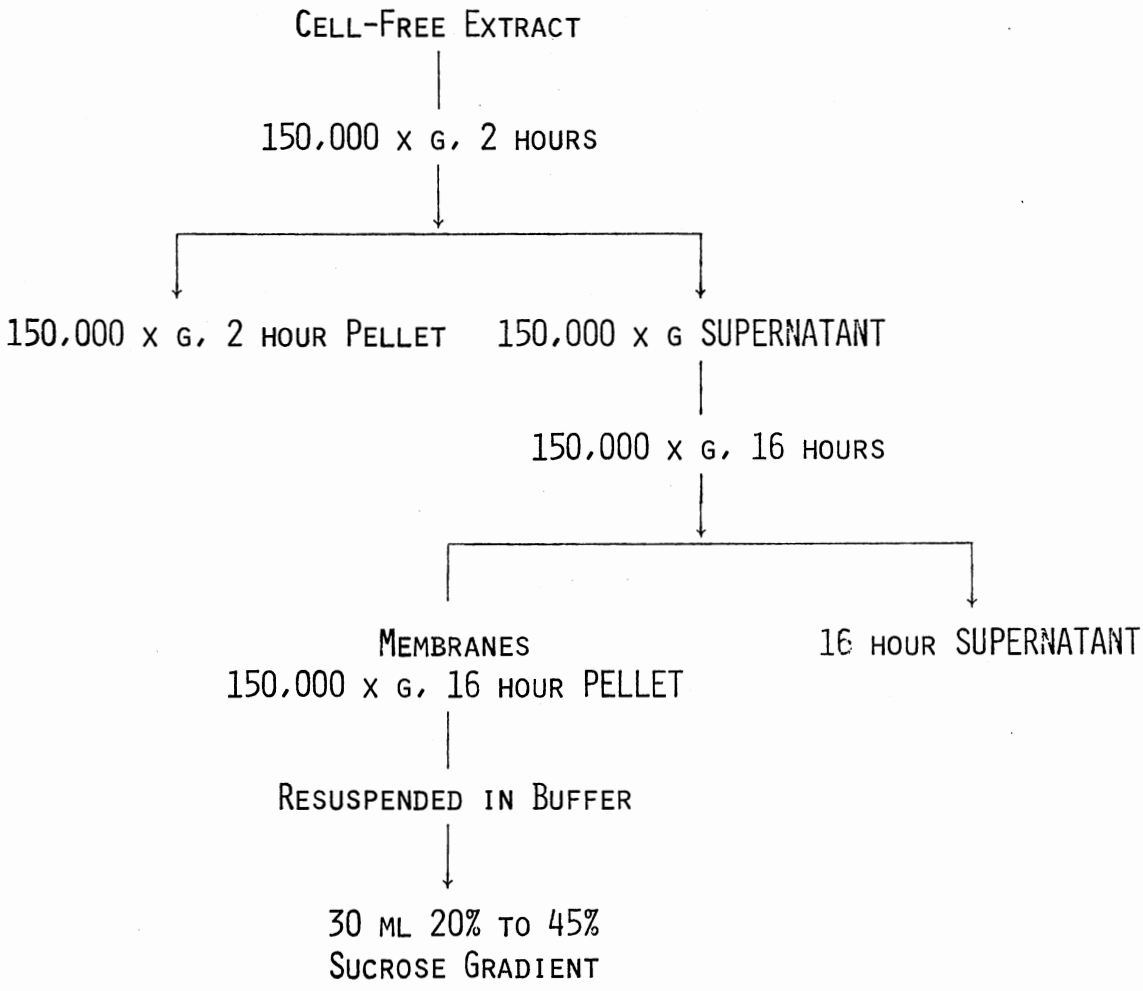

HOMOGENATE
6 LAYERS
CHEESECLOTH
3,000 X G, 20 MIN
3,000 X G PELLET
3,000 X G SUPERNATANT
30,000 X G, 20 MIN
30,000 X G PELLET
30,000 X G SUPERNATANT
(CELL-FREE EXTRACT)

Figure 3. Purification of Mevalonate Kinase by Differential Centrifugation


Cell-Free Extract
150,000 x g, 2 hours
150,000 x g, 2 hour Pellet
150,000 x g SUPERNATANT
150,000 x g, 16 hours
Membranes
150,000 x g, 16 hour PELLET
16 hour SUPERNATANT
Resuspended in Buffer
30 ml 20% to 45%
Sucrose Gradient

homogenized with five second burst five times. The mildly homogenized leaf tissue was pressed through six layers of cheese cloth and the exudate was centrifuged at 2,000 x g for 50 seconds. The pellet contained the whole chloroplasts. For experiments the whole chloroplasts were homogenized before centrifugation on a sucrose density gradient.

4. Sucrose Gradient

A discontinuous sucrose gradient of 20%-45% was used in all experiments. The sucrose solutions were made up in buffer containing 100 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, and 0.1 mM potassium mevalonate. The concentration of sucrose was checked with a refractometer.

The gradient consisted of 5.3 ml of each of the following sucrose concentrations: 20%, 25%, 30%, 34% and 38% layered on 3.2 ml of 45% sucrose solution. Fractions of either three or six ml were removed by using a density gradient fractionator and dialyzed against buffer containing 100 mM Tris ph 7.5, 10 mM $MgCl_2$, 0.5 mM DTT and 0.1 mM potassium mevalonate.

5. Assays

Protein concentrations were determined by a modification of the Lowry procedure as described by Hartree (68) using bovine serum albumin as a standard.

Alkaline phosphatase assays were performed as described for the E. coli enzyme (69).

Mevalonate kinase activity was measured by a radioactive assay based on a method described by Green and Levinthal (70). The assay

system included $MgCl_2$, ATP, dithiothreitol, β-glycerol phosphate and enzyme sources as indicated for individual experiments. Assays were run at 30°C for the time periods specified.

The reactions were performed in a total volume of 0.5 to 1.0 ml and were terminated by immersion in boiling water for three to five minutes. The precipitated protein was removed by centrifugation and the supernatant was applied to a Dowex-1 formate column for separation of products.

HMG-CoA reductase activity was measured by two similar radioactive assays. One assay was based on a method by Haber et al., (71) with some modifications. The reaction mixture in a volume of 1.0 ml contained 1 mM of EDTA, 1 mM of dithiothreitol, 10 mM of glucose-6-phosphate, 1 mM of disodium $NADP^+$, 2 units of glucose-6-phosphate dehydrogenase, and various amounts of 3-^{14}C-HMG-CoA and protein as indicated for individual experiments. Incubations were carried out at 37°C for the time specified in each experiment. The reaction was stopped by quick freezing in acetone and dry ice. Mevalonic acid lactone was formed by the addition of 200 µl of 2 N H_2SO_4. The reaction mixture was allowed to set at room temperature for 10 minutes in order for lactonization to occur. This mixture was lypholyzed, extracted with ether 4 times (1 x 9.5 ml and 3 x 4.5 ml) and the ether extracts were evaporated to dryness. The residue was dissolved in 0.5 ml of water and placed on a Dowex-1 formate column which was eluted with 5.0 ml of water. The eluate, which contained the mevalonalactone was collected in a scintillation vial and 10 ml of Instagel was added. The scintillation vial was then counted in a 3320 Packard scintillation spectrometer.

A second assay was used to detect other products formed. The reaction mixture was the same as described for the previous HMG-CoA reductase assay. After termination of the reaction by immersion in boiling water for three to five minutes, the precipitated protein was removed by centrifugation and the supernatant was applied to a Dowex-1 formate column. The elution was started by using three chambers containing 50 ml H_2O, 50 ml 1 N formic acid and 50 ml 2 N formic acid, followed by a discontinuous gradient consisting of 50 ml elutions of 4 N formic acid, 50 ml of 4 N formic acid and 0.4 N ammonium formate, and finally 50 ml of 4 N formic acid and 0.8 N ammonium formate. Using an ISCO "pup" fraction collector at 60 drops per fraction was obtained, collected in approximately 3 ml fractions and 100 µl of each fraction was removed for scintillation counting.

6. Chromatography

Descending paper chromatography for the identification of mevalonate, HMG and HMG-CoA was done on Whatman #1 paper. The solvent used was n-propanol:amminium hydroxide (7:3 v/v). Chromatography chambers were equilibrated with the solvent for at least 24 hours prior to use. The chromatograms were placed in the chamber and the system was allowed to equilibrate for an additional hour before introducing the elution solvent.

Ion exchange chromatography of mevalonate metabolites on Dowex-1 (formate form) columns was completed as described by Suzue (72) over 0.5 x 5.0 cm columns collecting 3.3 ml fractions. Stepwise elution of Dowex-1 formate columns was achieved with: a) 25 ml H_2O; b) 25 ml 2 N formic acid; c) 50 ml 4 N formic acid; d) 50 ml 0.4 M ammonium

formate in 4 N formic acid and e) 50 ml 0.8 ammonium formate in 4 N formic acid.

The resin for the Bio-Gel P-100 column was treated and poured as described in the Gel Chromatography manual by Bio-Rad Laboratories. The column was equilibrated with 100 mM Tris pH 7.5 prior to use.

7. Detection and Measurement of Radioactivity

Radioactivity on chromatograms was detected either by autoradiography or by cutting chromatograms into ½ inch strips and counting each strip in a toluene-ethanol scintillation counting cocktail. Radioactive elution profiles from ion exchange chromatography columns were obtained by counting an aliquot of each fraction in a toluene-ethanol or Instagel scintillation counting cocktail. The toluene-ethanol scintillation cocktail was composed of four grams of 2,5 diphenyloxazole (PPO) and 0.2 grams of 1,4-bis (2-(5-phenyloxazole))-benzene; phenyloxazolylphenyl (POPOP) in 400 ml sulfur free toluene and 600 ml absolute ethanol. All samples were counted in ten ml volumes.

A standard quench set was prepared utilizing ^{14}C benzoic-acid as the isotopic source with various amounts of reagent grade acetone used as a quencher. Samples counted in toluene-ethanol and Instagel cocktail were determined to be between 50 and 75 percent efficient.

8. Partial Purification of HMG-CoA Reductase and Mevalonate Kinase from N. Cataria Leaf and Callus Tissue

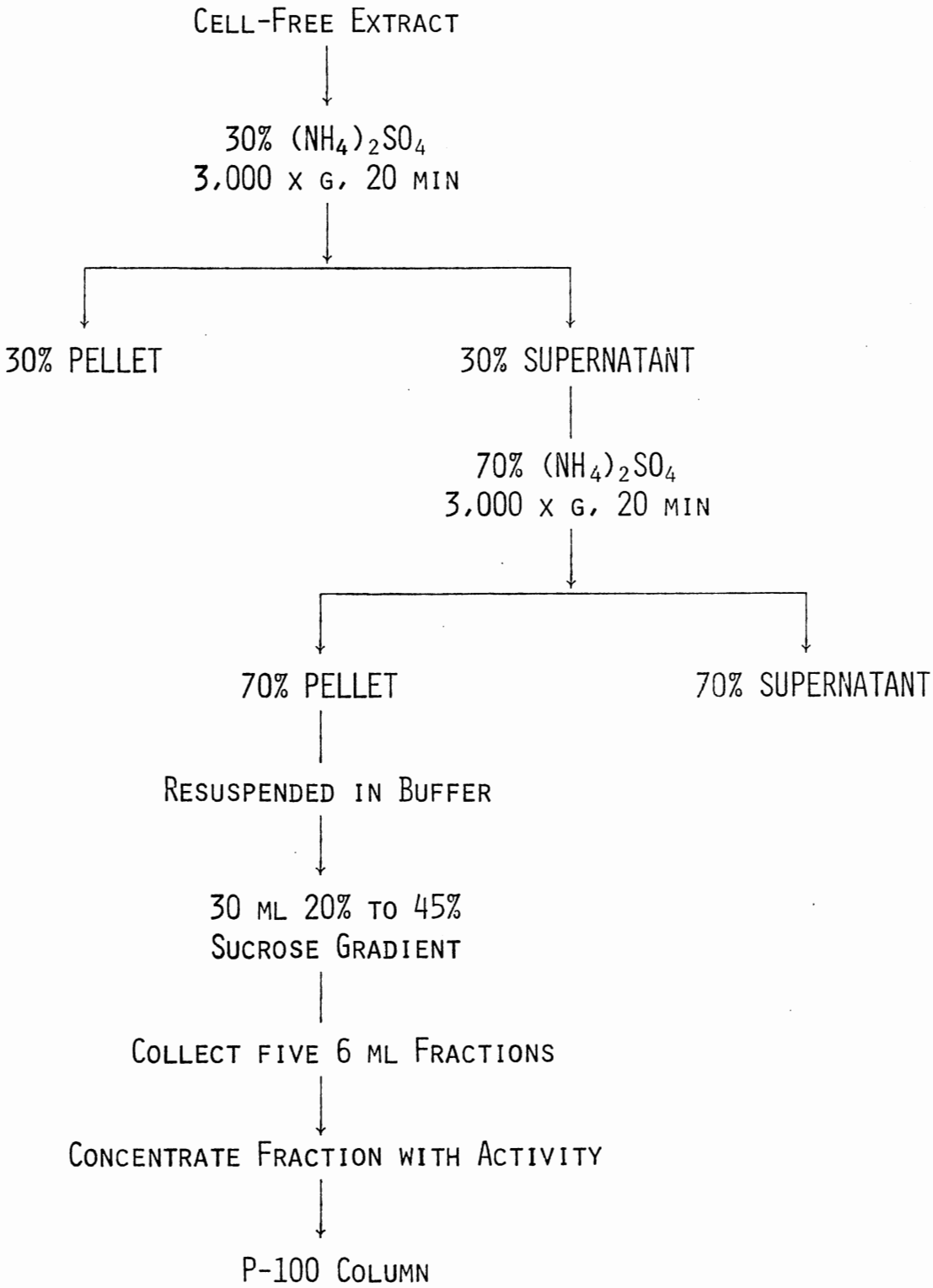
The cell-free extract was brought to 30% saturation (160 g/l) with

a-minium sulfate and centrifuged at 3,000 x g for 20 minutes. The 30% ammonium sulfate supernatant was brought to 70% saturation by the addition of 260 g/l of ammonium sulfate and centrifuged at 3,000 x g for 20 minutes. The 70% pellet was resuspended in a minimum volume of buffer (100 mM Tris pH 7.5, 10 mM $MgCl_2$, 1.0 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM potassium mevalonate) and dialyzed against the same buffer without EDTA. One ml of the dialyzed pellet solution (22 to 31 mg of protein) was placed on a 30 ml 20-45% discontinuous sucrose gradient. Six fractions of five mls each were collected. The fraction containing either the kinase or reductase activities were again dialyzed against the buffer previously described. The dialyzed fraction was concentrated by using a Diaflow ultrafilter apparatus. The concentrated fraction was applied to a Bio-Gel P-100 column eluted with the same buffer used in resuspending the 70% pellet and two ml fractions were collected. Figure 4 gives a detailed flow chart of the procedure.

9. Partial Purification of HMG-CoA Reductase and Mevalonate Kinase from Mammalian and Avian Tissue

The mammalian and avian tissues utilized were hog liver, turkey kidney and turkey liver. The cell-free extract was obtained by homogenizing the tissue in a blender using 15 second bursts until a smooth homogenate was obtained. The buffer used was the same as described for N. cataria tissue except 50 mM sucrose was used. The remaining steps of purification were carried out as described in Figure 4. The

Figure 4. Purification of Mevalonate Kinase and HMG-CoA Reductase by Ammonium Sulfate Fractionation


Cell-Free Extract
30% $(NH_4)_2SO_4$
3,000 x g, 20 min
30% Pellet
30% Supernatant
70% $(NH_4)_2SO_4$
3,000 x g, 20 min
70% Pellet
70% Supernatant
Resuspended in Buffer
30 ml 20% to 45%
Sucrose Gradient
Collect five 6 ml Fractions
Concentrate Fraction with Activity
P-100 Column

purification of mammalian and avian HMG-CoA reductase and mevalonate kinase was not carried out past the sucrose gradient step.

10. Partial Purification of Mevalonate Kinase from Yeast

Mevalonate kinase was solubilized from Fleischmann's active dry yeast for bakers by a modification of the autolysis procedure of Kirtley and Rudney (73). The liquid nitrogen freezing step prior to autolysis was omitted. One hundred grams of dry yeast was suspended in 200 ml of 100 mM Tris pH 7.5, 0.5 mM dithiotheritol, 1 mM EDTA, 10 mM $MgCl_2$ and 0.1 mM potassium mevalonate. The suspended yeast was stirred 12 hours at 4°C. The suspension was centrifuged at 20,000 x g for 20 minutes. The supernatant was assayed and discarded. The gummy precipitate was resuspended in 200 ml of the same buffer and stirred for another 48 hours at 4°C. The suspension was centrifuged as before. This step resulted in considerable portion of the enzyme being solubilized. The supernatant was next centrifuged at 30,000 x g for 20 minutes. The supernatant from the 30,000 x g centrifugation was then subjected to the steps described in Figure 4. The purification was terminated after the sucrose gradient step.

CHAPTER IV

RESULTS AND DISCUSSION

A. Mevalonate Activating Enzymes

1. Plants

a. Purification. The purification of mevalonate kinase from N. cataria leaf tissue outlined in Figure 3 is an extension of the method described by Downing and Mitchell (57). Mevalonate kinase, 5-phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase activities were observed in the 30 to 70 percent ammonium sulfate precipitate. Figure 5 gives a profile of products from mevalonate metabolism on a Dowex-1 formate column. A yield of 21 percent of the total units and a 2.1 fold purification was observed at the 30 to 70 percent ammonium sulfate precipitate as seen in Table I.

The 30-70 percent ammonium sulfate pellet was dialyzed and 1 ml (22 to 31 mg/ml) was layered on a 20-45% discontinuous sucrose gradient as described in methods. The gradient was centrifuged at 20,000 x g for three hours and five fractions of six mls each were collected. Figure 6 shows the elution profile of protein concentration and mevalonate kinase activity from the 20 to 45 percent discontinuous sucrose gradient. Mevalonate kinase is measured as total phosphorylated products rather than 5-phosphomevalonate. The assay mixture contained 10.0 mM $MgCl_2$, 5 mM ATP, 0.5 mM dithiothreitol, 1.0 mM EDTA,

Figure 5. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of 30 to 70 Percent Ammonium Sulfate Pellet from Leaf Tissue

Elution profile following assay of leaf 30 to 70 percent ammonium sulfate pellet under standard assay condition for mevalonate kinase. Elution of Dowex-1 formate columns:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 3.0 ml fractions

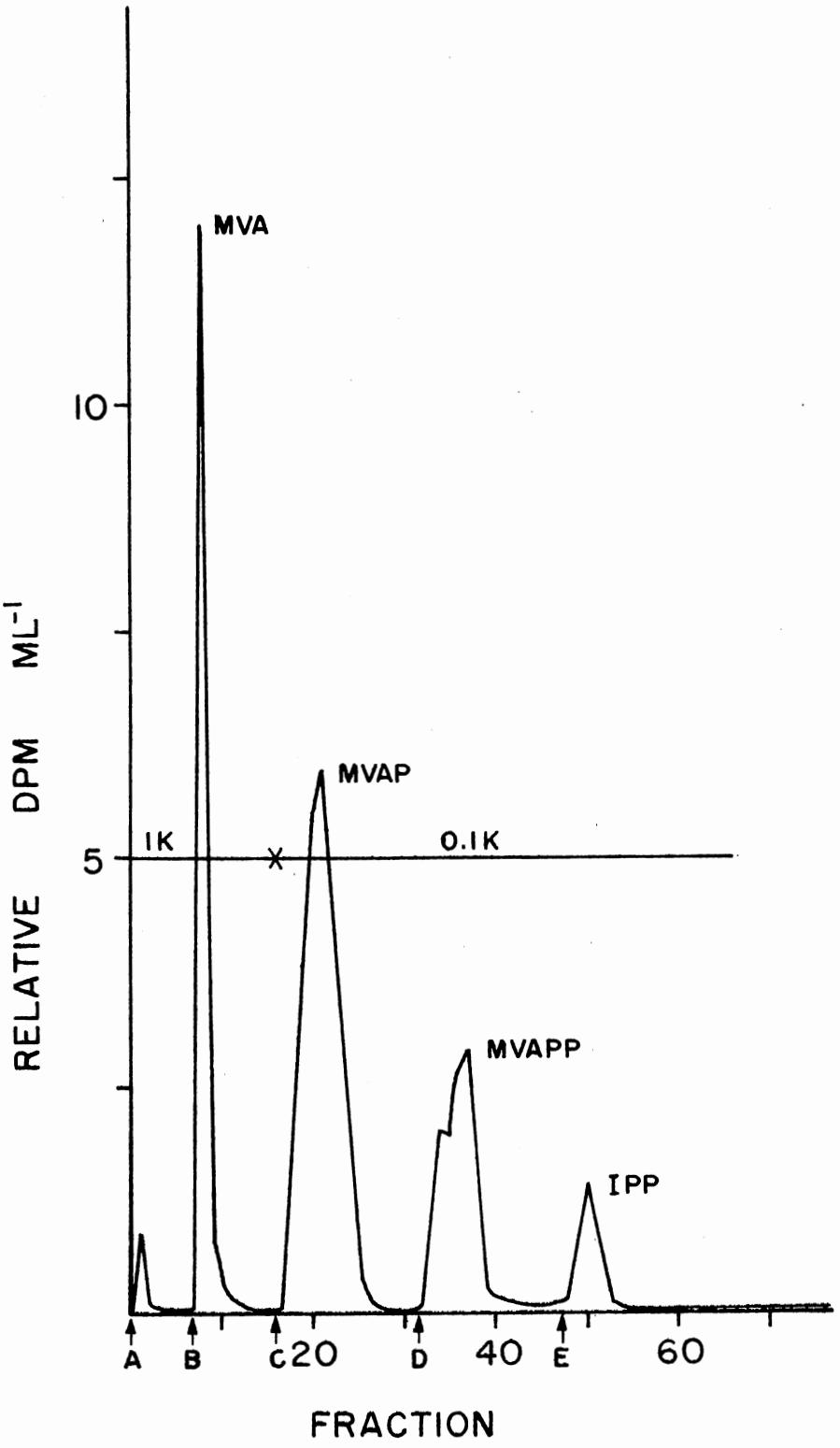

MVA
MVAP
MVAPP
IPP
1K
0.1K
10
5
RELATIVE DPM ML⁻¹
A
B
C
20
D
40
E
60
FRACTION

TABLE I

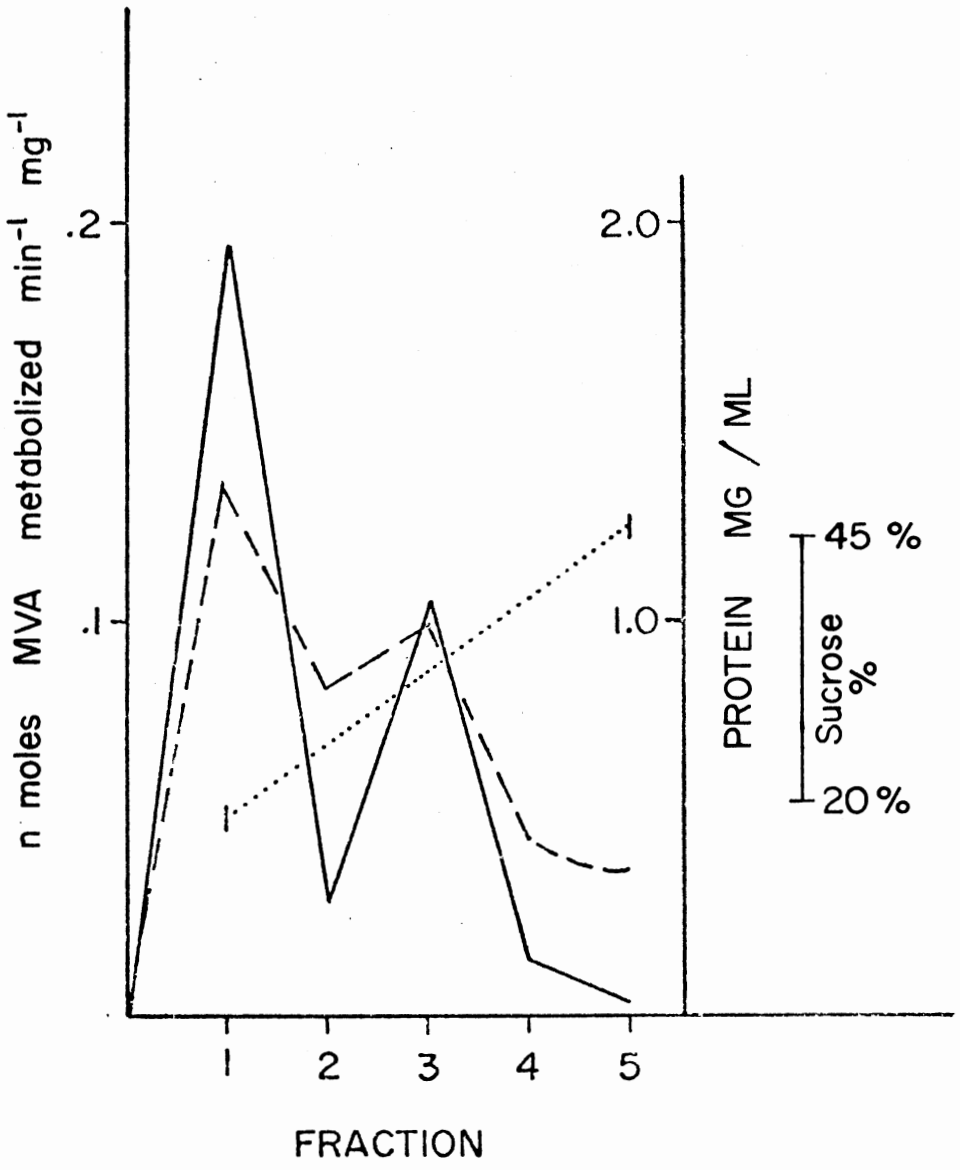
PURIFICATION OF MEVALONATE KINASE FROM N. CATARIA LEAF

Step	Volume ml	Concentration Units/ml	Total Units	Protein Mg/ml	Specific[(a)] Activity	Yield	Purification
Crude Homogenate	60.0	410	24,500	17.8	.024	100.0%	1.00
3,000 x g Supernatant	55.0	422	23,210	15.0	.028	94.0%	1.19
30,000 x g Supernatant	52.0	486	25,272	14.0	.035	102.0%	1.47
Ammonium Sulfate Fractionation - - - - -							
30% Supernatant	47.0	392	18,424	9.2	.043	75.0%	1.81
30% Pellet	5.3	0	0	2.3	.0	0.0	0.0
70% Supernatant	44.0	15	660	6.2	.002	3.0%	0.10
70% Pellet	10.2	990	10,098	9.7	.051	41.2%	2.14
Sucrose Gradient - - - - -							
Fraction #1	18.0	258	4,644	1.33	.194	18.9%	8.2
Fraction #3	18.0	105	1,890	1.02	.103	7.8%	4.4
P-100 Column - - - - -							
Fraction #10	9.0	427	3,843	0.42	1.068	15.6%	45.3

[(a)] Specific Activity Units are given in nanomoles of mevalonate metabolized min^{-1} mg^{-1}.

Figure 6. Protein and Mevalonate Kinase Activity Profile from 20 to 45 Percent Sucrose Gradient

Centrifugation of a 30 to 70 percent ammonium sulfate precipitate from leaf tissue. Protein (---), Mevalonate kinase (——)


n moles MVA metabolized min⁻¹ mg⁻¹
.2
.1
2.0
1.0
PROTEIN MG / ML
45 %
Sucrose %
20 %
1
2
3
4
5
FRACTION

110 mM DL-[2-^{14}C] mevalonate (sp. act. 4.6-5.9 x 10^3 DPM/μmole) and 10 μM β-glycerol phosphate in a total volume of one ml. Figure 7 shows the effect of 10 μM β-glycerol phosphate on the mevalonate kinase activity isolated from the first fraction of the sucrose gradient.

Table II shows the effect that 10 μM β-glycerol phosphate has on the two mevalonate metabolizing systems found in the sucrose gradient. Fraction one shows a four-fold increase in product formation whereas fraction three product formation remains virtually unchanged. These data suggest that fraction one contains contaminating non-specific phosphatase enzymes since the addition of β-glycerol phosphate, a phosphatase inhibitor, increases the amount of phosphorylated products formed. Likewise fraction three is relatively free of non-specific phosphatase activities.

TABLE II

EFFECT OF β-GLYCEROL PHOSPHATE

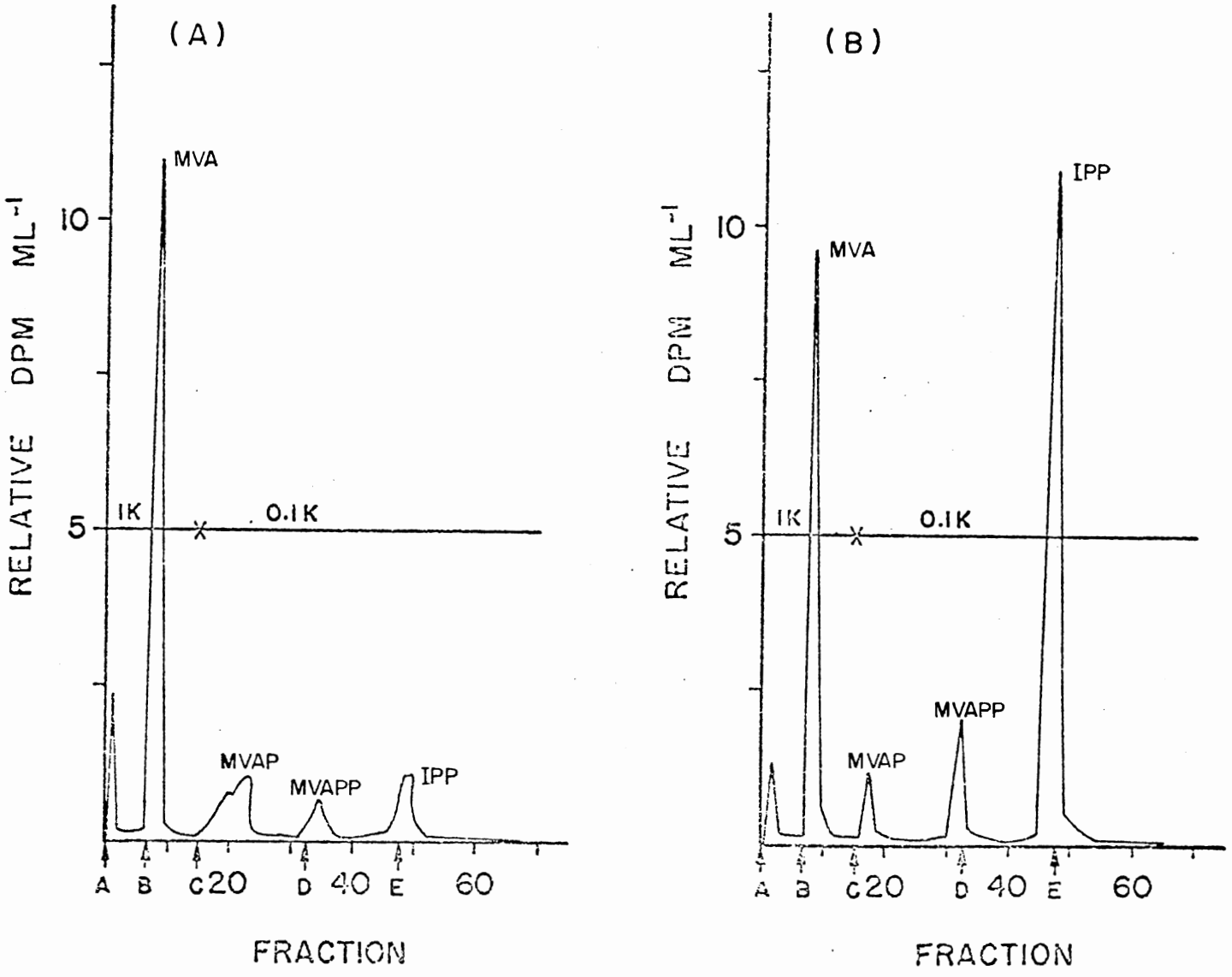
	-β-Glycerol Phosphate nmoles/min mg	+β-Glycerol Phosphate nmoles/min mg
Fraction 1	.018	.081
Fraction 3	.086	.089

Figure 7. Effect of β-Glycerol Phosphate on Mevalonate Kinase Activity From Fraction One of 20 to 45 Percent Sucrose Gradient Centrifugation of 30 to 70 Percent Ammonium Sulfate Precipitate of Leaf Tissue

Dowex-1 formate elution profiles following assay of fraction one of discontinuous 20 to 45 percent sucrose gradient (A) under standard assay conditions, (B) under standard assay conditions plus 10 μM β-glycerol phosphate. Elution of Dowex-1 formate columns:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 3.0 ml fractions


(A)
RELATIVE DPM ML⁻¹
10
5
MVA
1K
0.1K
MVAP
MVAPP
IPP
A B C 20 D 40 E 60
FRACTION
(B)
RELATIVE DPM ML⁻¹
10
5
IPP
MVA
1K
0.1K
MVAPP
MVAP
A B C 20 D 40 E 60
FRACTION

Fractions one and three also contain 5-phosphomevalonate kinase as well as mevalonate kinase activity. Fraction one contains 5-pyrophosphomevalonate decarboxylase activity, but fraction three contains very little of this activity (Figure 8).

Fraction one from the sucrose gradient was dialyzed against the same buffer as previously described, concentrated to 1.5 ml on a Diaflow-membrane apparatus and run on a Bio-Gel P-100 column (2 x 40 cm) which was equilibrated with 100 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM mevalonate. The elution profile is shown in Figure 9. Only one peak of mevalonate kinase activity was observed. The kinase activity eluted in the void volume of the P-100 column. The partially purified mevalonate kinase as reported in Table I, had a specific activity of 1.068 nmoles of mevalonate metabolized per minute per mg of protein. This represented a purification of nearly 50 fold.

The mevalonate kinase obtained from the P-100 column void volume was found to be stable at 4°C for up to three weeks. The enzyme was also stable to lyophilization and could be stored in this form.

Co-purification of mevalonate kinase, 5-phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase through the Bio-Gel P-100 column chromatography step is demonstrated by the elution profile of the products on Dowex-1 formate column (Figure 10). This observation indicates that the enzymes are either all physically similar and of high molecular weight or associated in an enzyme-like complex. The suggestion of a high molecular weight multi-enzyme complex is consistent with the ideas expressed by Banthorpe et al., (4) and Francis (5) "that the enzymes involved in monoterpene biosynthesis may be associated in a multi-enzyme complex with a high degree of spatial

Figure 8. Effect of β-Glycerol Phosphate on Mevalonate Kinase Activity from Fraction Three of 20 to 45 Percent Sucrose Gradient Centrifugation of 30 to 70 Percent Ammonium Sulfate Precipitate of Leaf Tissue

Dowex-1 formate elution profiles following assay of fraction one of discontinuous 20 to 45 percent sucrose gradient (A) under standard assay conditions, (B) under standard assay conditions plus 10 μM β-glycerol phosphate. Elution of Dowex-1 formate column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 3.0 ml fractions

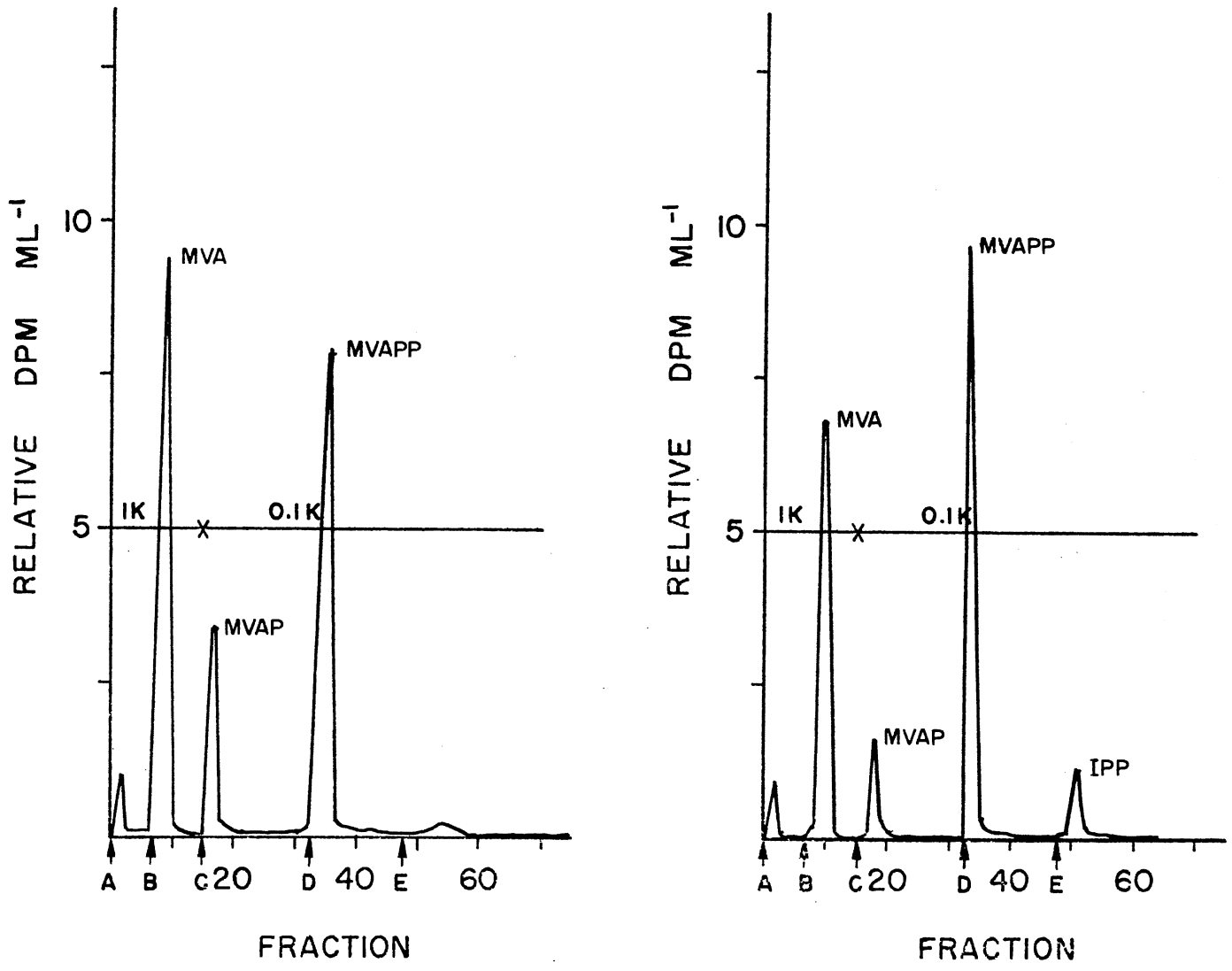

RELATIVE DPM ML-1
10
5
MVA
MVAPP
MVAP
IK
0.1K
A
B
C
20
D
40
E
60
FRACTION
RELATIVE DPM ML-1
10
5
MVAPP
MVA
IK
0.1K
MVAP
IPP
A
B
C
20
D
40
E
60
FRACTION

Figure 9. Protein and Mevalonate Kinase Activity Profile from Bio-Gel P-100 Column of Leaf Tissue

Protein (——), Mevalonate kinase (---)

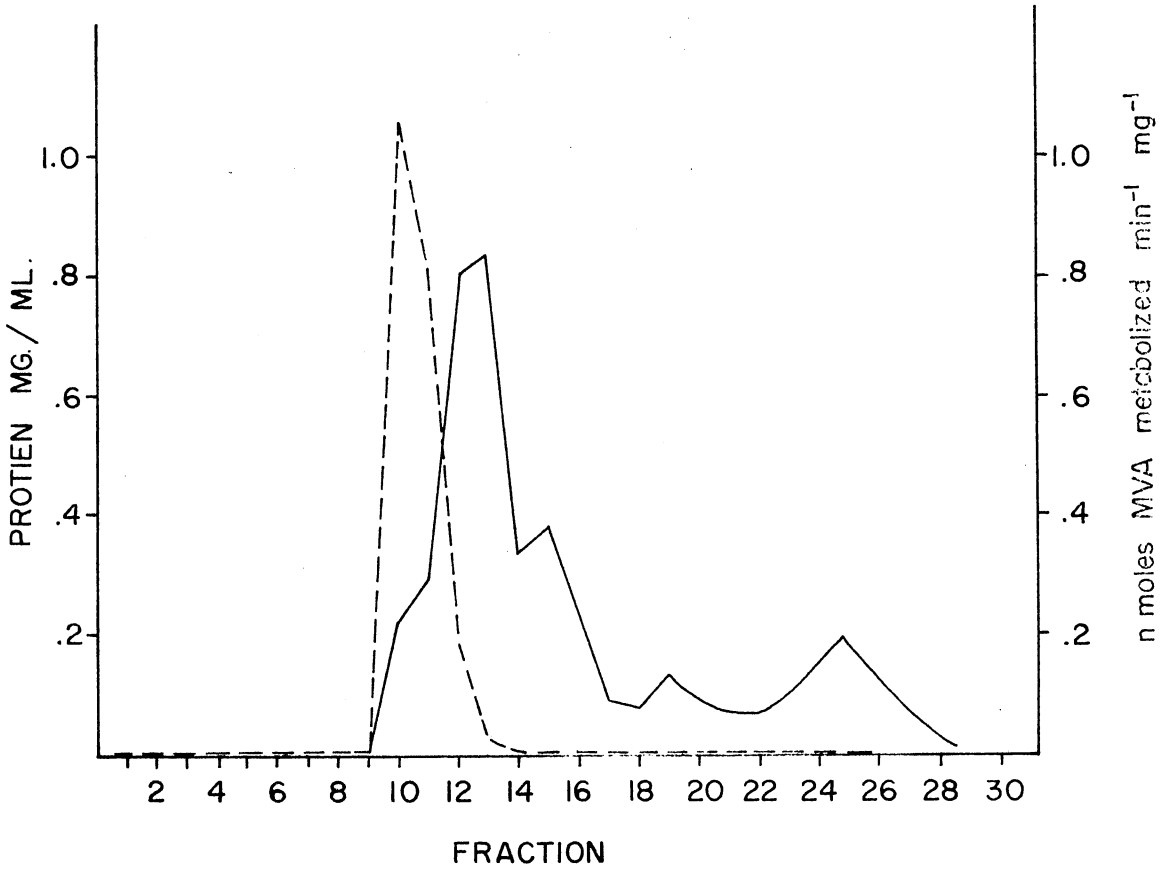
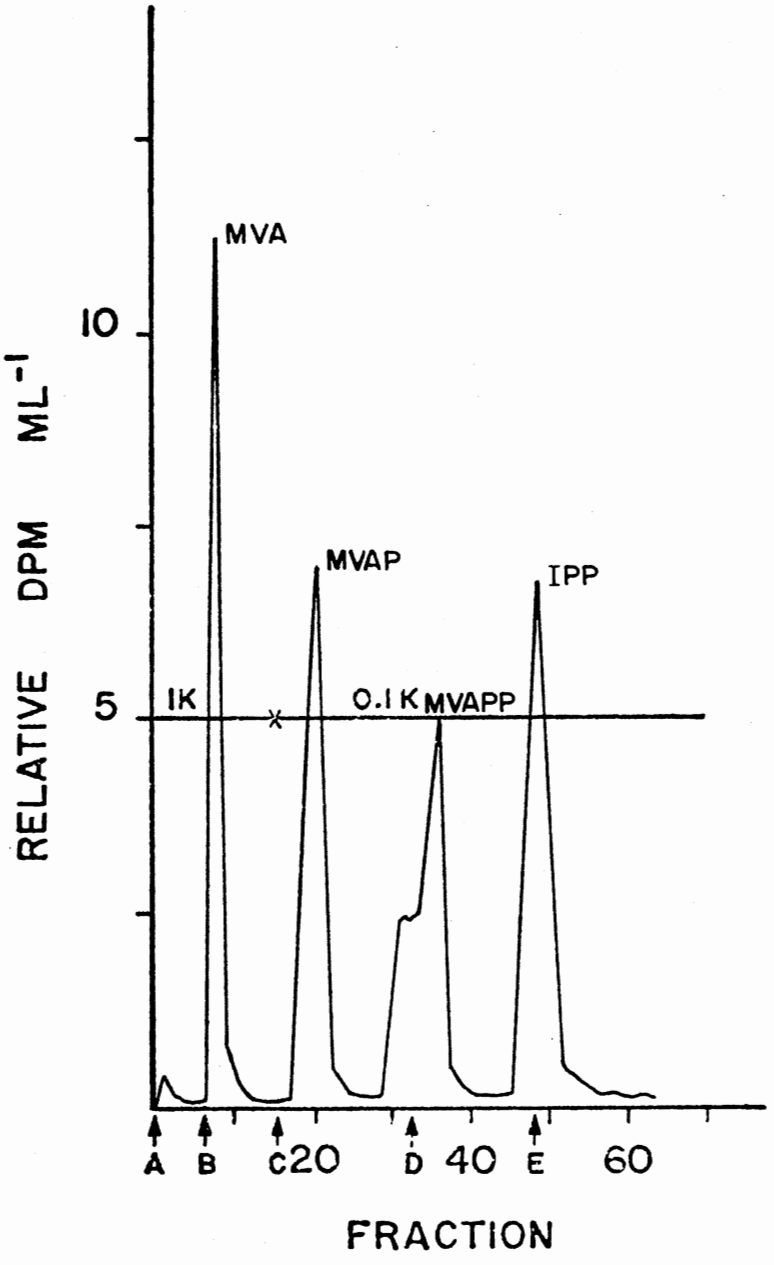

PROTIEN MG. / ML.
1.0
.8
.6
.4
.2
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
FRACTION
n moles MVA metabolized min⁻¹ mg⁻¹
1.0
.8
.6
.4
.2

Figure 10. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of Fraction 10 from Bio-Gel P-100 Column of Leaf Tissue

Elution profile following mevalonate kinase assay of fraction 10 from Bio-Gel P-100 column under standard assay conditions. Elution of Dowex-1 column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 3.0 ml fractions


RELATIVE DPM ML⁻¹
10
5
MVA
MVAP
IPP
IK
0.1 K
MVAPP
A
B
C
20
D
40
E
60
FRACTION

organization for efficient substrate circulation." Mevalonate kinase from a number of sources has been reported to have a molecular weight of approximately 100,000 daltons (29). This corresponds very well with the observation that mevalonate kinase activity voids a Bio-Gel P-100 column, which indicates a minimum molecular weight of 100,000 daltons.

b. Intracellular Location. The observation of two different mevalonate metabolizing systems in the plant leaf tissue leads one to the question of the intracellular location of these two systems. The following experiments were attempted in an effort to localize these activities.

The procedure as described in Figure 2 was used to obtain microsomal membranes. The 30,000 x g supernatant was obtained in the same manner as described in the previous section. The 30,000 x g supernatant contained 93% of the total units as indicated in Table III. The 30,000 x g supernatant was centrifuged at 150,000 x g for two hours. The 150,000 x g 2 hour pellet was resuspended in 100 mM Tris pH 7.5, 10 mM $MgCl_2$, 1.0 mM EDTA, 0.5 mM dithiothreitol and 0.1 mM mevalonate and dialyzed against the same buffer without EDTA. The 2 hour pellet contained very little mevalonate kinase activity as shown in Figure 11. There was also no alkaline phosphatase activity in the 150,000 x g pellet (2 hour).

The 150,000 x g supernatant was then centrifuged at 150,000 x g for 16 hours. The pellet was resuspended and dialyzed as previously described. The 150,000 x g 16 hour pellet also contained alkaline phosphatase activity. For this reason all assays were carried out in the presence of 10 μM β-glycerol phosphate. Figure 12 indicates that

TABLE III

MEMBRANE ISOLATION

Step	Volume ml	Concentration Units/ml	Total Units	Protein Mg/ml	Specific[(a)] Activity	Yield	Specific[(b)] Activity
Crude Homogenate	60	391	23,460	16.6	.0235	100.0%	--
30,000 x g Supernatant	54	405	21,870	13.0	.0312	93.0%	--
150,000 x g 2 Hr Pel	3	44	132	9.0	.049	0.6%	--
150,000 x g 16 Hr Pel	4	2580	10,320	7.4	.1395	44.4%	1.85
150,000 x g 16 Hr Sup	46	1	46	8.0	.002	0.2%	0.46
Sucrose Gradient - - - - -							
Fraction 1	24	360	8,640	0.91	.1758	36.8	1.61
2	24	6	144	0.32	4.7	--	--
3	24	3	72	0.36	2.2	--	--
4	24	2	48	0.24	4.0	--	--
5	24	0	0	0.29	0.0	0	--

(a) Specific Activity Units are given in nanomoles of mevalonate formation min^{-1} mg^{-1}

(b) Specific Activity Units are given in nanomoles of p-NO_2 phenyl phosphate hydrolyzed min^{-1} mg^{-1}

Figure 11. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of Pellet from 2 Hour 150,000 x g Centrifugation of Leaf Tissue

Elution profile following mevalonate kinase assay of pellet from 150,000 x g centrifugation for 2 hours under standard assay conditions. Elution of Dowex-1 column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 3.0 ml fractions

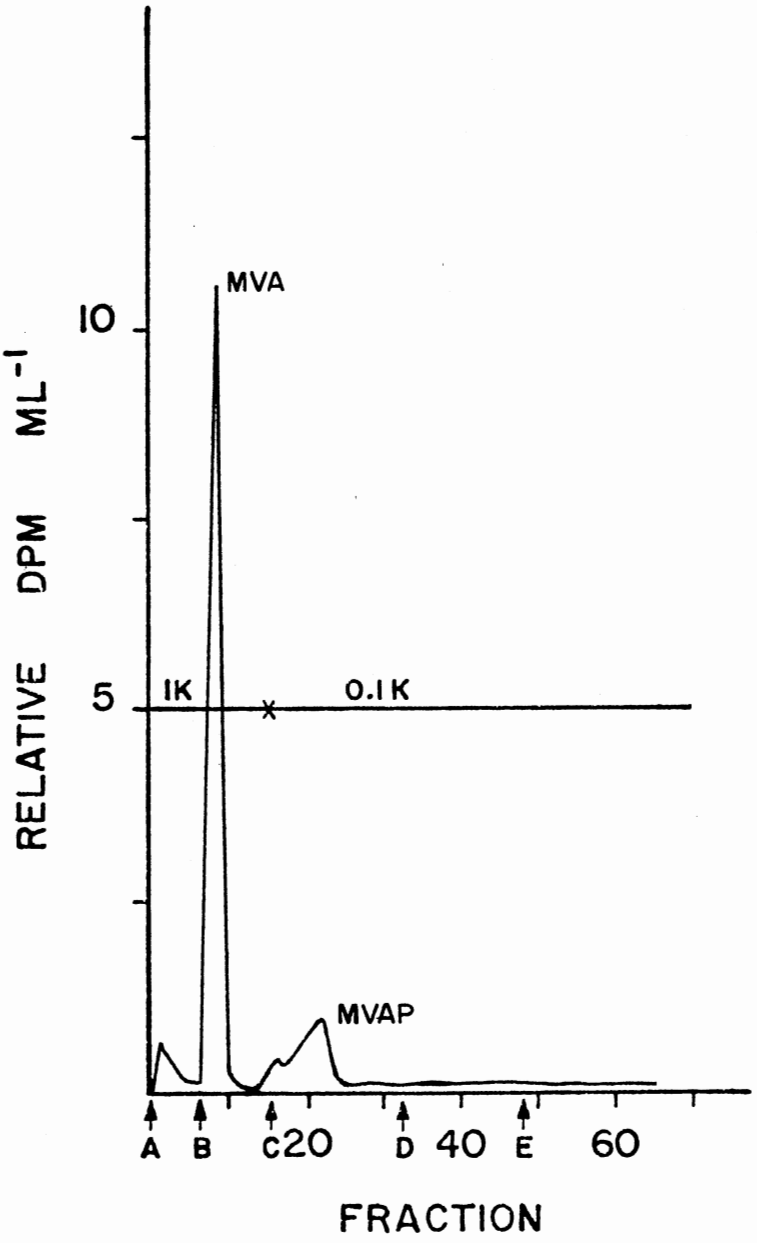
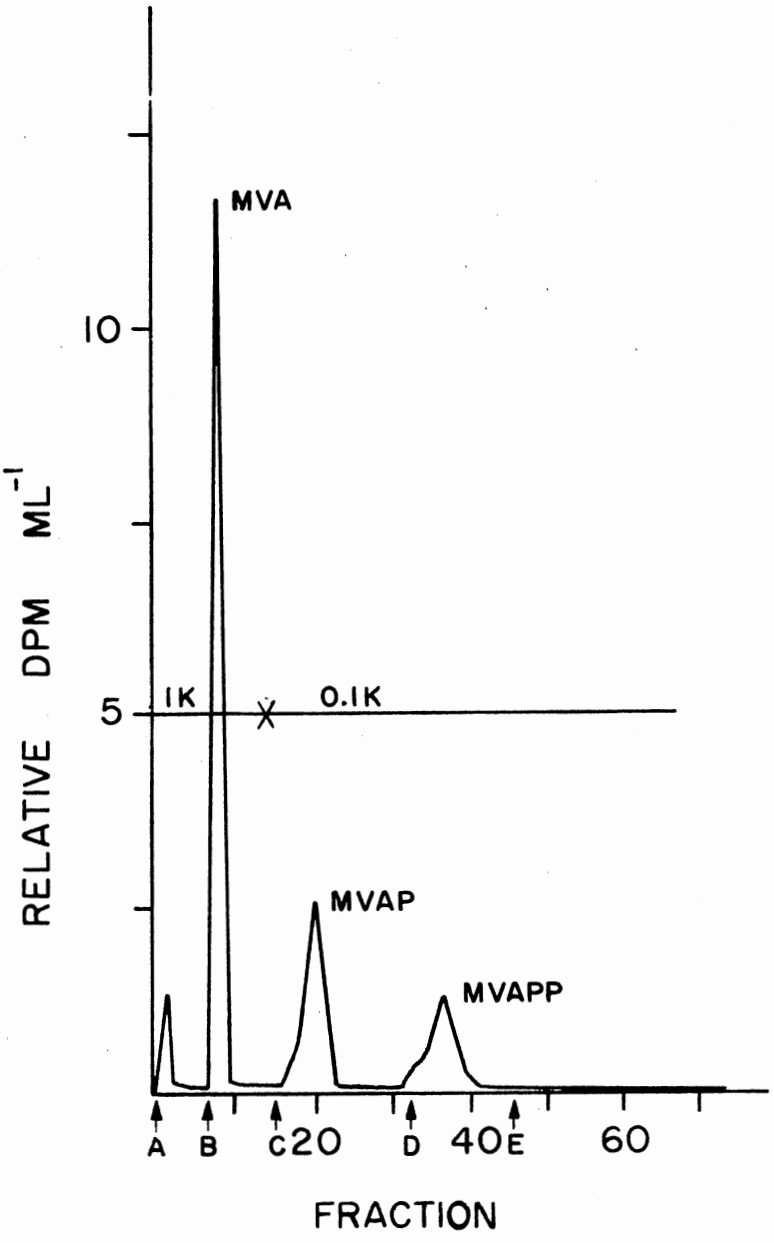

RELATIVE DPM ML-1
10
5
MVA
1K
0.1K
MVAP
A
B
C
20
D
40
E
60
FRACTION

Figure 12. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of Pellet from 16 Hour 150,000 x g Centrifugation of Leaf Tissue

Elution profile following mevalonate kinase assay of pellet from 150,000 x g centrifugation for 16 hours under standard assay conditions. Elution of Dowex-1 column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 3.0 ml fractions


MVA
10
5
1K
0.1K
MVAP
MVAPP
RELATIVE DPM ML^{-1}
A
B
C
20
D
40
E
60
FRACTION

5-phosphomevalonate kinase as well as mevalonate kinase activity is present in the 16 hour pellet. The ratio of 5-phosphomevalonate to 5-pyrophosphatemevalonate is 32:18. This is the first report of mevalonate kinase being associated with a particulate fraction in plant tissue. The fact that a pellet was obtained after 16 hours at 150,000 x g also suggests that the protein (enzymes) are very large and are perhaps aggregated.

One ml of the dialyzed 16 hour pellet were layered on a 30 ml 20-45% sucrose gradient. The gradient was centrifuged at 20,000 x g for three hours and five fractions of six mls was collected. The fractions were dialyzed in similar manner as for the 2 hour pellet and then assayed for mevalonate kinase and alkaline phosphatase. Figure 13 and Table III indicate that the bulk of mevalonate kinase activity and alkaline phosphatase are both in fraction one. The profile of products on the Dowex-1 formate column (Figure 14) shows that 5-phosphomevalonate kinase is also present in fraction one. The ratio of 5-phosphomevalonate to 5-pyrophosphomevalonate is 31:19. This ratio of products is virtually the same as obtained from the resuspended 16 hour pellet which was 32:18. The presence of mevalonate kinase and 5-phosphomevalonate kinase in the same fraction throughout purification and the observation that the ratio of products remains constant suggest the possibility that either mevalonate kinase and 5-phosphomevalonate kinase may be associated in some form or the reaction is catalyzed by the same enzyme or protein. This suggestion needs further study.

Figure 13. Protein and Mevalonate Kinase Activity Profile from 20 to 45 Percent Sucrose Gradient Centrifugation of 16 Hour Pellet from Leaf Tissue

Protein (---), Mevalonate Kinase (——)

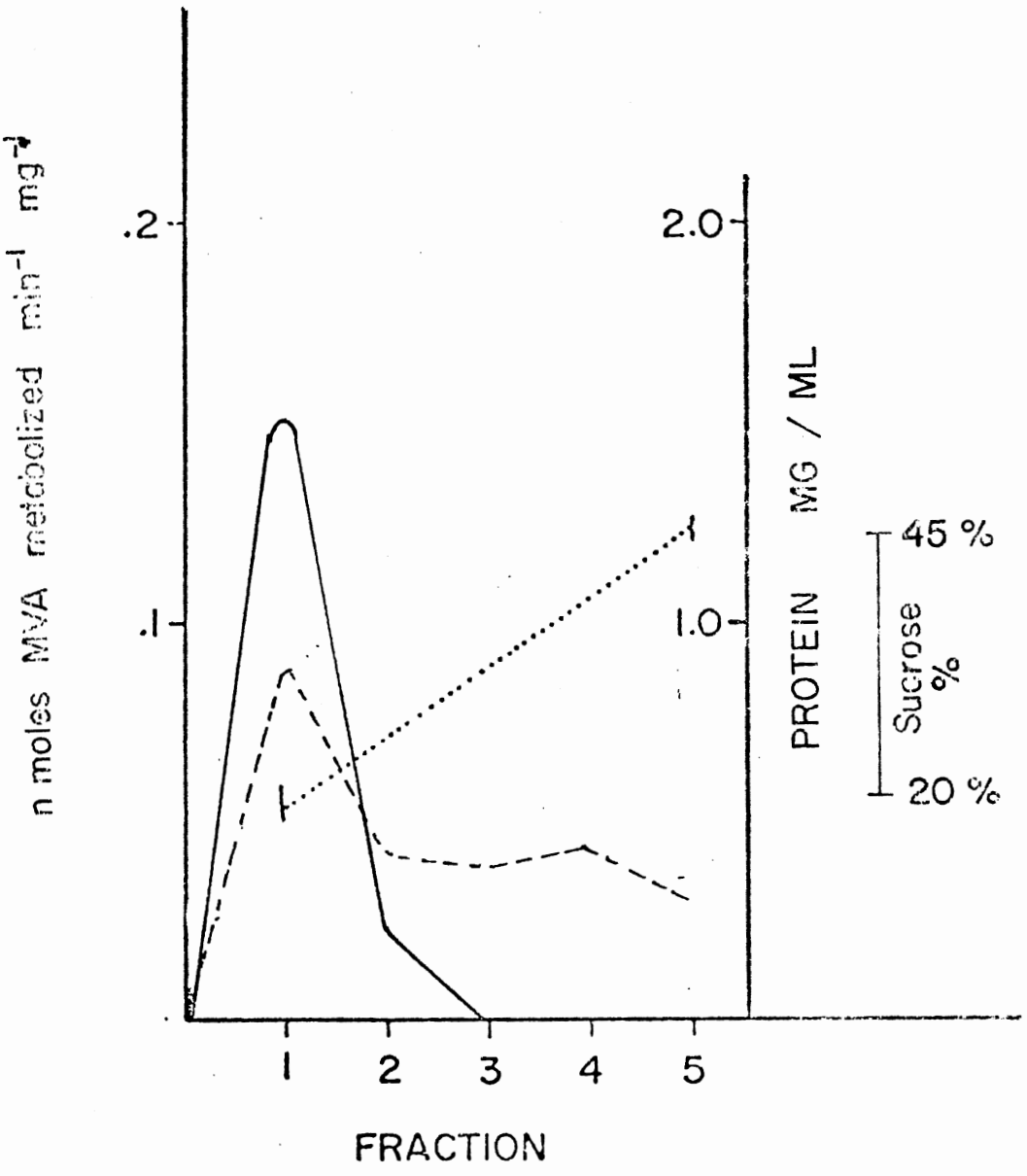
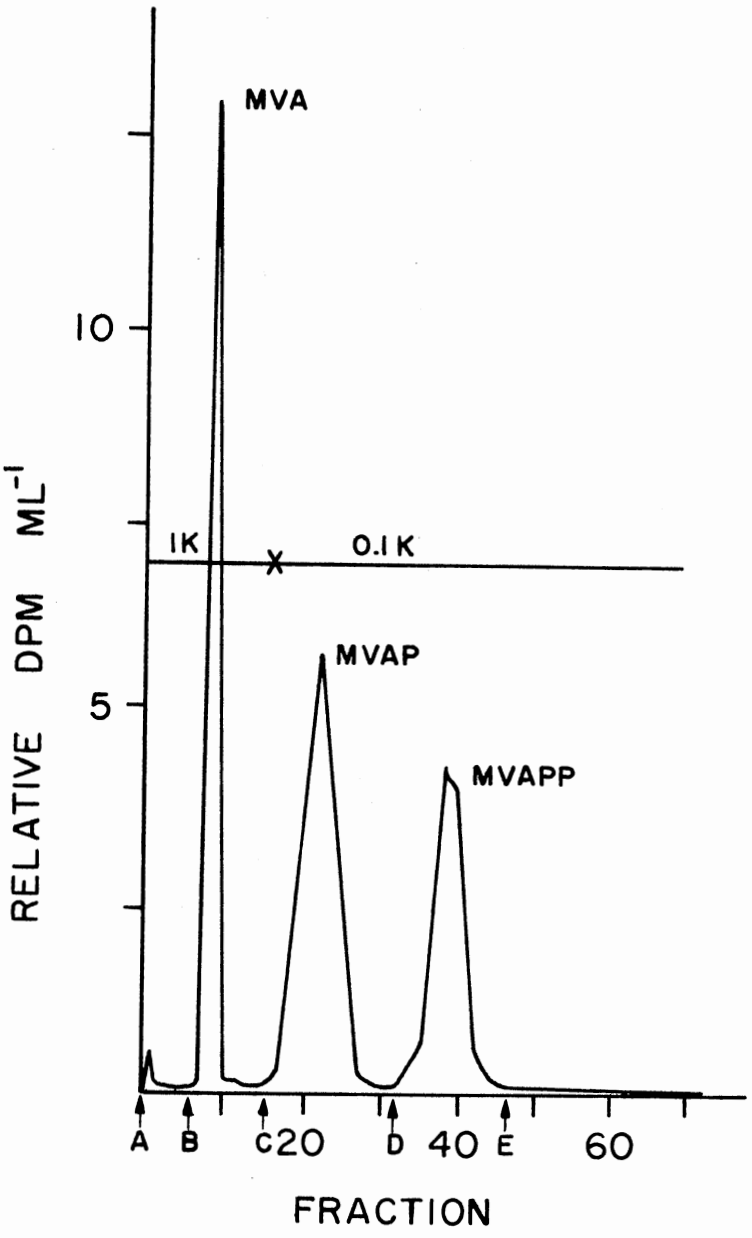

n moles MVA metabolized min⁻¹ mg⁻¹
.2
.1
2.0
1.0
PROTEIN MG / ML
45 %
Sucrose %
20 %
1
2
3
4
5
FRACTION

Figure 14. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of Fraction One from 20 to 45 Percent Sucrose Gradient Centrifugation of 16 Hour Pellet from Leaf Tissue

Elution profile following mevalonate kinase assay of fraction one from 20 to 45 percent discontinuous sucrose gradient of 16 hour pellet under standard assay conditions. Elution of Dowex-1 column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 3.0 ml fractions


MVA
10
1K
0.1K
MVAP
5
MVAPP
RELATIVE DPM ML-1
A
B
C
20
D
40
E
60
FRACTION

2. Chloroplast Isolation

Whole chloroplasts were isolated by a modified method of Bassham and Jensen (67). Leaf tissue was homogenized in 50 mM MES pH 6.1, 20 mM NaCl, 330 mM sorbitol, 2 mM $NaNO_3$, 2 mM EDTA, 2 mM Na isoascorbate, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 0.5 mM K_2HPO_4. The leaf homogenate was centrifuged at 2,000 x g for 50 seconds. The 2,000 x g pellet was resuspended in 1 ml of 50 mM HEPES pH 6.7, 20 mM NaCl, 330 mM sorbitol, 2 mM $NaNO_3$, 2 mM EDTA, 2 mM Na isoascorbate, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 0.5 mM K_2HPO_4 and centrifuged again at 2,000 x g for 50 seconds. Examination by light microscopy showed whole chloroplasts. The pellet was resuspended as before and homogenized. The homogenized chloroplasts were placed on a 30 ml 20-45% sucrose gradient and centrifuged at 20,000 x g for three hours. Five fractions of six mls each were collected and assayed for mevalonate kinase activity.

Mevalonate kinase activity was found to be present only in fraction three. Figure 15 shows the product profile from a Dowex-1 formate column of the third fraction. Fraction three was shown to contain both mevalonate kinase and 5-phosphomevalonate kinase activities. These findings show that the mevalonate kinase activities are located in the microsomal membranes and chloroplasts in the leaf tissue of N. cataria. Rodgers, Shah and Goodwin (46) have also reported mevalonate kinase activity in the chloroplast fraction.

3. Callus Tissue from N. Cataria

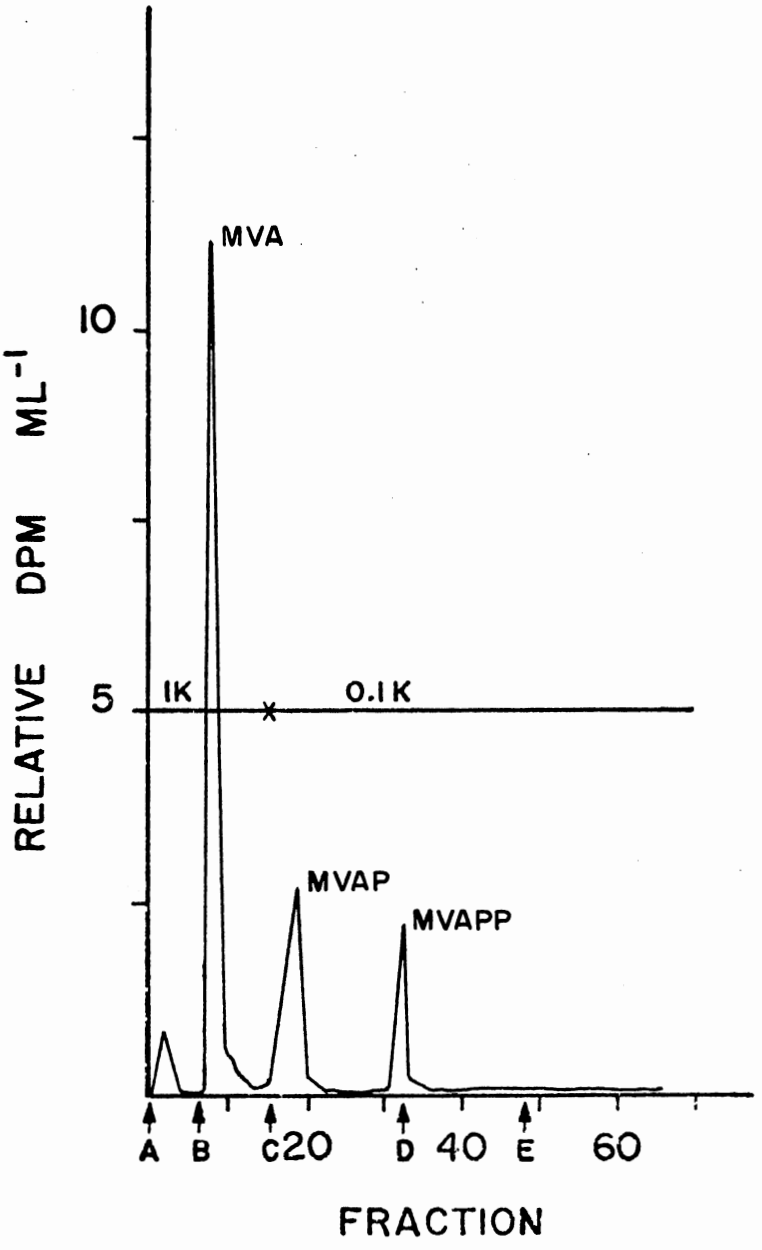
Callus tissue from N. cataria was homogenized in a similar fashion as described for leaf tissue. The homogenate was centrifuged at 3,000

Figure 15. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of Fraction Three from 20 to 45 Percent Sucrose Gradient Centrifugation of Homogenized Chloroplasts from Leaf Tissue

Elution profile following mevalonate kinase assay of fraction 3 from 20 to 45 percent discontinuous sucrose gradient of homogenized chloroplasts under standard assay conditions. Elution of Dowex-1 column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 3.0 ml fractions


RELATIVE DPM ML⁻¹
10
5
MVA
1K
0.1K
MVAP
MVAPP
A
B
C
20
D
40
E
60
FRACTION

x g for 20 minutes. The pellet was discarded and the supernatant was centrifuged at 30,000 x g for 20 minutes. The 30,000 x g supernatant was dialyzed and 1.5 ml of the 30,000 x g supernatant was applied to a 30 ml 20-45% sucrose gradient. Five fractions of six mls each were collected, dialyzed and assayed for mevalonate kinase activity. Figure 16 is a profile of protein and mevalonate kinase activity in each of the fractions. Only one mevalonate kinase activity profile was observed, that being in fraction one which is the activity corresponding to the microsomal activity described in the leaf tissue. The product profile of fraction one, Figure 17, indicates that 5-phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase are also present.

Callus tissue is markedly lacking in chloroplast. If one of the two mevalonate kinase activities is in the chloroplast, one could not expect to find two mevalonate kinase activities in callus tissue. The observation of only one mevalonate kinase activity in callus tissue lends further support to the idea that terpenoid compounds are synthesized in the chloroplasts.

4. Comparison of Mevalonate Activating Enzymes from N. Cataria Leaf and Callus, Hog Liver and Yeast

Mevalonate kinase activity was found to be in the 30-70% ammonium sulfate precipitate in hog liver and yeast tissue.

The 30-70% pellet from both sources was resuspended and dialyzed as described from the plant tissue. Then one ml of the dialyzed pellet was placed on 30 ml 20-45% sucrose gradient. Five fractions

Figure 16. Protein and Mevalonate Kinase Activity Profile from 20 to 45 Percent Sucrose Gradient Centrifugation of 30 to 70 Percent Ammonium Sulfate Precipitate from Callus

Protein (---), Mevalonate Kinase (——)

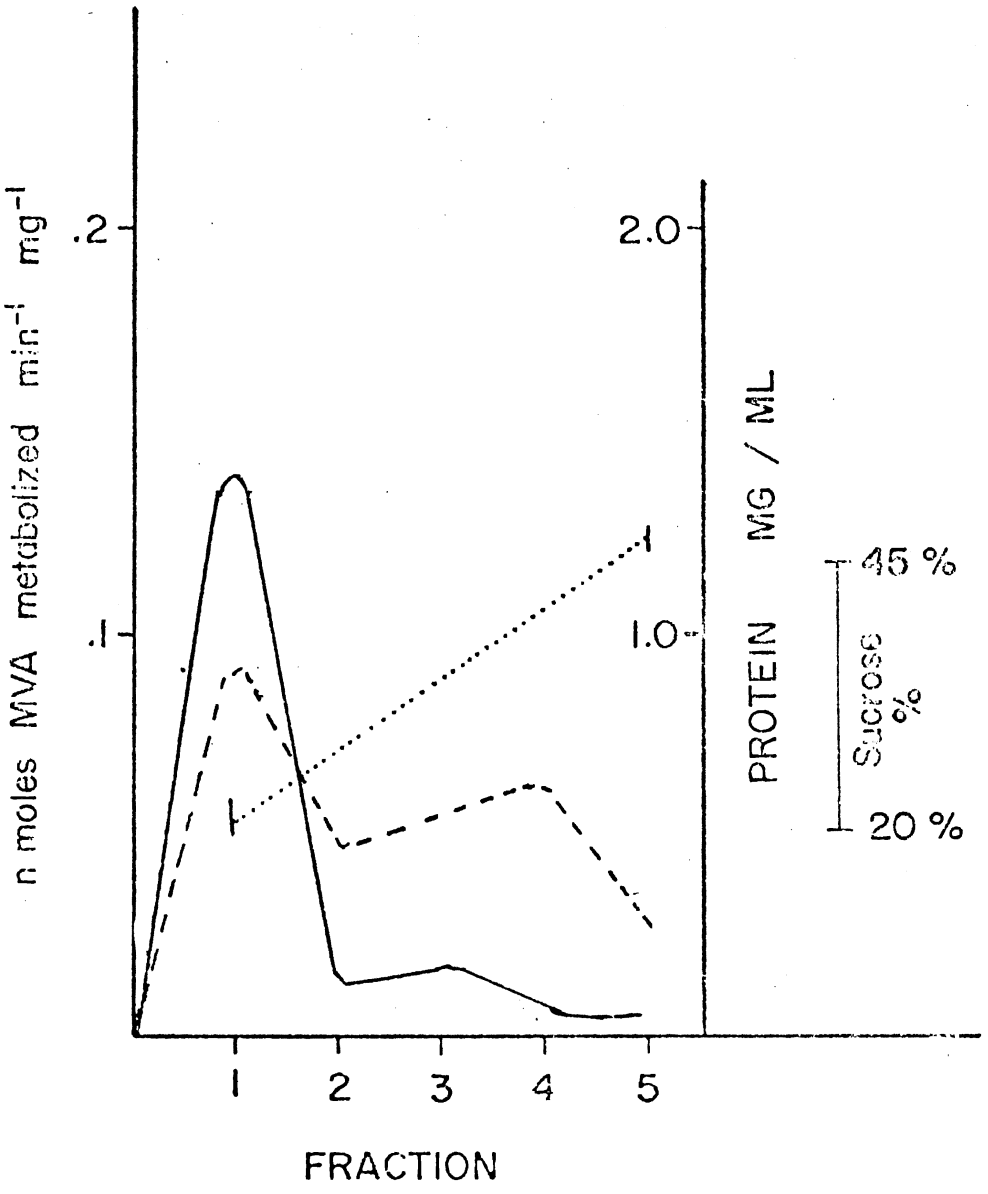
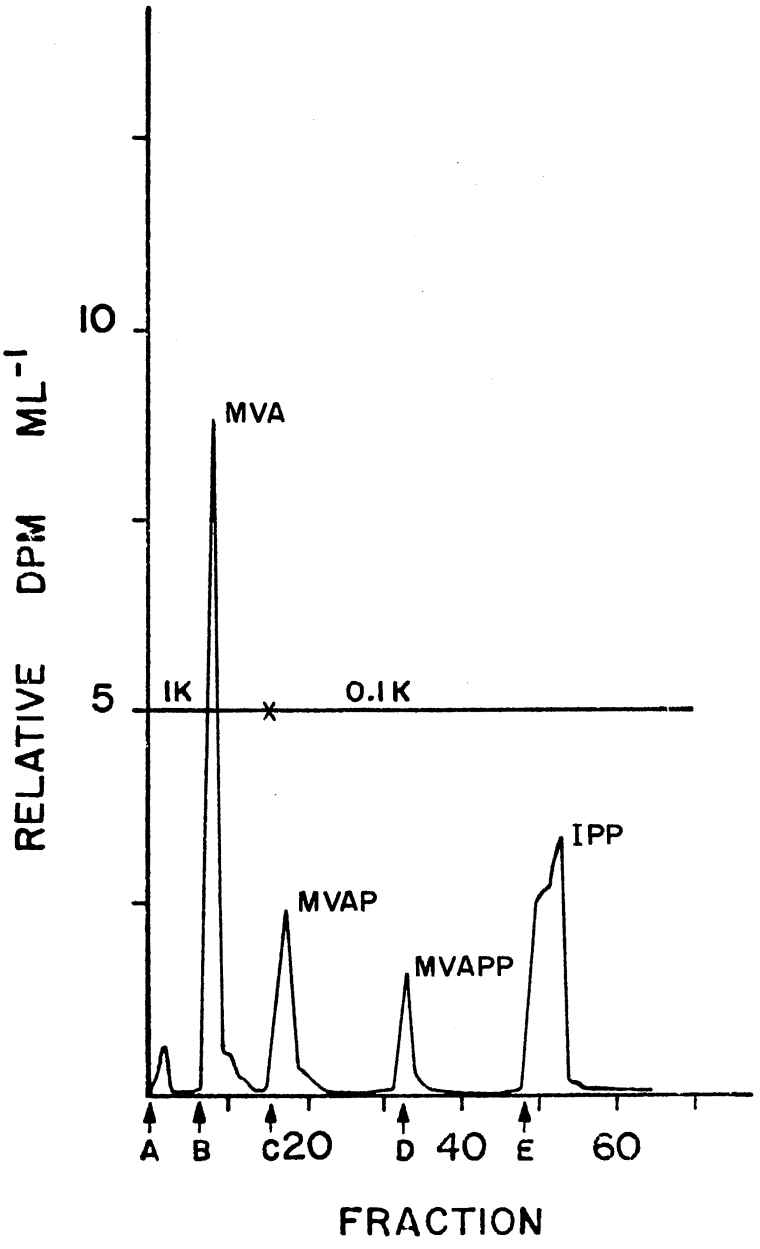

n moles MVA metabolized min⁻¹ mg⁻¹
.2
.1
2.0
1.0
PROTEIN MG / ML
45 %
Sucrose %
20 %
1
2
3
4
5
FRACTION

Figure 17. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of Fraction One from 20 to 45 Percent Sucrose Gradient Centrifugation of 30 to 70 Percent Ammonium Sulfate Precipitate from Callus

Elution profile following mevalonate kinase assay of fraction one from 20 to 45 percent discontinuous sucrose gradient of callus under standard assay conditions. Elution of Dowex-1 column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 30 ml fractions


RELATIVE DPM ML⁻¹
10
5
MVA
1K
0.1K
MVAP
MVAPP
IPP
A
B
C
20
D
40
E
60
FRACTION

were collected and assayed for mevalonate kinase activity. Figure 18 shows the profile of protein and mevalonate kinase activity from the gradient of the yeast tissue. Just as in the callus, only one mevalonate kinase activity was observed, that being fraction one. Figure 19 is the profile from hog liver and again one major peak of activity is in fraction one. The product profiles of the fractions with activity are shown in Figure 20. The yeast (Figure 20 A) like the plant system shows some decarboxylase activity, but the hog liver (Figure 20 B) does not.

5. Mevalonate Kinase from Turkey Liver and Kidney

Cell-free extracts from quick frozen (liquid nitrogen) turkey kidney and liver tissue was obtained by homogenizing 100 grams of tissue in a blender with a 100 ml homogenization buffer. (The homogenization buffer was the same as the buffer described in Preparation of Cell-Free Extracts with the exception that 50 mM sucrose was used.)

Cell-free extracts from turkey kidney were subjected to the procedure described in Figure 3. The mevalonate kinase activity was found in the 30 to 70% ammonium sulfate precipitate. The 30 to 70% ammonium sulfate pellet was resuspended, dialyzed and placed on a 30 ml 20-45% sucrose gradient as described for the mevalonate kinase isolated from N. cataria (6.4 mg/ml).

The product profile from the Dowex-1 formate column, shown in Figure 21, indicates that both mevalonate kinase and 5-phosphomevalonate kinase activity are present (1.2 mg/ml). The ratio of 5-phosphomevalonate to 5-pyrophosphomevalonate is 57:43 or 1.33.

Figure 18. Protein and Mevalonate Kinase Activity Profile from 20 to 45 Percent Sucrose Gradient Centrifugation of 30 to 70 Percent Ammonium Sulfate Precipitate from Yeast Autolysates

Protein (---), Mevalonate Kinase (——)

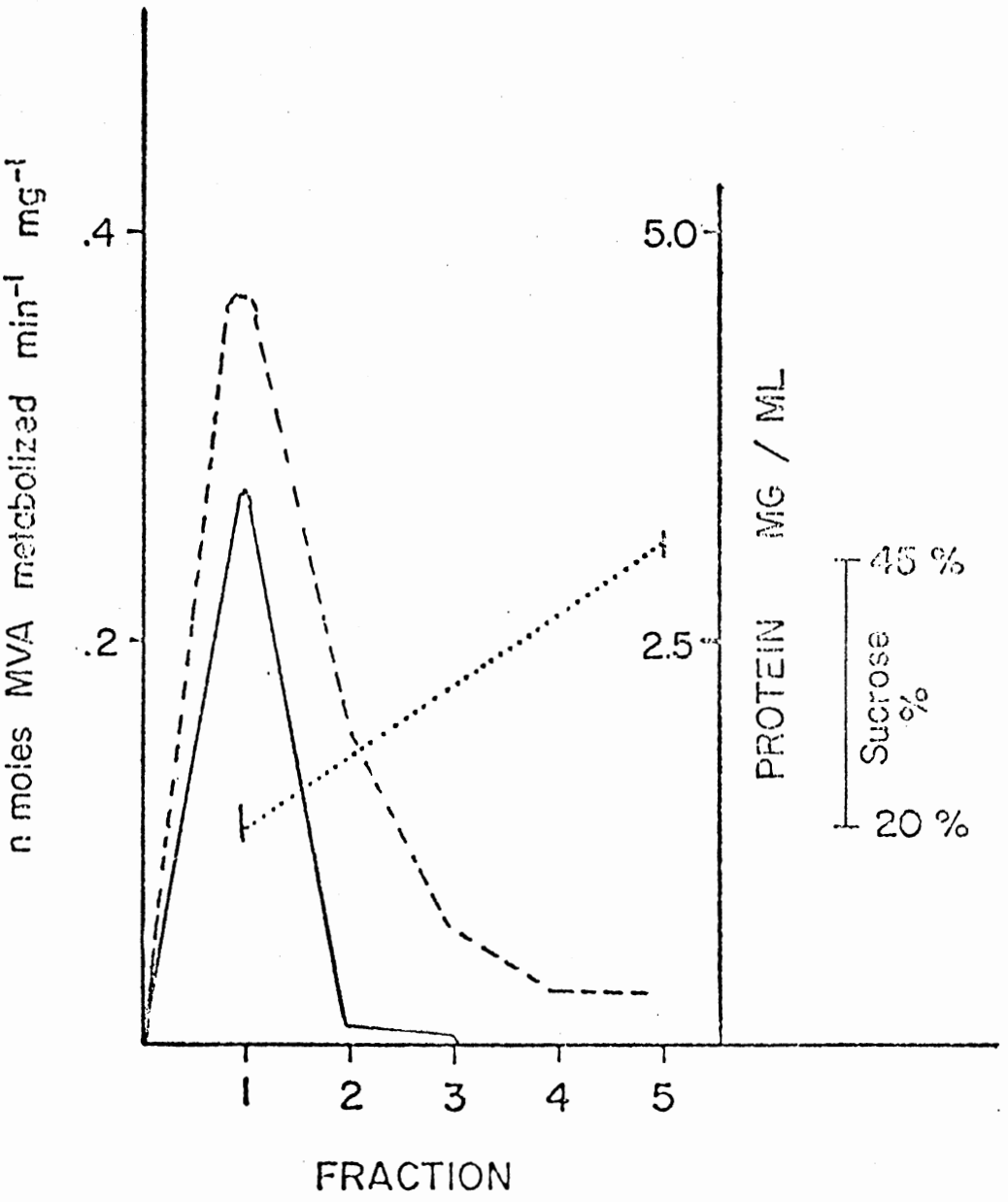

n moles MVA metabolized min⁻¹ mg⁻¹
.4
.2
5.0
2.5
PROTEIN MG / ML
45 %
Sucrose %
20 %
1
2
3
4
5
FRACTION

Figure 19. Protein and Mevalonate Kinase Activity Profile from 20 to 45 Percent Sucrose Gradient Centrifugation of 30 to 70 Percent Ammonium Sulfate Precipitate from Hog Liver Tissue

Protein (---), Mevalonate Kinase (——)

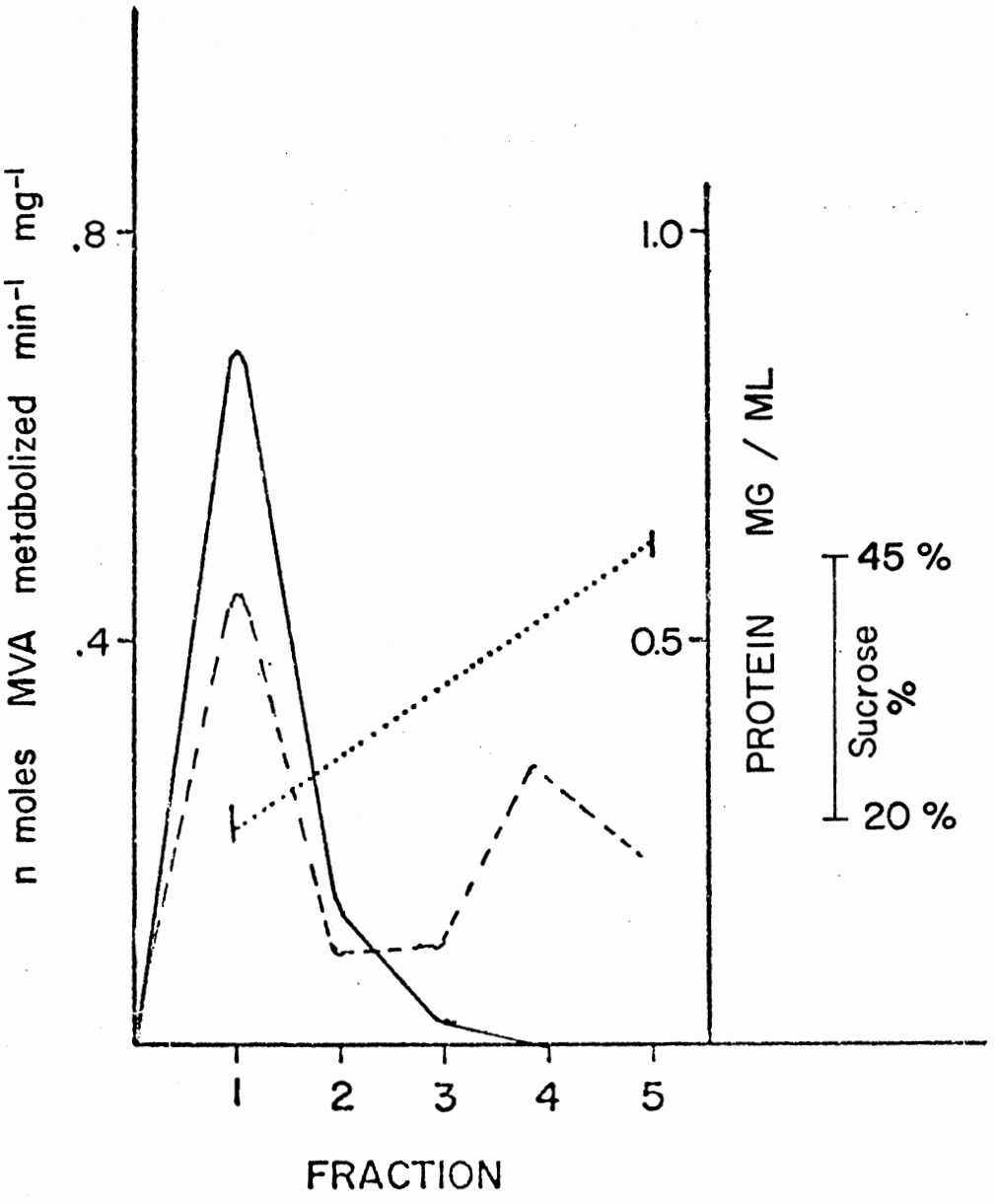

n moles MVA metabolized min⁻¹ mg⁻¹
.8
.4
1.0
0.5
PROTEIN MG / ML
45 %
Sucrose %
20 %
1
2
3
4
5
FRACTION

Figure 20. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of Fraction One from 20 to 45 Percent Sucrose Gradient Centrifugation of (A) Yeast and (B) Hog Liver

Elution profiles following assay for mevalonate kinase of (A) yeast and (B) hog liver fraction one from 20 to 45 percent discontinuous sucrose gradient under standard assay conditions. Elution of Dowex-1 column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 30 ml fractions

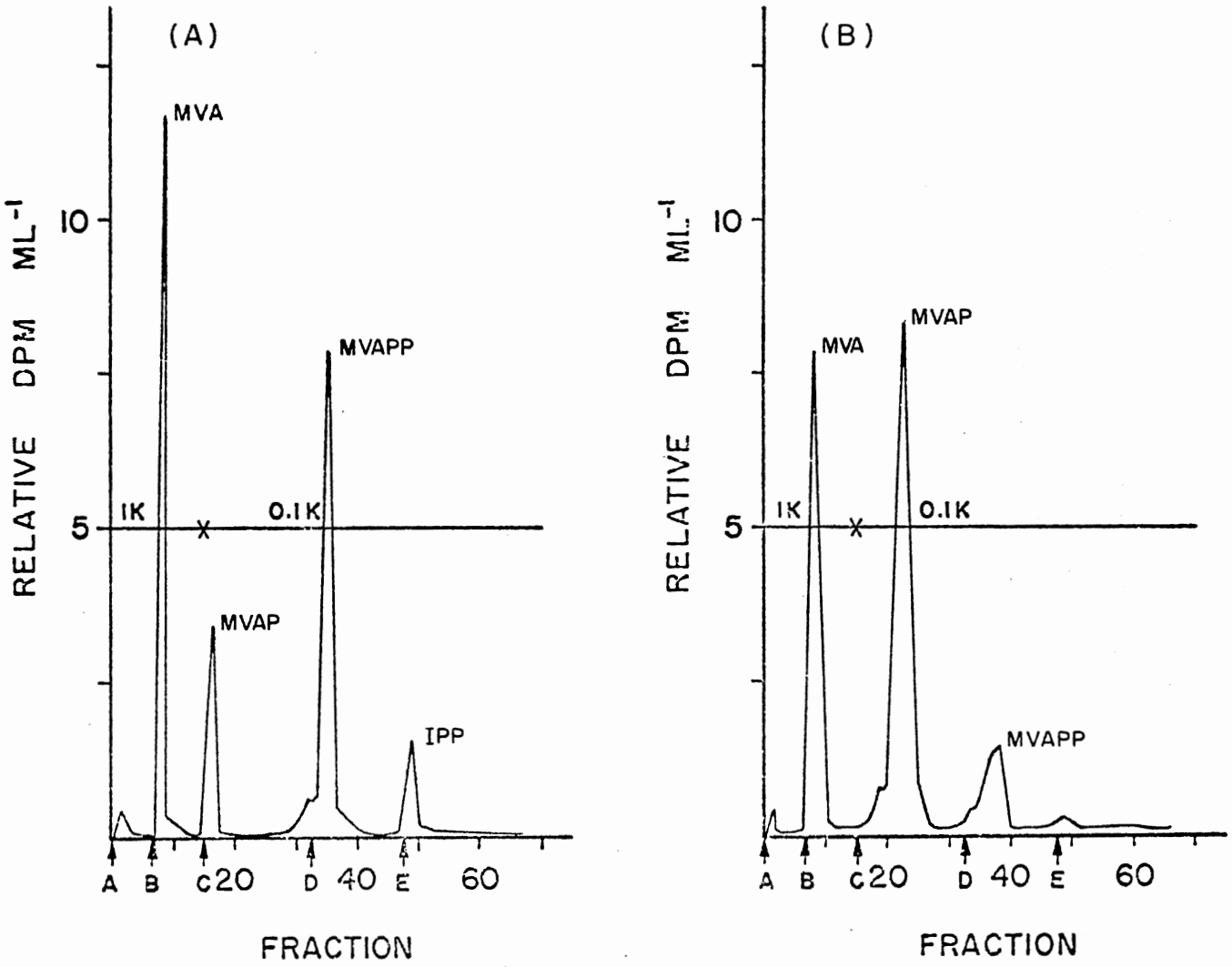
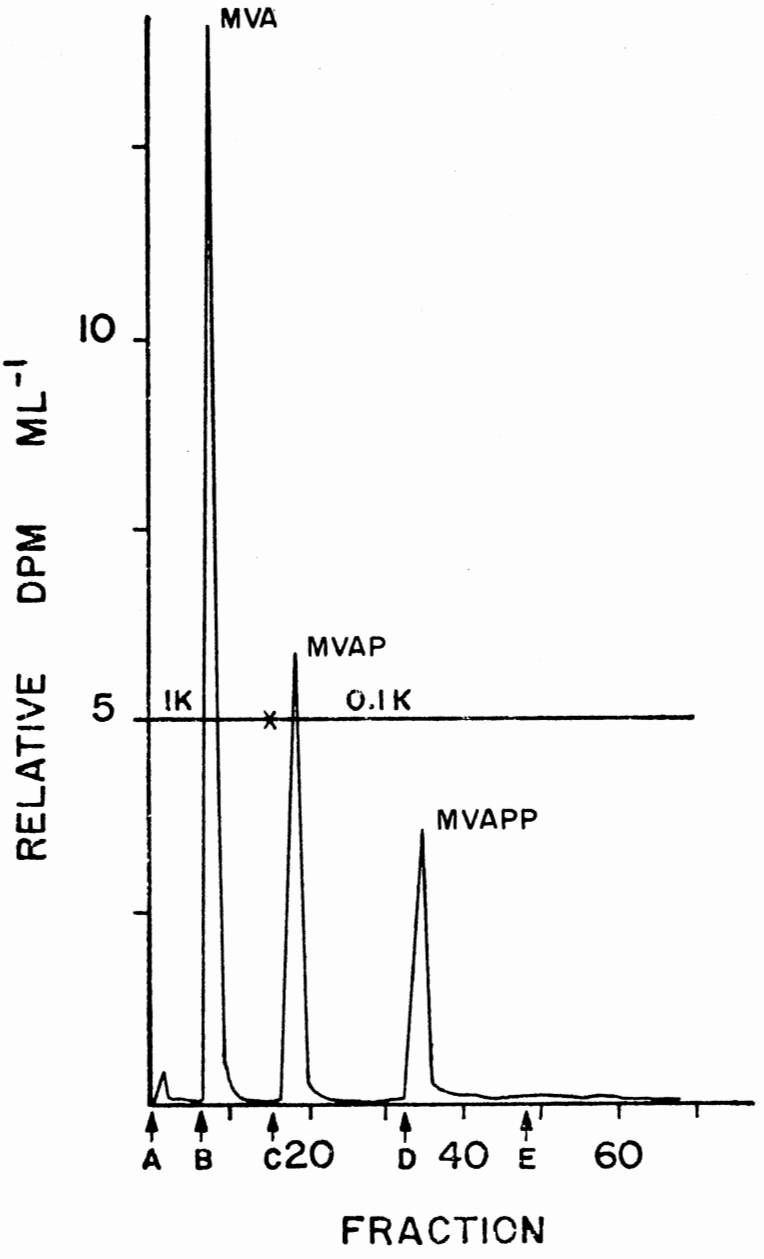

(A)
MVA
MVAPP
MVAP
IPP
1K
0.1K
RELATIVE DPM ML-1
10
5
A B C 20 D 40 E 60
FRACTION
(B)
MVA
MVAP
MVAPP
1K
0.1K
RELATIVE DPM ML-1
10
5
A B C 20 D 40 E 60
FRACTION

Figure 21. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of the 30 to 70 Percent Ammonium Sulfate Precipitate from Turkey Kidney

Elution profile following assay for mevalonate kinase of turkey kidney 30 to 70 percent ammonium sulfate precipitate under standard assay conditions. Elution of Dowex-1 column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 30 ml fractions


MVA
10
5
RELATIVE DPM ML⁻¹
MVAP
1K
0.1K
MVAPP
A
B
C
20
D
40
E
60
FRACTION

The mevalonate kinase activity was found to be in fraction one from the sucrose gradient. The product profile shown in Figure 20, indicates that very little 5-phosphomevalonate kinase activity remains in fraction one. The ratio of 5-phosphomevalonate to 5-pyrophosphomevalonate is 83:17 or 4.88 and is very different in the product ratio observed in the 30-70% ammonium sulfate pellet. This difference in product ratios is strikingly different than that obtained from N. cataria in which the two purification steps had identical product ratios. This would give an indication that the physical relationships of the enzyme systems of mevalonate metabolism is very different in N. cataria.

Turkey liver was removed and quick frozen in liquid nitrogen. The frozen tissue was allowed to thaw out and then 100 g of tissue was homogenized with 100 ml of homogenation buffer. (Same buffer as described for turkey kidney preparation.) This crude homogenate was subjected to the purification procedure as described in Figure 3. Mevalonate kinase activity was found to be present in the 30-70% ammonium sulfate precipitate and this precipitate also contained 5-phosphomevalonate kinase (9.1 mg/ml). This is shown in Figure 22. Again 5-phosphomevalonate is present which indicates the presence of mevalonate phosphate kinase.

B. HMG-CoA Reductase

1. Assay

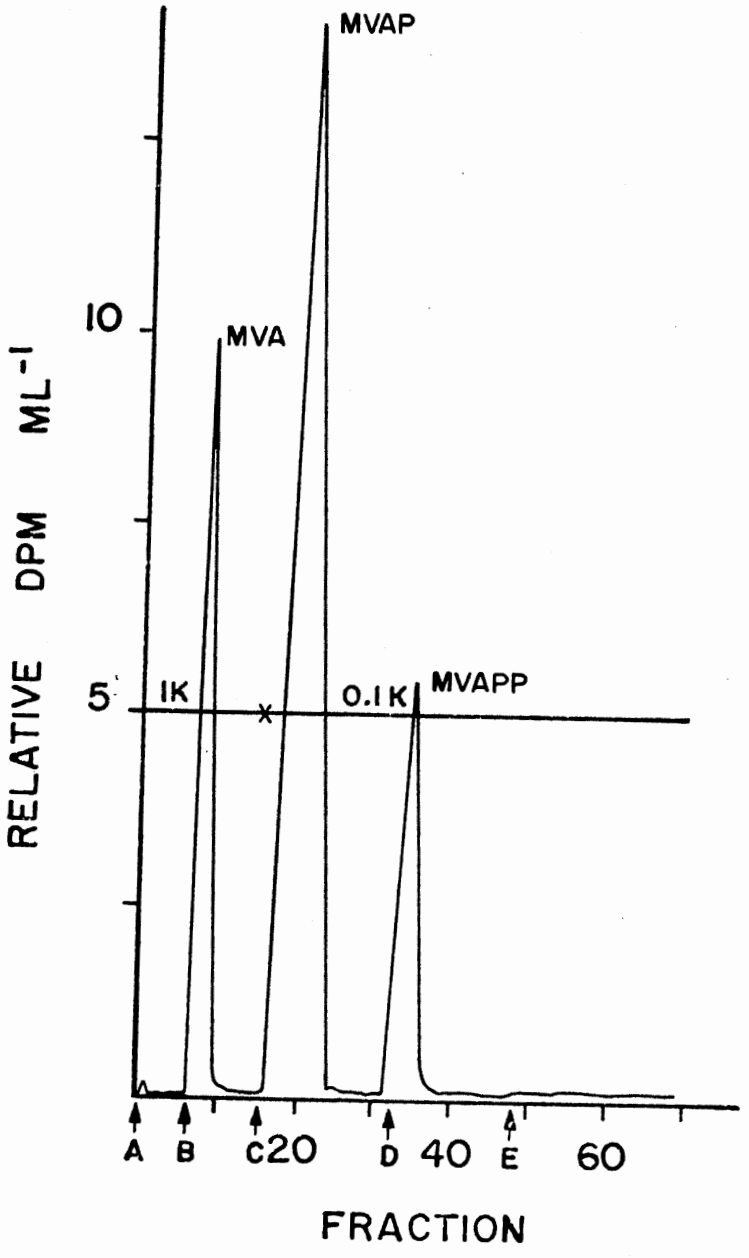
β-Hydroxy-β-methylglutaryl-CoA reductase (HMG-CoA reductase) catalyses the reduction by NADPH of HMG-CoA to mevalonate. The

Figure 22. Dowex-1 Formate Elution Pattern: Mevalonate Kinase Assay of Fraction One from 20 to 45 Percent Sucrose Gradient Centrifugation of the 30 to 70 Percent Ammonium Sulfate Precipitate from Turkey Kidney Tissue

Elution profile following assay for mevalonate kinase of turkey kidney fraction one of 20 to 45 percent discontinuous gradient under standard assay conditions. Elution of Dowex-1 column:

A. 25 ml of water
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 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

Collect approximately 3.0 ml fractions


MVAP
MVA
10
5
1K
0.1K
MVAPP
RELATIVE DPM ML⁻¹
A
B
C
20
D
40
E
60
FRACTION

purified enzyme can be assayed spectrophotometrically (74) but in crude preparations it must be assayed radiochemically. The common problem of all radiochemical procedures currently used for assaying this enzyme is the separation of the radioactively labelled substrate HMG-CoA, HMG or other degradation products from the reaction product mevalonate or mevalonolactone.

The separation of the substrate and the product has been achieved by:

1. Enzymatic phosphorylation of mevalonate followed by ether extraction of HMG (75, 76).
2. Gas-liquid chromatography (77, 78, 79, 80).
3. Thin layer chromatography (81, 82, 83, 84).
4. Thin-layer electrophoresis (85, 86).
5. Lactionization of mevalonate and isolation (71).

A method of column chromatography was devised to separate HMG-CoA and its breakdown products from the reaction product mevalonate.

The reaction was performed as described in the methods section. A concentration of 15 μM for [3-^{14}C] HMG-CoA (specific activity 26.5 mCi/mmole) was used. The reaction mixture was allowed to incubate for 30 minutes at 37°C and terminated by placing the reaction containers in boiling water. The protein was removed by centrifugation and the remaining reaction mixture was placed on a Dowex-1 formate column (23 x .55 cm). The upper end of the column was tightly connected to a small glass funnel. The column was washed with a few ml of water and then the reaction mixture was applied. The column was eluted with a 150 ml gradient consisting of three chambers containing 50 ml of H_2O, 50 ml of N formic acid and 50 ml of 2 N formic acid. The gradient was

followed by stepwise elutions with 50 ml of 4 N formic acid, 50 ml of 4 N formic acid and 0.4 N ammonium formate, and 50 ml of 4 N formic acid and 0.8 N ammonium formate. Fractions of 3 ml volumes were collected and 100 µl was removed for scintillation counting.

Figure 23 gives the elution profile of 3-^{14}C-HMG-CoA when applied directly to the column. Figure 24 shows the elution profile of 3-^{14}C-HMG-CoA after incubation at 37°C for 30 minutes (reaction conditions) in 100 mM Tris pH 7.5. The appearance of a second peak is observed. This peak coinsides with the peak one obtains when HMG-CoA is incubated with 100 mM NaOH for three hours at 50°C. (See Figure 25.) Paper chromatography of this peak confirmed that it was hydroxymethylglutarate. Figure 26 shows the elution profile of mevalonate (2-^{14}C). The mixture of 2-^{14}C-mevalonate and 3-^{14}C-HMG-CoA which was treated with 100 mM NaOH for three hours was applied to the column. Two peaks were observed, one corresponding to mevalonate and the other to HMG (Figure 27).

Paper chromatography of each peak was run to confirm that the two peaks were mevalonate acid and HMG respectively. Pooled fractions containing these two peaks were combined to give two fractions. These fractions were evaporated in vacuo dryness and then resuspended in 200 µl of 10 mM Tris pH 7.5. Twenty µl of each fraction was applied to 30 inch by 2 inch strips and chromatographed in n-propanol:ammonium hydroxide (7:3) as previously described. After the solvent front had moved 20 inches from the origin, the chromatogram was removed, allowed to air dry, cut into ¼ inch strips and then placed in a scintillation vial with 10 ml of toluene ethanol cocktail. Standards of 2-^{14}C-mevalonate and 3-^{14}C-HMG were done in a similar manner.

Figure 23. Dowex-1 Elution Pattern of HMG-CoA

Elution of Dowex-1 column:

AG. Linear gradient of 0-2 N formic acid using three chambers containing 50 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid
C. 50 ml of 4 N formic acid
D. 50 ml of 4 N formic acid + 0.4 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

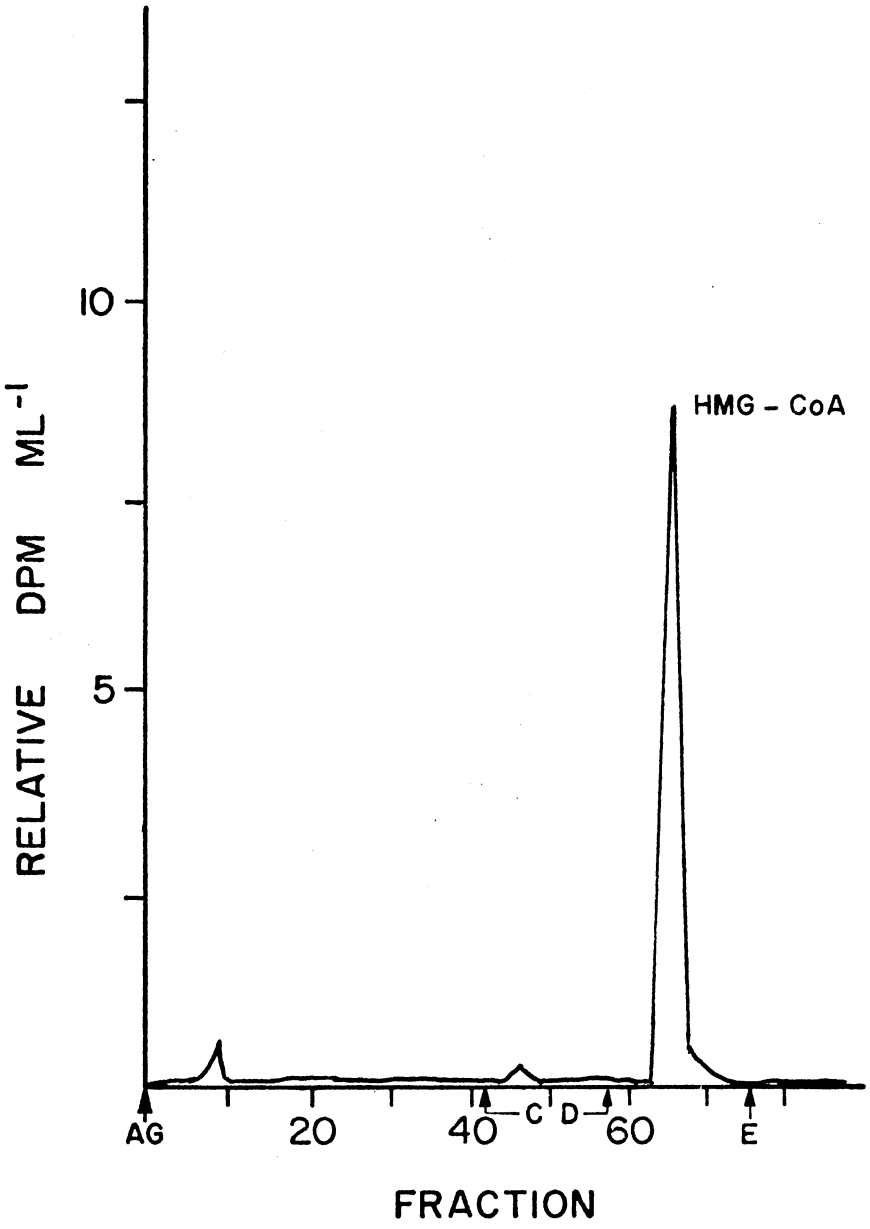

RELATIVE DPM ML⁻¹
10
5
HMG - CoA
AG
20
40
C
D
60
E
FRACTION

Figure 24. Dowex-1 Elution Pattern of HMG-CoA After Incubation at 37°C for 30 Minutes pH 7.5

Elution of Dowex-1 column:

AG. Linear gradient of 0-2 N formic acid using three chambers containing 30 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid
C. 50 ml of 4 N formic acid
D. 50 ml of 4 N formic acid + 0.4 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

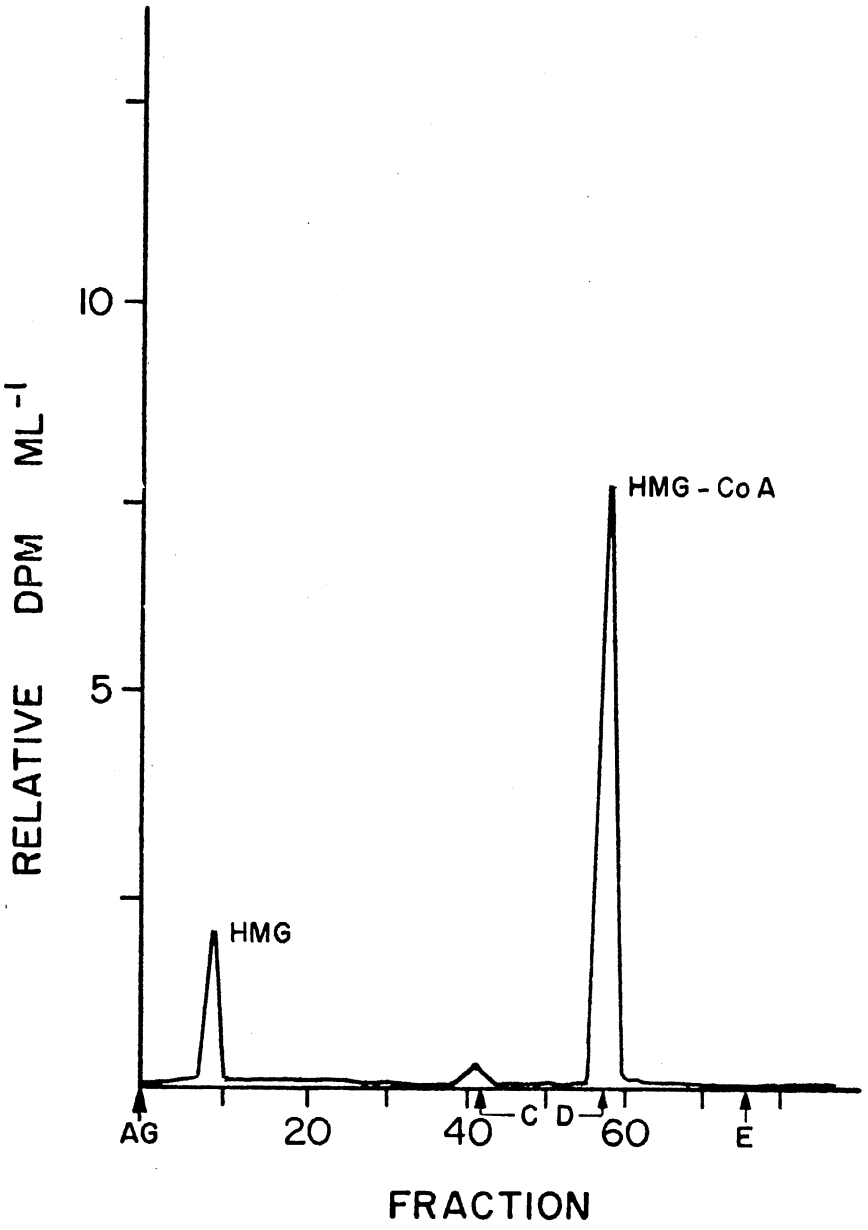

RELATIVE DPM ML-1
10
5
HMG - Co A
HMG
AG
20
40
C
D
60
E
FRACTION

Figure 25. Dowex-1 Elution Pattern of HMG-CoA After Incubation at 50°C for Three Hours in 100 mM NaOH

Elution of Dowex-1 column:

AG. Linear gradient of 0-2 N formic acid using three chambers containing 30 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid
C. 50 ml of 4 N formic acid
D. 50 ml of 4 N formic acid + 0.4 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

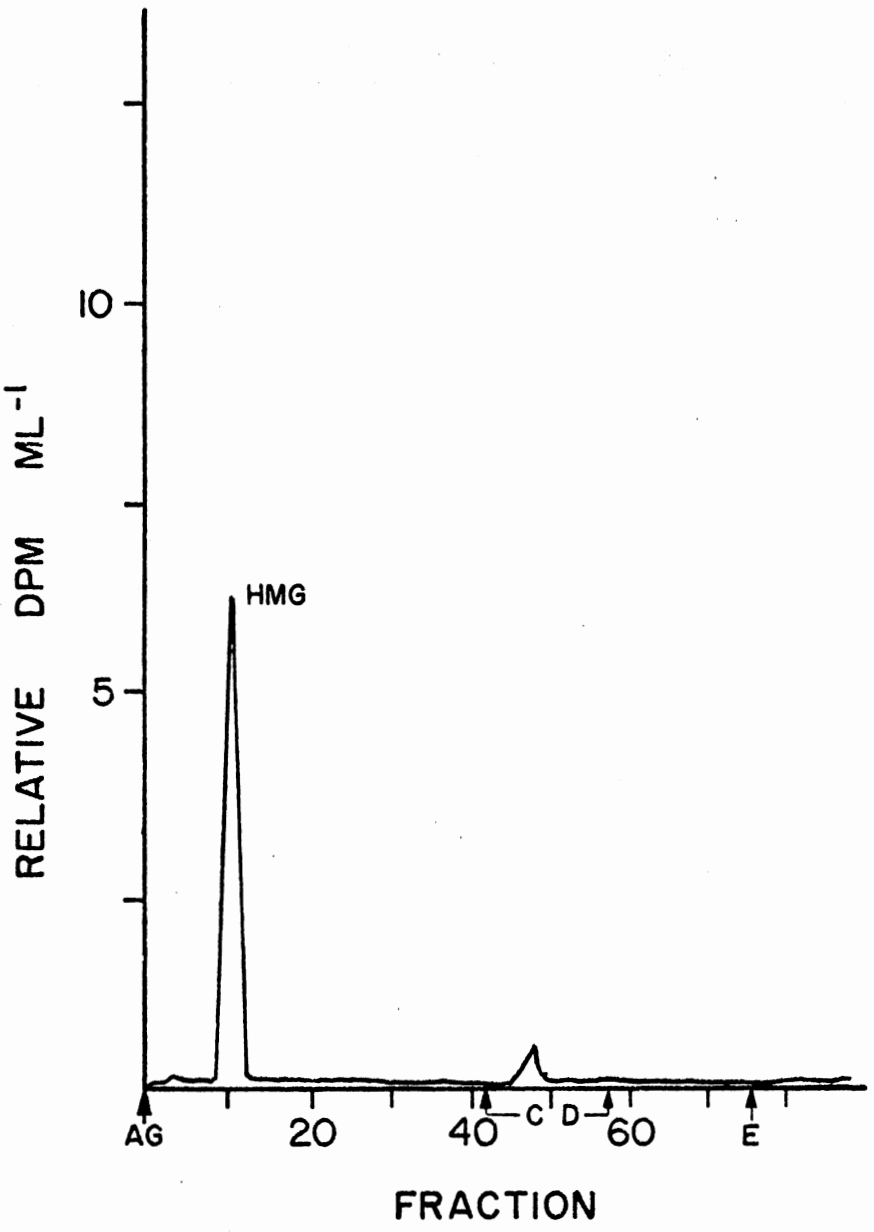

RELATIVE DPM ML-1
10
5
HMG
AG
20
40
C
D
60
E
FRACTION

Figure 26. Dowex-1 Elution Pattern of Mevalonate

Elution of Dowex-1 column:

AG. Linear gradient of 0-2 N formic acid using three chambers containing 30 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid
C. 50 ml of 4 N formic acid
D. 50 ml of 4 N formic acid + 0.4 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

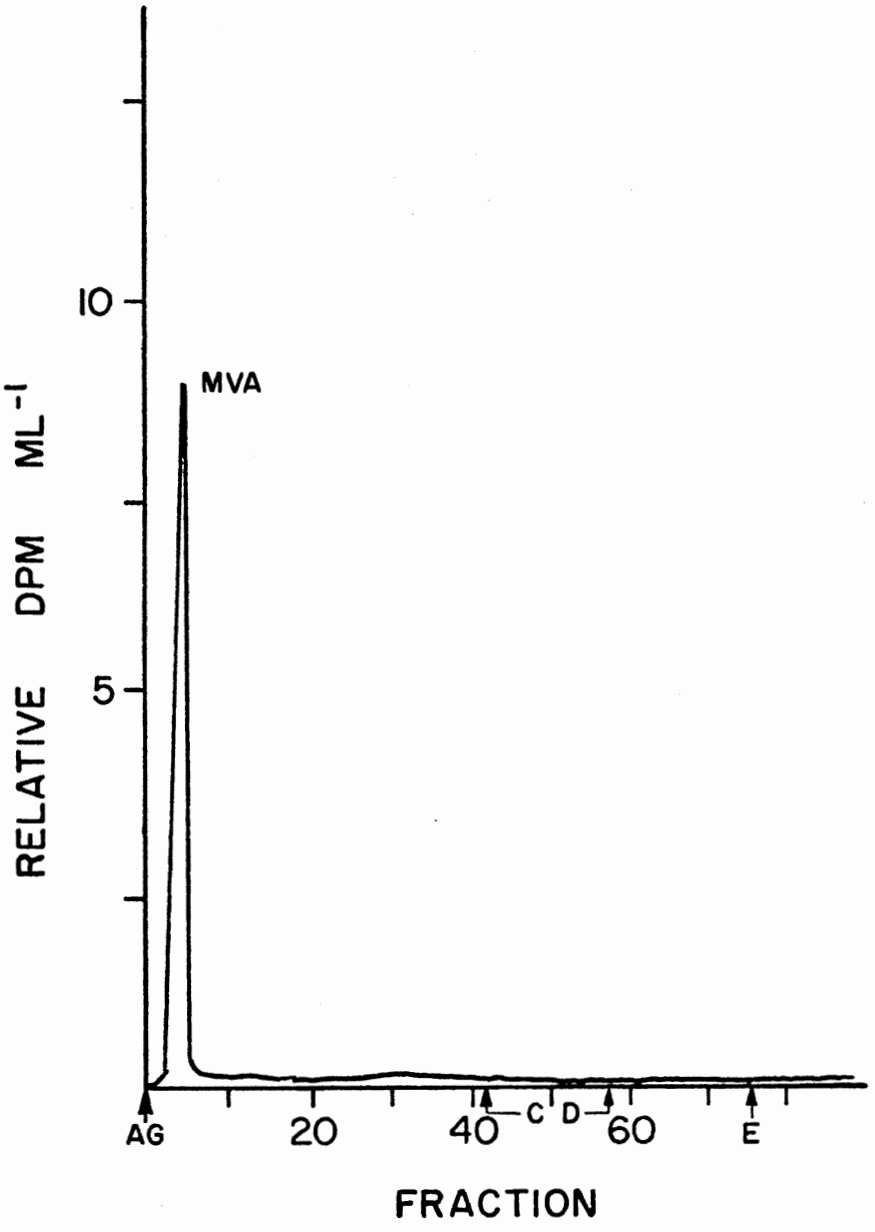

RELATIVE DPM ML^{-1}
10
5
MVA
AG
20
40
C
D
60
E
FRACTION

Figure 27. Dowex-1 Elution Pattern of Mevalonate and HMG-CoA After Incubation at 50°C for Three Hours in 100 mM NaOH

Elution of Dowex-1 column:

AG. Linear gradient of 0-2 N formic acid using three chambers containing 30 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid
C. 50 ml of 4 N formic acid
D. 50 ml of 4 N formic acid + 0.4 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

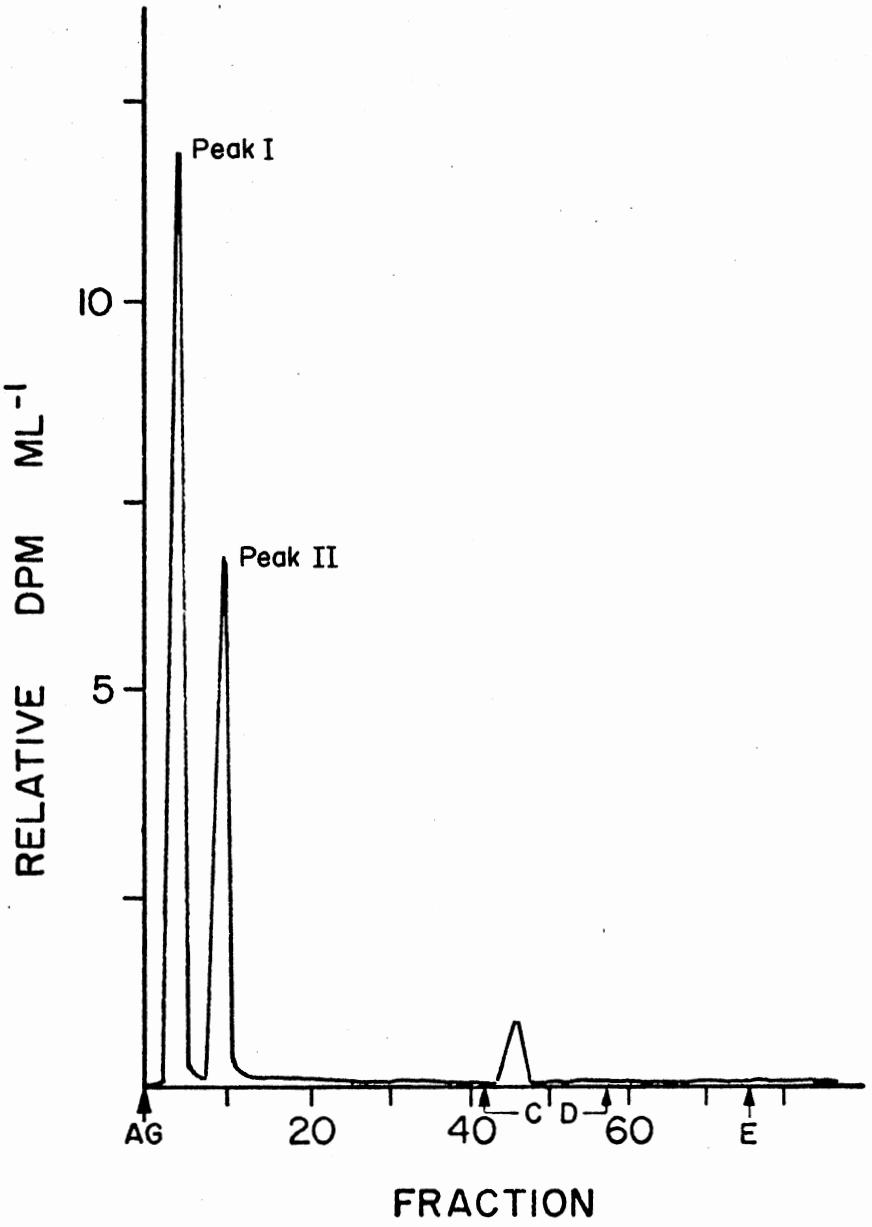

Peak I
Peak II
10
5
RELATIVE DPM ML⁻¹
AG
20
40
C
D
60
E
FRACTION

Table IV shows the R_f values for the two peaks as well as mevalonate and HMG.

TABLE IV

R_f VALUES OF PEAK I & II, MEVALONATE AND β-HYDROXYMETHYLGLUTARATE

2-^{14}C-mevalonate	.69 - .76
3-^{14}C-HMG	.17 - .25
Peak 1	
Fraction 4-5 Dowex-1 formate	.72 - .78
Peak 2	
Fraction 8-11 Dowex-1 formate	.20 - .26

Since mevalonate and HMG have similar R_f values on paper as peak 1 and peak 2 respectively, and also since mevalonate elutes in the same fraction as peak 1 on Dowex-1 and HMG elution is identical to peak 2 it would appear that mevalonate and HMG can be separated and identified - peak 1 being mevalonate and peak 2 as HMG. This would be advantageous if one has low levels of HMG-CoA reductase activity. This procedure separates all of the products and an investigation is able to determine the products without worrying about losing radioactivity by extraction procedures.

2. HMG-CoA Reductase Activity from N. Cataria

Fresh N. cataria leaf tissue (60 g) was used for this experiment. The cell-free extract was obtained as described in methods. The purification procedure described in Figure 2 was used, with the final step being the 20-45% sucrose gradient.

Table V indicates that the bulk of the HMG-CoA reductase activity occurs in the 30-70% ammonium sulfate precipitate (Figure 28). When the 30-70% pellet was applied to a 20-45% discontinuous sucrose gradient, two activities are observed on the sucrose gradient (Figure 29). This is similar to the data one observes for mevalonate kinase activity. However, the second activity of HMG-CoA reductase and mevalonate kinase do not overlap.

3. HMG-CoA Reductase Activity in Chloroplasts

Chloroplasts were isolated from N. cataria leaf tissue in similar manner as described for mevalonate kinase. The chloroplasts (9.8 mg/ml) were resuspended in 1.0 ml of 100 mM Tris pH 7.5, 1.0 mM EDTA, 1.0 mM dithiothreitol and homogenized. The homogenized chloroplasts were placed on a 20-45% discontinuous sucrose gradient and centrifuged at 20,000 x g for three hours. Five fractions of six ml each were collected and assayed for HMG-CoA reductase activity. Only one peak HMG-CoA reductase activity was observed - that being in fraction four as shown in Figure 30. This activity corresponds to the second activity observed with the 30-70% ammonium sulfate precipitate of the whole leaf was centrifuged in a 20-45% discontinuous sucrose gradient.

TABLE V

PARTIAL PURIFICATION OF HMG-CoA REDUCTASE

Step	Volume ml	Concentration Units/ml	Total Units	Protein Mg/ml	Specific Activity[a]	Yield	Purification
1) Crude Homogenate	60	274	16.440	19.1	.014	100.0%	1.00
3,000 x g Supernatant	56	271	15,176	16.2	.017	92.0%	1.17
30,000 x g Supernatant	53	269	14,257	15.0	.018	87.0%	1.25
30,000 x g Pellet	3.1	60	186	4.6	.013	1.1%	0.91
2) Ammonium Sulfate Fractionation - - - - -							
30% Supernatant	48	260	12,480	8.8	.030	76.0%	2.06
30% Pellet	4.6	29.8	137	4.3	.007	0.8%	0.48
70% Supernatant	45	8.9	401	5.7	.002	0	3.45
70% Pellet	9.6	890	8,544	7.8	.050	52.0	
3) Sucrose Gradients of 70% Pellet - - - - -							
Fraction 1	30	72.6	2,187	1.25	.058	13.2	4.05
Fraction 2	30	10.3	309	.60	.017	1.4	1.20
Fraction 3	30	18.1	543	.44	.041	3.3	2.87
Fraction 4	30	57.1	1,713	.31	.084	10.4	12.84
Fraction 5	30	34.5	1,035	.79	.044	6.3	3.05

(a) Specific Activity Units are given in nanomoles of mevalonate formation min^{-1} mg^{-1} of protein

Figure 28. Dowex-1 Formate Elution Pattern: HMG-CoA Reductase Assay of 30 to 70 Percent Ammonium Sulfate Precipitate from Leaf Tissue

Elution profile following assay for HMG-CoA reductase of leaf 30 to 70 percent ammonium sulfate precipitate under standard assay conditions. Elution of Dowex-1 column:

AG. Linear gradient of 0-2 N formic acid using three chambers containing 50 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid
C. 50 ml of 4 N formic acid
D. 50 ml of 4 N formic acid + 0.4 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

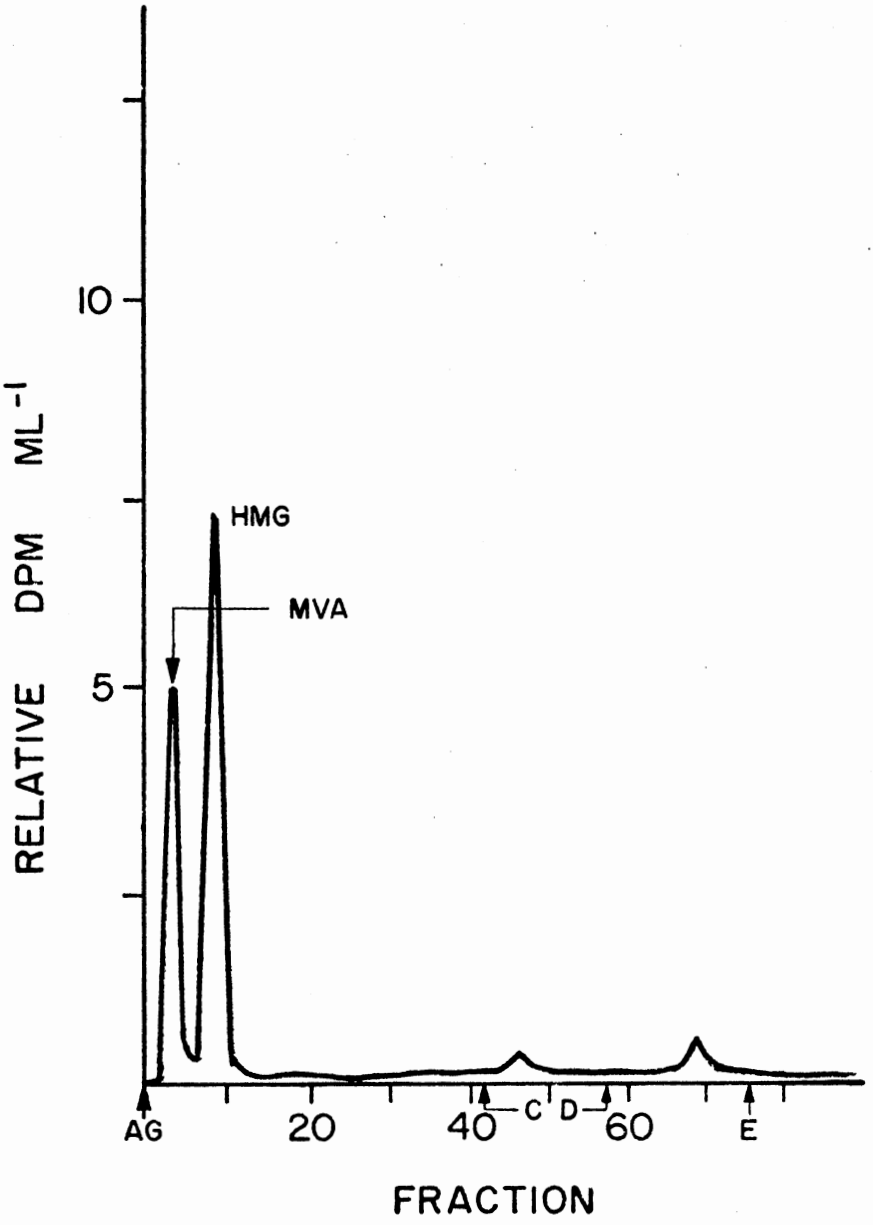

RELATIVE DPM ML⁻¹
10
5
HMG
MVA
AG
20
40
C
D
60
E
FRACTION

Figure 29. Protein and HMG-CoA Reductase Activity Profile from 20 to 45 Percent Sucrose Gradient Centrifugation of 30 to 70 Percent Ammonium Sulfate Precipitate from Leaf Tissue

Protein (---), HMG-CoA Reductase (——)

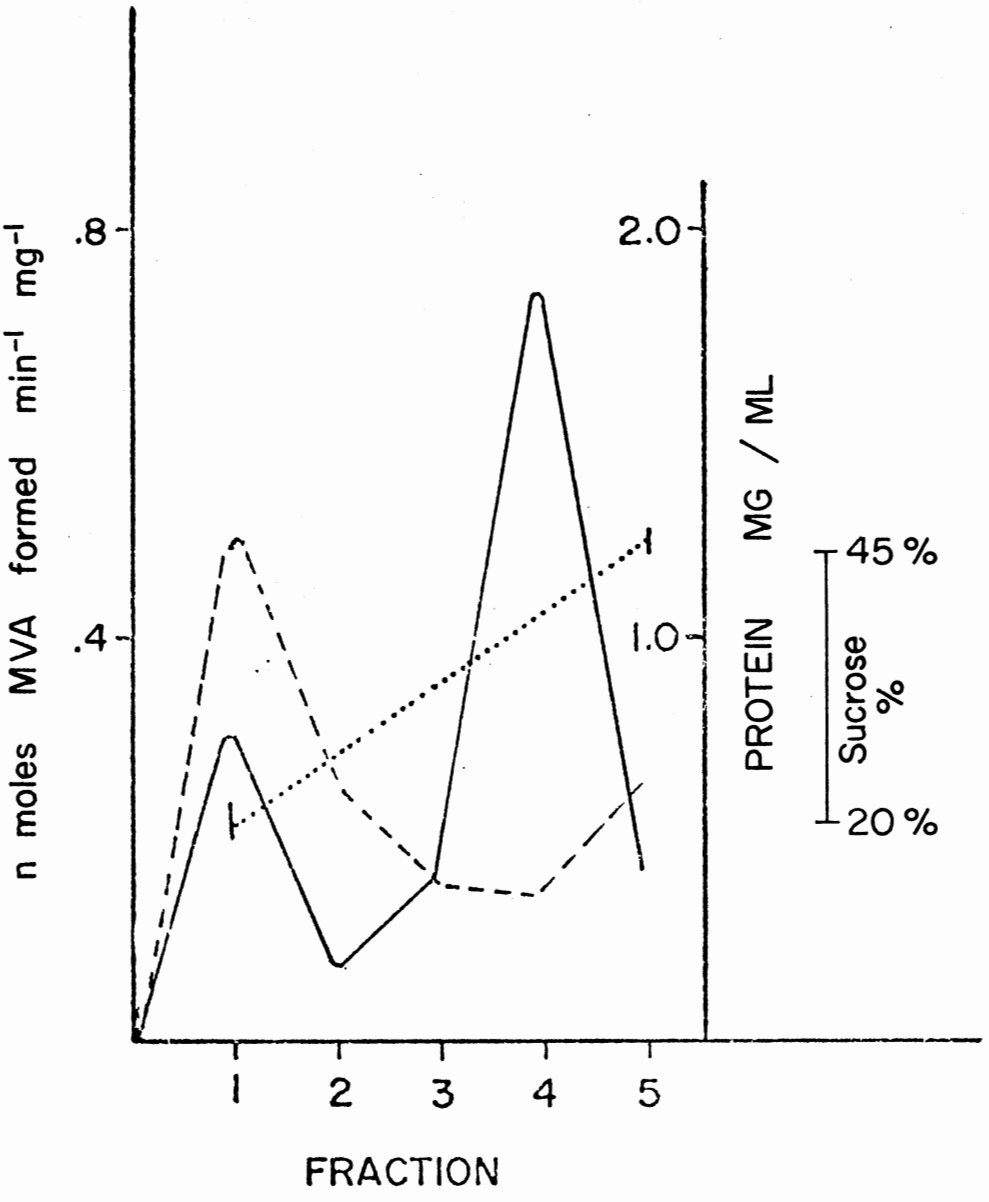
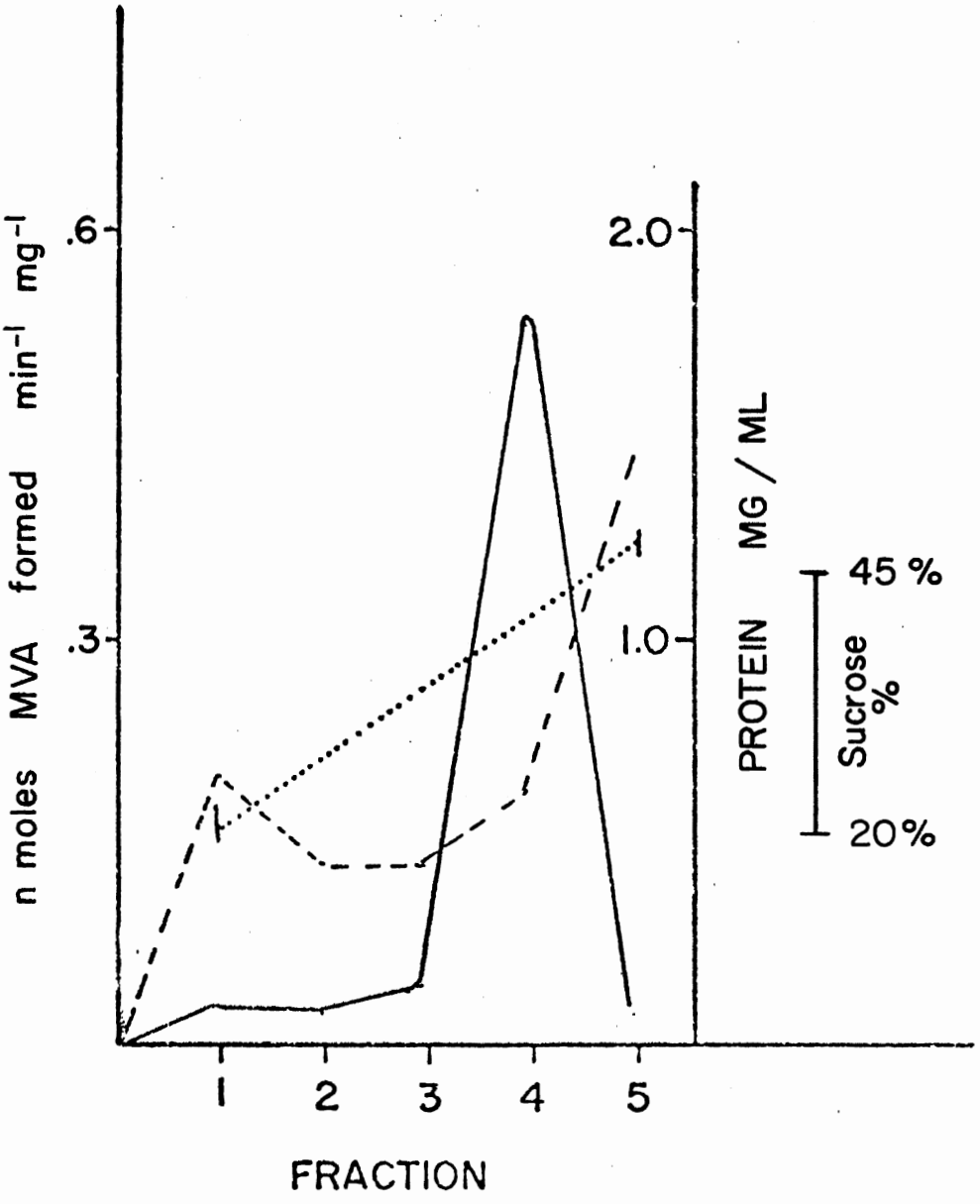

n moles MVA formed min⁻¹ mg⁻¹
.8
.4
2.0
1.0
PROTEIN MG / ML
45 %
Sucrose %
20 %
1
2
3
4
5
FRACTION

Figure 30. Protein and HMG-CoA Reductase Activity Profile from 20 to 45 Percent Sucrose Gradient Centrifugation of Homogenized Chloroplasts

Protein (---), HMG-CoA Reductase (——)


n moles MVA formed min⁻¹ mg⁻¹
.6
.3
2.0
1.0
PROTEIN MG / ML
45 %
Sucrose %
20%
1
2
3
4
5
FRACTION

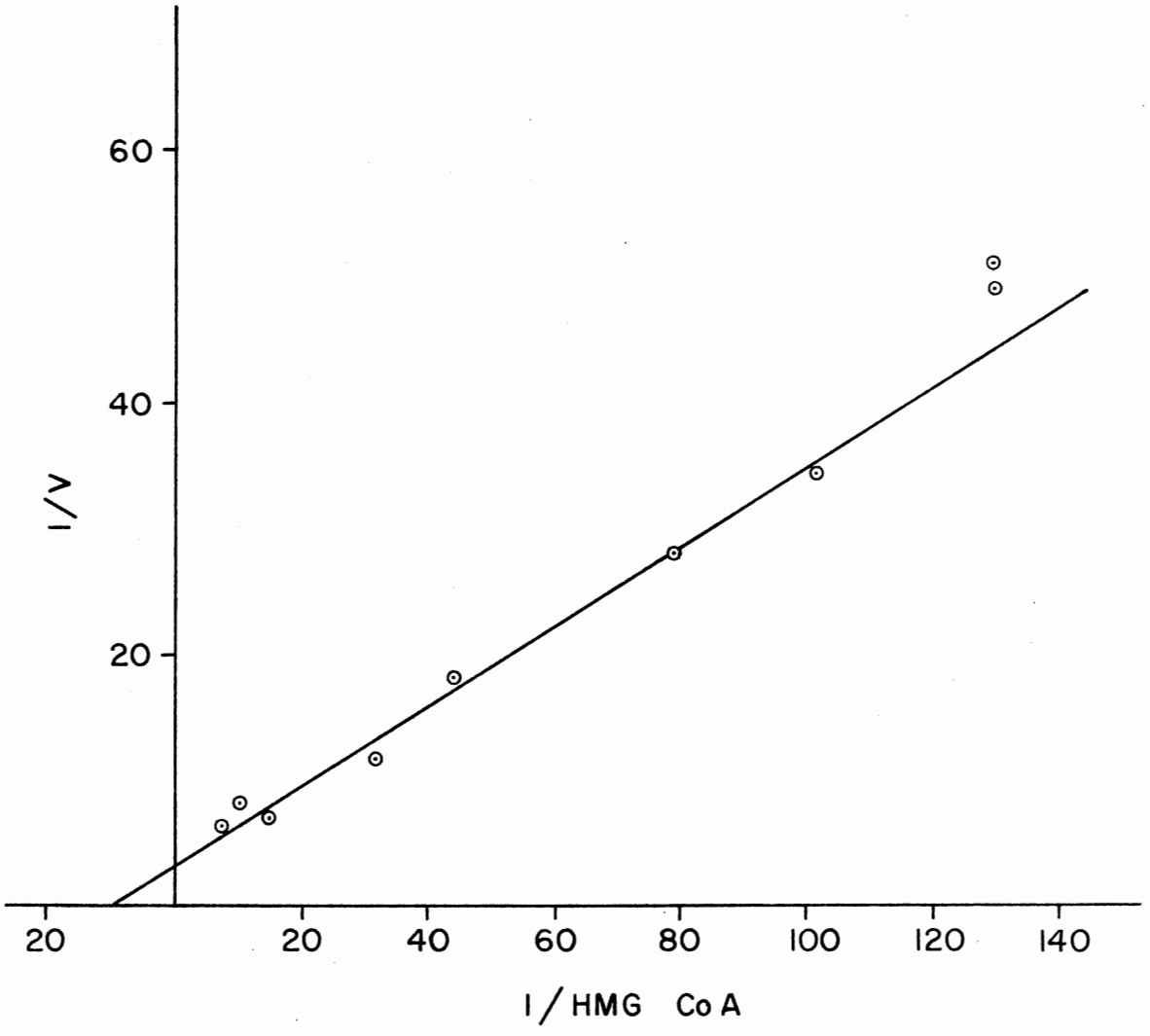
4. Kinetic Studies

Fraction four from the sucrose gradient of the homogenized chloroplasts was dialyzed against 100 mM Tris pH 7.5 and 1.0 mM dithiothreitol and concentrated to 1.5 ml on diaflow membrane apparatus. The concentrated fraction four had a concentration of 1.54 mg/ml and 184.1 units/mg and was purified 12.8 fold.

Stock solutions of HMG-CoA were mixed with 3-^{14}C-HMG-CoA to give specific activities of 0.51, 1.01, 3.77, 5.03, 9.14, 11.97, 15.08 micromoles per microcurie. Assays were incubated in a total volume of 1.0 ml at 37 C for 30 minutes. The incubation mixture of 100 mM Tris pH 7.5, 1.0 mM EDTA, 1.0 mM dithiothreitol, 10.0 mM glucose-6-phosphate dehydrogenase and the enzyme source was preincubated for 10 minutes to allow for the formation of NADPH. The reaction was started by the addition of the labelled substrate HMG-CoA in various amounts. The reaction was terminated by freezing in dry ice/acetone. The mevalonate formed was then converted to mevalonolactone by the addition of 200 µl of 2 N H_2SO_4 and allowing it to stand at room temperature for 10 minutes. The reaction mixture was lyophilyzed, extracted with ether, evaporated to dryness and resuspended as described in methods. The resuspended extract was applied to Dowex-1 formate column and eluted with 5 ml of water to remove the mevalonolactone. The eluent was collected as one five ml fraction and 10 ml of Insta-gel was added and counted with a scintillation counter. Velocity of the reaction was determined by calculating the number of nmoles of mevalonate lactone formed per minute. The results are presented as a Lineweaver-Burk plot in Figure 31.

Figure 31. Lineweaver-Burk Plot of 1/Velocity vs. 1/HMG-CoA Reductase

The assay system utilized was the standard system except for varying concentrations of HMG-CoA.


60
40
20
1/V
20
20
40
60
80
100
120
140
1/HMG CoA

The plot is a best-fit line to the data as obtained from a least square analysis.

The apparent Km for HMG-CoA as calculated from the Lineweaver-Burk plot shown in Figure 30 is 113 μM.

5. HMG-CoA Reductase in Turkey

Cell-free extracts from quick frozen turkey kidney tissue were obtained by homogenizing 100 grams of tissue in a blender with 100 ml of buffer. (Buffer was the same as the buffer described in Preparation of Cell-Free Extracts with the exception that 50 mM sucrose was used.)

The crude supernatant was assayed by separating the products on a Dowex-1 formate ionexchange column with a gradient elution. The product mevalonate was formed as well as a small unknown peak. Figure 28 gives product profile of the crude supernatant.

Cell-free extracts from turkey kidney were subjected to the procedure described in Figure 3. The HMG-CoA reductase activity was found to be in the 30-70% ammonium sulfate precipitate. The precipitate was resuspended in 3 ml of 100 mM Tris pH 7.5, 1.0 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM mevalonate to give a total volume of 6 ml. The resuspended pellet was divided into two 3 ml fractions and one portion was dialyzed against 10 mM Tris pH 7.5, 0.5 mM dithiothreitol. The dialyzed fractions were assayed for HMG-CoA reductase activity as described in methods. Figures 32 and 33 show the effect of assaying in the presence of 0.1 mM mevalonate and without mevalonate. Without mevalonate a peak appears that elutes off a Dowex-1 formate column at a very low ionic strength. This peak is only slightly detectable in the presence of mevalonate. In the presence of 0.1 mM mevalonate 1.5

Figure 32. Dowex-1 Formate Elution Pattern: Crude Homogenate HMG-CoA Reductase Assay of Turkey Kidney

Elution profile following assay for HMG-CoA reductase of turkey kidney crude homogenate under standard assay conditions. Elution of Dowex-1 column:

AG. Linear gradient of 0-2 N formic acid using three chambers containing 50 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid
C. 50 ml of 4 N formic acid
D. 50 ml of 4 N formic acid + 0.4 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate

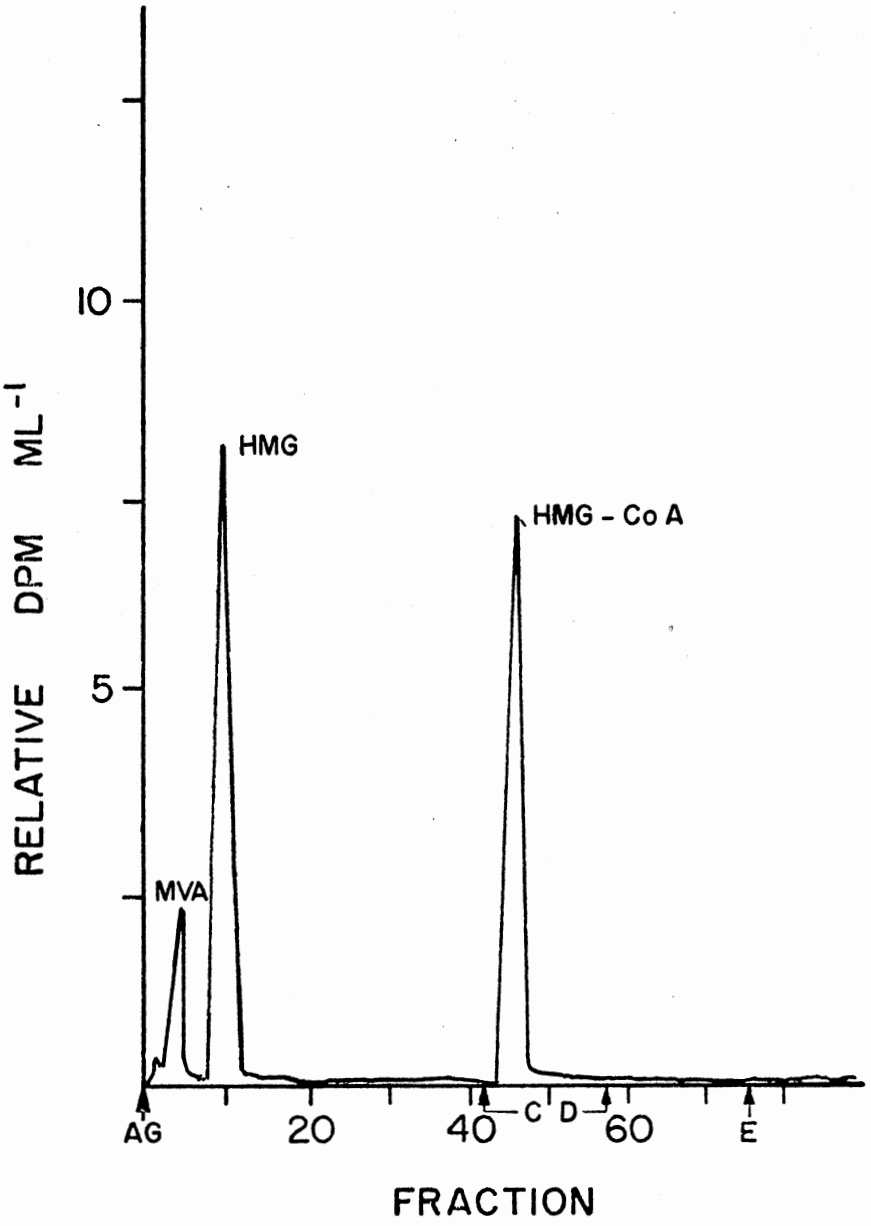
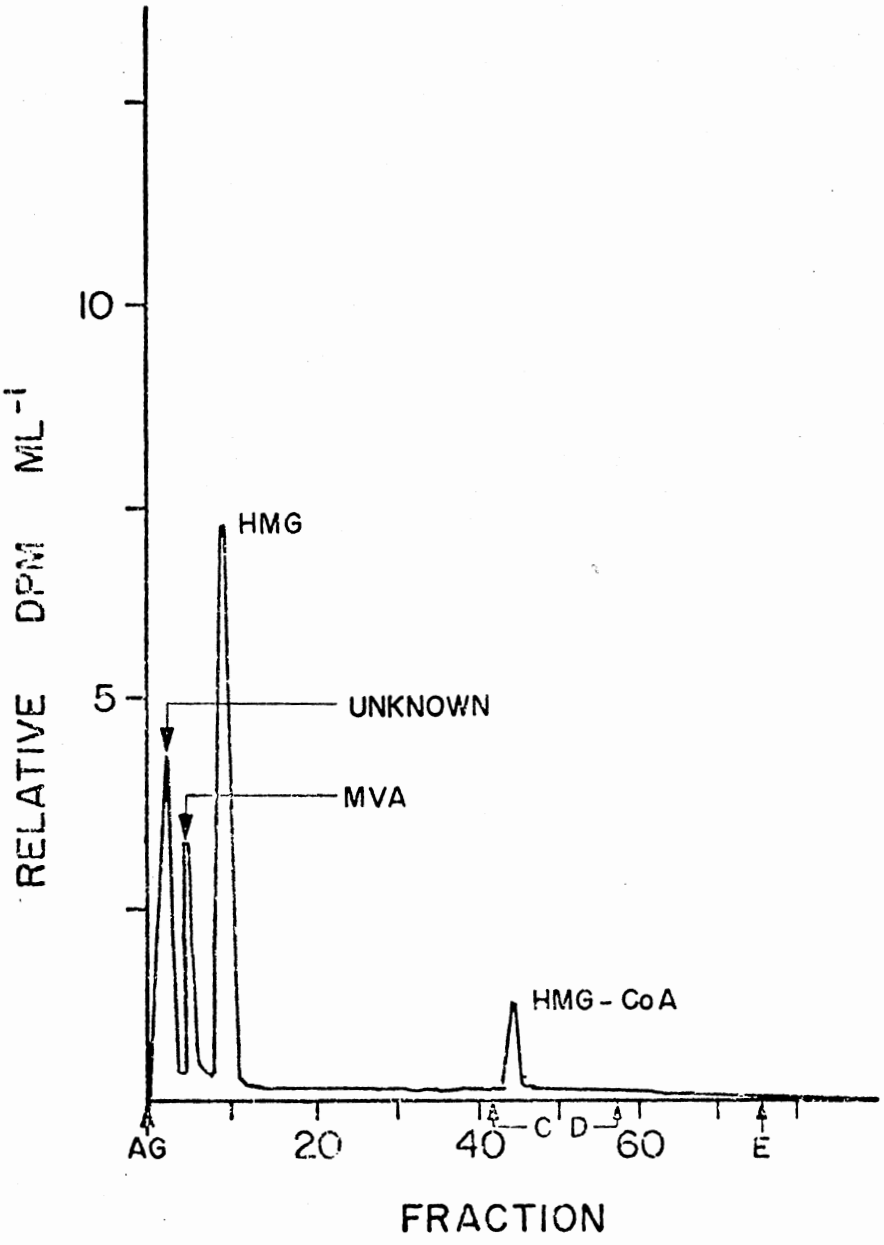

RELATIVE DPM ML⁻¹
10
5
HMG
HMG - Co A
MVA
AG
20
40
C
D
60
E
FRACTION

Figure 33. Dowex-1 Formate Pattern: HMG-CoA Reductase Assay of 30 to 70 Percent Ammonium Sulfate Precipitate from Turkey Kidney in the Presence of 0.1 mM Mevalonate

Elution profiles following assay of turkey kidney 30 to 70 percent ammonium sulfate precipitate in the presence of 0.1 mM mevalonate (A) and with no mevalonate present (B) and all other assay conditions standard. Elution of Dowex-1 column:

AG. Linear gradient of 0-2 N formic acid using three chambers containing 50 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid
C. 50 ml of 4 N formic acid
D. 50 ml of 4 N formic acid + 0.4 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate


RELATIVE DPM ML⁻¹
10
5
HMG
UNKNOWN
MVA
HMG - CoA
AG
20
40
C
D
60
E
FRACTION

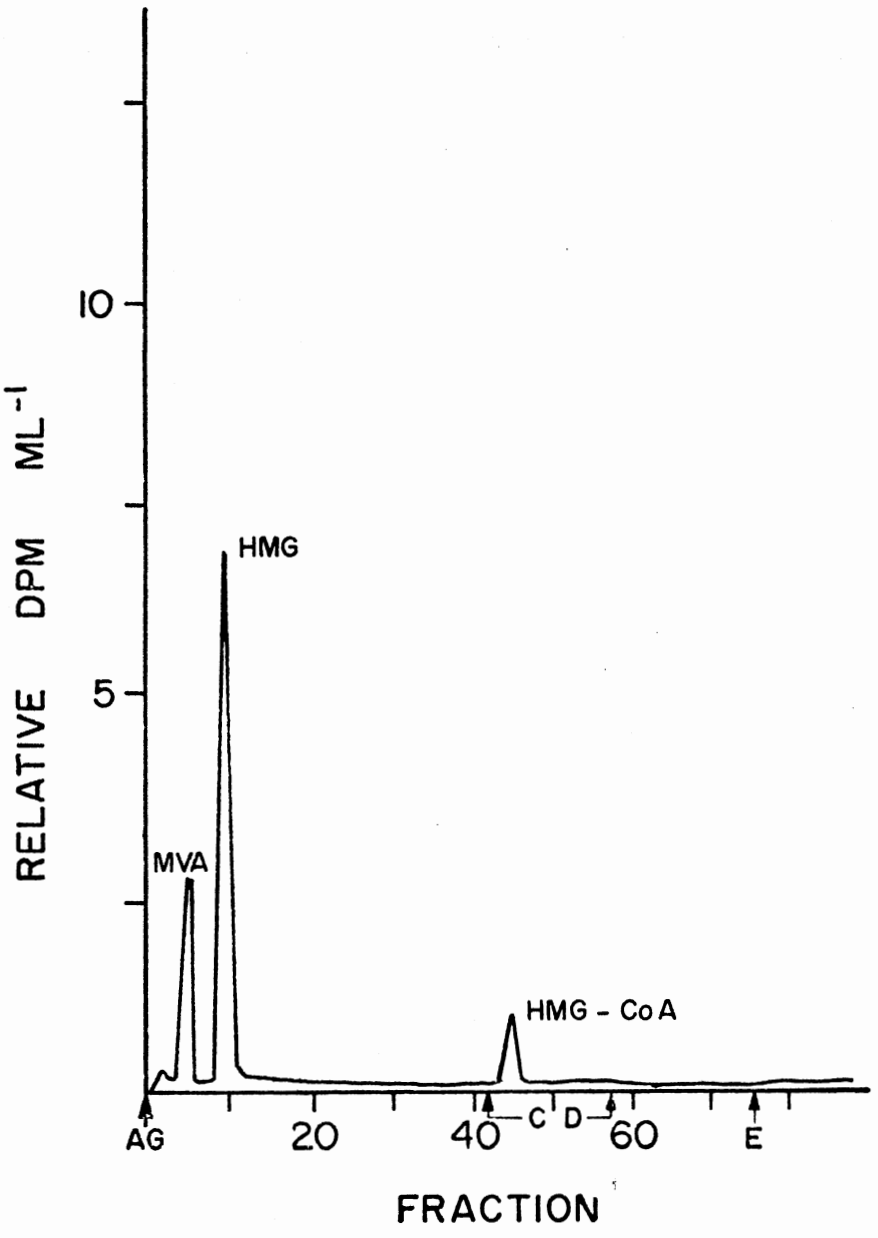
nmoles of mevalonate were formed, without mevalonate present 1.57 nmoles of mevalonate were formed. There is apparently very little effect by the product at the level used in this study. One ml of the 70% pellet dialyzed against 0.1 mM mevalonate was placed on a 30 ml 20-45% sucrose gradient as described for the mevalonate kinase isolated from N. cataria. Five fractions of six ml each were collected and dialyzed against the same buffer used in the previous dialysis.

The dialyzed fractions were then assayed for HMG-CoA reductase as described for the 70% pellet. The first fraction was the only fraction that showed activity. Figure 34 shows the product profile of this fraction. The specific activity of HMG-CoA reductase in this fraction was 261 pmoles/mg min as compared to a specific activity of 46.0 pmoles/mg min for the cell-free extract. This indicates a purification of 5.67 fold.

Figure 34. Dowex-1 Formate Elution Pattern: HMG-CoA Reductase Assay of Fraction One of 20 to 45 Percent Sucrose Gradient of 30 to 70 Percent Ammonium Sulfate Precipitate from Turkey Kidney

Elution profile following assay for HMG-CoA reductase of turkey kidney fraction one of 20 to 45 Percent discontinuous sucrose gradient under standard assay conditions. Elution of Dowex-1 column:

AG. Linear gradient of 0-2 N formic acid using three chambers containing 50 ml water, 50 ml 1 N formic acid and 50 ml 2 N formic acid
C. 50 ml of 4 N formic acid
D. 50 ml of 4 N formic acid + 0.4 N ammonium formate
E. 50 ml of 4 N formic acid + 0.8 N ammonium formate


RELATIVE DPM ML-1
10
5
HMG
MVA
HMG - Co A
AG
20
40
C
D
60
E
FRACTION

CHAPTER V

SUMMARY

The mevalonate activating enzymes mevalonate kinase, 5-phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase co-purify through 30 to 70 percent ammonium sulfate precipitate, 20 to 45 percent discontinuous sucrose gradient, and Bio-Gel P-100 column, suggesting that the molecular weight of the individual enzymes of mevalonate activating system or the enzyme complex is in excess of 100,000 daltons. In N. cataria leaf tissue mevalonase kinase and 5-phosphomevalonate kinase were found to be present in both the light microsomal membrane fraction and in the chloroplasts. Callus tissue from N. cataria was found to contain only one mevalonate activating system which corresponds to the extra-chloroplastidic enzyme system. The callus tissue did not contain chloroplasts. Turkey liver and kidney, hog liver and yeast autolysates were all shown to have only one system.

Another interesting note that gives support to the possibility that the mevalonate activating enzymes may be a complex in N. cataria leaf tissue is the fact that the ratio of the products 5-phosphomevalonate and 5-pyrophosphomevalonate in the microsomal system remains constant throughout purification from the 30 to 70 percent ammonium sulfate precipitate through the P-100 column. The mammalian systems showed wide variation in the ratio of these two products, which is characteristic of non-associated enzymes.

In N. cataria leaf tissue two activities of HMG-CoA reductase were also observed with one activity being localized in the chloroplasts. The chloroplastidic HMG-CoA reductase exhibited a Km for HMG-CoA of 113 μM. The chloroplastidic HMG-CoA reductase could be separated from the chloroplastidic mevalonate kinase by the use of a sucrose density gradient. However, the extra-chloroplastidic (microsomal membrane) HMG-CoA reductase and mevalonate kinase activities both appear in the first fraction of the 20 to 45 percent sucrose gradient. HMG-CoA reductase from turkey kidney showed only one activity profile which was located at the top of the 20-45% sucrose density gradient.

SELECTED BIBLIOGRAPHY

1. Siperstein, M. D. (1970) Curr. Top. Cell. Regul., 2, 65-100.

2. Block, K. (1965) Science, 150, 19-28.

3. Dietschy, J. M., Wilson, J. D. (1970) N. Eng. J. Med., 282, 1128-1138, 1179-1183, 1241-1249.

4. Banthrope, D. V., Charlwood, B. V. & Francis, M. S. O. (1972) Chem. Rev., 72, 115-155.

5. Francis, M. J. O. (1971) in "Aspects of Terpenoid Chemistry and Biochemistry" (Goodwin, T. W., ed.), pp. 29-51, Academic Press, New York.

6. Popjak, G. (1969) Methods in Enzymol., 15, 393-454.

7. Yuan, C. & Block, K. (1959) J. Bio. Chem., 234, 2605-2608.

8. Foole, C. D. & Wold, F. (1963) Biochemistry, 2, 1254-1258.

9. Bonniger, C. & Popjak, G. (1967) Biochem. J., 105, 545-547.

10. Logan, D. M. (1972) Lipid Res., 13, 137-138.

11. Gould, R. G. & Popjak, G. (1957) Biochem. J., 66, 51-54.

12. Bucher, N. L. R., McGarrahan, K., Gould, E. & Loud, A. V. (1959) J. Biol. Chem., 234, 262-270.

13. Siperstein, M. D. & Guest, M. J. (1960) J. Clin. Invest., 39, 642-652.

14. Siperstein, M. D., Guest, N. M. & Dietschy, J. M. (1966) J. Biol. Chem., 241, 597-601.

15. Siperstein, M. D. & Fagen, V. M. (1966) J. Biol. Chem., 241, 602-609.

16. Skakukida, H., Shediac, C. C. & Siperstein, M. D. (1963) J. Clin. Invest., 42, 1521-1531.

17. Wiess, H. J. & Dietschy, J. M. (1969) J. Clin. Invest., 48,

18. Linn, T. (1967) J. Biol. Chem., 242, 990-996.

19. Dietschy, J. M. & Siperstein, M. D. (1967) J. Lipid Res., 8, 97-114.

20. Dietschy, J. M. & Siperstein, M. D. (1965) J. Clin. Invest., 44, 1311-1327.

21. Kandutsch, A. A. & Saucier, S. E. (1969) J. Biol. Chem., 244, 2299-2306.

22. Back, P., Hamprecht, B. & Lynen, F. (1969) Arch. Biochem. Biophys., 133, 11-19.

23. Shapiro, D. S. & Rodwell, V. W. (1969) Biochem. Biophys. Res. Commun., 37, 867-877.

24. Shefer, S., Hauser, S., Lapar, V. & Mosbach, E. H. (1972) J. Lipid Res., 13, 401-412.

25. Goldfarb, S. & Pitot, H. C. (1971) Cancer Res., 31, 1874-1884.

26. Danielsson, H. (1972) Steroids, 20, 63-73.

27. Rodwell, V. W., Nordstrom, J. L. & Mitchelen, J. J. (1976) Adv. Lipid Res., 14, 1-74.

28. Lakshmanan, M. R., Nepokroeff, C. M., Ness, G. C., Dugan, R. E. & Porter, J. W. (1973) Biochem. Biophys. Res. Commun., 50, 704.

29. Huber, J. Guder, W. Latzin, S. & Hamprecht, B. (1973) Hoppe-Seyler's Z. Physiol. Chem., 353, 313-323.

30. Nepokroeff, C. M., Lakshmanan, M. R., Ness, G. C., Dugan, R. E. & Porter, J. W. (1974) Arch. Biochem. Biophys., 160, 387-397.

31. Edwards, P. A. (1973) J. Biol. Chem., 248, 2912-2922.

32. Brown, M. S. & Goldstein, J. L. (1974) J. Biol. Chem., 249, 7306-7316.

33. Horton, B. J., Horton, J. D. & Sabine, J. R. (1972) Ear. J. Cancer, 8, 437.

34. Horton, B. J. & Sabine, J. R. (1971) Ear. J. Cancer, 1, 459-469.

35. Horton, B. J. & Sabine, J. R. (1973) Ear. J. Cancer, 9, 1-10.

36. Sabine, J. R. (1969) Biochem. Biophys. Acta, 176, 600-610.

37. Horton, B. J., Horton, J. D. & Pitot, H. C. (1973) Cancer Res., 33, 1301.

38. Suzuko, H., Oba, K. & Uritani, J. (1975) Physiol. Plant Path., 7, 265-276.

39. Beytia, E. D. & Porter, J. W. (1976) Annual Review of Biochem., 113-142.

40. Shirnizn, I., Nagai, J., Hatanaka, H. & Katsaki, H. (1973) Biochem. Biophys. Acta, 296, 310-320.

41. Qureshi, N., Dugan, R. E., Nimmannit, S., Wu, W. & Porter, J. W. (1976) Biochemistry, 15, 4185-4190.

42. Bucher, N. R., Overath, P. & Lynen, L. (1960) Biochem. Biophys. Acta, 40, 491-501.

43. Shimizu, I., Nagai, J., Hatanaka, H., Saito, E. & Katsaki, H. (1971) Journal of Biochem., 70, 175-177.

44. Beedle, A. S., Munday, K. A. & Salunkhe, D. K. (1972) Biochem. Journal, 142, 57-64.

45. Shapiro, D. J. & Rodwell, V. W. (1972) Biochemistry, 11, 1042.

46. Rodgers, L. J., Shah, S. P. J. & Goodwin, T. W. (1966) Biochem. J., 100, 14c-17c.

47. Rodgers, L. J., Shah, S. P. J. & Goodwin, T. W. (1966) Biochem. J., 99, 381-388.

48. Tchen, T. T. (1958) J. Biol. Chem., 233, 1100-1103.

49. Loomis, W. D. & Battaile, J. (1963) Biochem. Biophys. Acta, 67, 54-63.

50. Williamson, I. P. & Kekwich, R. G. O. (1965) Biochem. J., 96, 862-871.

51. Rogers, L. J., Shah, S. P. J. & Goodwin, T. W. (1966) Biochem. J., 100, 14c-17c.

52. Rogers, L. J., Shah, S. P. J. & Goodwin, T. W. (1966) Biochem. J., 99, 381-388.

53. Pollard, C. J., Bonner, J., Haagen-Smit, A. J. & Nimmo, C. C. (1966) Plant Physiol., 41, 66-70.

54. Gray, J. C. & Kedwick, R. G. O. (1973) Biochem. J., 133, 335-347.

55. Potty, V. H. & Bruemmer, J. H. (1970) Phytochemistry, 9, 99-105.

56. Garcia-Peregrin, E., Suarez, M. D. & Mayor, F. (1973) FEBS Letters, 30, 15-17.

57. Downing, M. R. & Mitchell, E. D. (1974) Phytochemistry, 13, 1419-1421.

58. Beytia, E., Dorsey, J. K., Marr, J., Cleland, W. W. & Porter, J. W. (1970) J. Biol. Chem., 245, 5450-5458.

59. Popjak, G. (1969) Methods in Enzymol., 15, 393-454.

60. Gray, J. C. & Kekwick, R. G. O. (1973) Arch. Biochem. Biophys., 159, 458-462.

61. Flint, A. P. F. (1970) Biochem. J., 120, 145-150.

62. Gray, J. C. & Kekwick, R. G. O. (1972) Biochem. Biophys. Acta, 279, 290-296.

63. Skilleter, D. N. & Kekwick, R. G. O. (1971) Biochem. J., 124, 407-417.

64. Garcia-Peregrin, E. & Mayor, F. (1970) Rev. Espan. Fisiol., 26, 209-216.

65. Bloch, K., Chaykin, S., Phillips, A. H. & DeWaard, A. (1959) J. Biol. Chem., 234, 2595-2604.

66. Tchen, T. T. (1962) Methods in Enzymol., 5, 489-499.

67. Jensen, R. G. & Bassham, J. A. (1960) Proc. Natl. Acad. Sci., 56, 1095-1101.

68. Hartree, E. F. (1972) Anal. Biochem., 48, 422-427.

69. "Worthington Enzyme Manual" (1972) Worthington Biochemical Corp., Freehold, New Jersey, pp. 73-74.

70. Green, T. R. & Levinthal, O. S. (1970) Anal. Biochem., 38, 130-138.

71. Huber, J., Latzin, S. & Hamprecht, B. (1973) Hoppe-Seyler's Z. Physiol. Chem., 354, 1645-1647.

72. Suzue, G., Orihara, K., Morishima, H. & Tanaka, S. (1964) Radioisotopes, 13, 300-303.

73. Kirtley, M. E. & Rudney, H. (1967) Biochemistry, 6, 230-238.

74. Chesterton, C. J. & Kekwick, R. G. O. (1968) Arch. Biochem. Biophys., 125, 76-85.

75. Knappe, J., Ringelmann, E. & Lynen, F. (1959) Biochem. Z., 332, 195-213.

76. Bucher, N. L. R., Overath, P. & Lynen, F. (1960) Biochem. Biophys. Acta, 40, 491-501.

77. Siperstein, M. D., Fagan, V. M. & Dietschy, J. M. (1966) J. Biol. Chem., 241, 597-601.

78. Guchhait, R. B. & Porter, J. W. (1966) Anal. Biochem., 15, 509-516.

79. Linn, T. C. (1967) J. Biol. Chem., 242, 984-989.

80. Kandutsch, A. A. & Saucier, S. E. (1969) J. Biol. Chem., 244, 2299-2305.

81. Hamprecht, B. & Lynen, F. (1970) Eur. J. Biochem., 14, 323-326.

82. Shapiro, D. J., Inblum, R. L. & Rodwell, V. W. (1969) Anal. Biochem., 31, 383-390.

83. Goldfarb, S. & Pitot, H. C. (1971) J. Lipid Res., 12, 512-515.

84. Higgens, M., Kawachi, T. & Rudney, H. (1971) Biochem. Biophys. Res. Commun., 45, 138-144.

85. Shefer, S., Hauser, S., Lapar, V. & Mosbach, E. H. (1972) J. Lipid Res., 13, 402-412.

86. Berndt, J. & Gaummert, R. (1970) Hoppe-Seyler's Z. Physiol. Chem., 351, 1297-1298.

VITA

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Doctor of Philosophy

Thesis: THE OCCURRENCE AND SOME PROPERTIES OF 3-HYDROXY-3-METHYL-GLUTARYL COENZYME A REDUCTASE AND MEVALONATE KINASE IN NEPETA CATARIA LEAF TISSUE

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