MEVALONATE METABOLISM IN CELL-FREE SYSTEMS FROM *N. CATARIA* (CATNIP) LEAF AND STATIC CYTOCULTURE TISSUE

By

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Dedicated to
Chris and Heather

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CHAPTER I

INTRODUCTION

The biosynthesis of monoterpenes in higher plants has been the subject of a large number of investigations. These investigations have involved many species of plants and a variety of precursors were used, including CO_2, acetate, mevalonate, geranylpyrophosphate and nerylpyrophosphate. It is generally accepted that higher plants have evolved such that there are two sites (compartments) of monoterpene biosynthesis. In some plants these two sites are: 1) the chloroplastic site which is involved in the production of chloroplastic pigments, and 2) an extra-chloroplastic site for the biosynthesis of essential oils.

The biosynthesis of nepetalactone, the major constituent of *N. cataria* essential oil, is of interest because it is produced in large amounts by *N. cataria* leaves and it is a methylcyclopentane monoterpene. The mechanism of cyclization of acyclic precursors to the five and six membered rings in unknown and is still under investigation. In flowering *N. cataria* plants, mevalonate has been shown to be incorporated into nepetalactone; however, the per cent incorporation is very low. Several biogenic schemes propose geranylpyrophosphate as a precursor of nepetalactone.

Oil glands on the leaf surface of *N. cataria* leaves have been shown to contain essential oils. At this time it remains to be

determined if the oils are synthesized in the gland, or synthesized elsewhere in the plant and transported to the gland for storage.

Mevalonate has been shown to be a precursor of geraniol and geranylpyrophosphate as well as nerol and nerylpyrophosphate in cell-free systems. Since geranylpyrophosphate is a possible precursor of nepetalactone the following objectives were undertaken in this study: 1) to follow the time course of $^{14}CO_2$ incorporation into ^{14}C-nepetalactone in N. cataria seedlings; 2) to develop a cell-free system from N. cataria leaves capable of metabolizing mevalonate; 3) to identify the products, and thus the enzymes, of mevalonate metabolism in cell-free systems of N. cataria; 4) to observe properties of the enzymes involved in mevalonate metabolism; 5) to initiate and maintain cell cultures of N. cataria tissue; and 6) to study the enzymes involved in mevalonate metabolism in cell-free systems from cultured cells for comparison with those from leaf tissue.

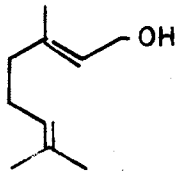
CHAPTER II

LITERATURE REVIEW

A. Monoterpene Structure and Biosynthesis

Monoterpenes are compounds whose structure can be derived either directly or indirectly from the condensation of two isoprene units, they are major constituents of many essential oils and are predominately products of secondary metabolism in plants. Monoterpenes are usually isolated by steam distillation or solvent extraction of leaves or fruit and are rarely found in roots or bark (1). Monoterpenes can also be isolated in non-steam distillable forms such as glucosides of geraniol (2), nerol (3), methylcyclopentane monoterpenes (2) and other terpenes (1). A variety of monoterpene structures have been elucidated, some of which are illustrated in Figure 1. These include: the simple acyclics such as geraniol (I), nerol (II) and linalool (III); the monocyclics such as menthol (IV), αterpineol (V) and limonene (VI) all composed of a cyclohexane ring structure or irodial (VII) with a cyclopentane ring structure; αpinene (VIII), nepetalactone (IX), dihydronepetalactone (X), and 5,9-dehydronepetalactone (XI) are bicyclic monoterpenes (1, 2, 4, 5, 6).

Figure 1. Monoterpene Structures
I. geraniol; II. nerol; III. linalool;
IV. menthol; V. α terpineol; VI. limonene;
VII. irodial; VIII. αpinene; IX. nepetalactone;
X. dihydronepetalactone; XI. 5,9-dehydronepetalactone



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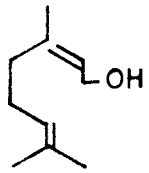

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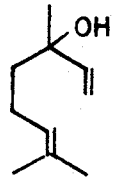

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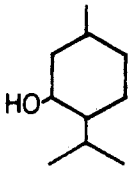

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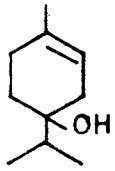

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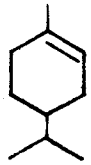

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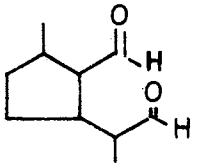

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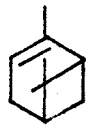

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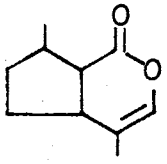

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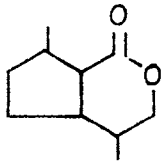

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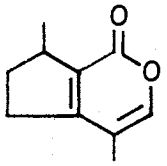


XI

The traditional view of monoterpenes was that these compounds were waste products or dead-end metabolites of secondary plant metabolism. It has been reported, at least in peppermint plants, that essential oils are not dead-ends in that certain pools of essential oils turn over rather rapidly (7, 8, 9). The storage pools of oils (i.e., in the oil glands) turn over more slowly (8) with no loss of oil from storage glands due to evaporation (7). During catabolism of the essential oils the number of empty oil glands on leaf surfaces increased (7).

Since $^{14}CO_2$ is the only isotopic compound that can be administered to plants under physiological conditions, many studies of monoterpene synthesis involve growing whole plants or stem sections in the presence of this gas (1, 7, 9, 10, 11). The discovery of mevalonic acid (XII) in 1956, followed by the observation that ^{14}C-mevalonate could be incorporated into cholesterol, added impetus to the search for the biological equivalent of the isoprene unit (12). Since the discovery of mevalonate and its incorporation into higher terpenoids a number of investigations using ^{14}C-mevalonate as a precursor of monoterpenes in plants have been reported. Banthorpe (3) studied the incorporation of ^{14}C-mevalonate into β-D-glucosides of geraniol (I) and nerol (II) in whole flower heads. Croteau et al. (8, 9, 11) have studied the incorporation of $^{14}CO_2$, ^{14}C-glucose, ^{14}C-sucrose and ^{14}C-mevalonate into mono- and sesqui-terpenes of peppermint. Incorporation of ^{14}C-mevalonate into geraniol (I), nerol (II) and other prenols in a cell-free system from pine has been reported (13), and whole flower heads have been shown to incorporate label from ^{14}C-mevalonate into geraniol (I) and nerol (II), both as free alcohols and as β-glucosides (14).

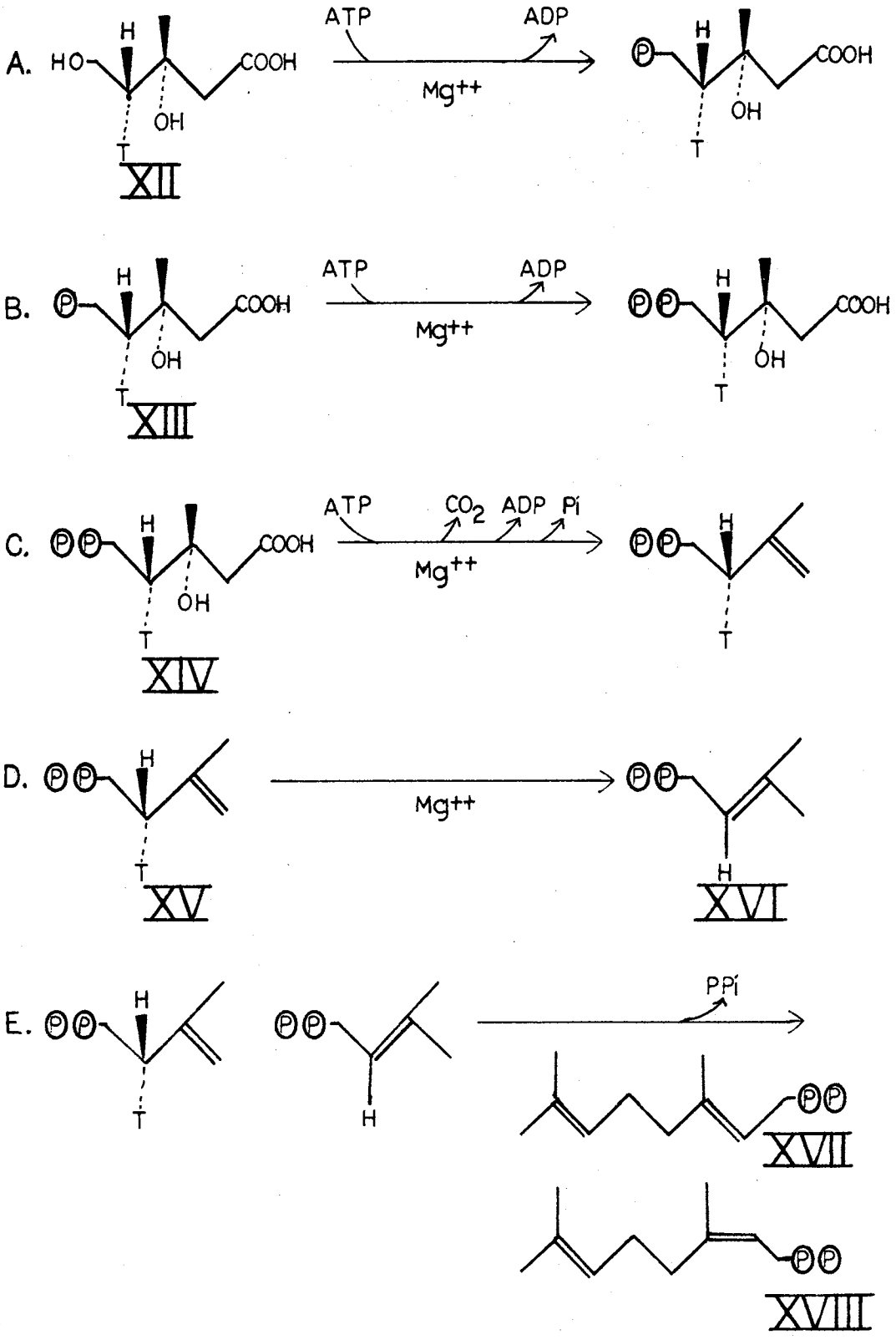
Wedgwood iris incorporated ^{14}C-mevalonate into terpenes either in excised shoots or in a cell-free system (15). ^{14}C-mevalonate was fed to N. cataria plants and low levels of label were incorporated into nepetalactone (IX) (16).

The first steps of mevalonate incorporation into monoterpenes have been reviewed (1, 2, 4, 12) and are illustrated in Figure 2. These reactions will be discussed further in the next section. 3-R-mevalonate (XII) is the isomer metabolized by biological systems, the 4-S^{3}H is also illustrated in Figure 2 to show the stereochemistry of isomerization. Mevalonate (XII) is phosphorylated to 5-physpho-mevalonate (XIII), 5-phosphomevalonate is further phosphorylated to 5-pyrophosphomevalonate (XIV) and 5-pyrophosphomevalonate is decarboxylated to isopentenyl pyrophosphate (XV). An isomerization, with loss of the 4-S^{3}H from mevalonic acid (2-R^{3}H of isopentenyl pyrophosphate), is observed in the formation of dimethylallyl pyrophosphate (XVI). A simple head to tail condensation of isopentenyl pyrophosphate (XV) and dimethylallyl pyrophosphate (XVI) results in the formation of the simple acyclic monoterpene pyrophosphates of geraniol (XVII) and nerol (XVIII).

The cyclization of acyclic monoterpenes has been discussed (1, 2, 4) but to date no experimental evidence has been presented for a mechanism of cyclization. It is proposed that nerol (II) derivatives cyclize to cyclohexane ring systems better than geraniol (I) derivatives (1, 2, 4, 17, 18), the pyrophosphate of linalool (III) has also been proposed as a precursor of cyclic monoterpenes (1). In cell-free systems from peppermint, nerylpyrophosphate (XVIII) has been shown to cyclize to αterpineol (V) (19). The isomerization of

Figure 2. Mevalonate Pathway

A: Mevalonate Kinase
B: 5-Phosphomevalonate Kinase
C: 5-Pyrophosphomevalonate Decarboxylase
D: Isopentenyl Pyrophosphate Isomerase
E. Prenyltransferase


A.
HO
H
COOH
OH
T
XII
ATP
ADP
Mg++
P
B.
XIII
C.
PP
XIV
CO2
Pi
D.
XV
XVI
E.
PPi
XVII
XVIII

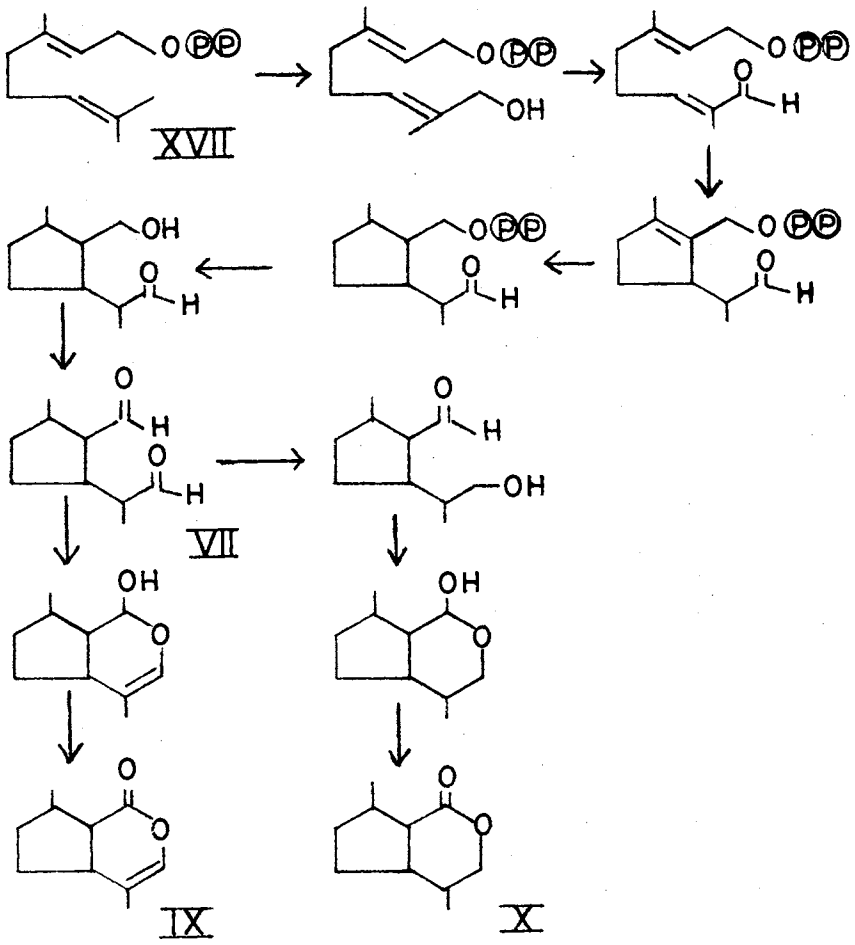
geranylpyrophosphate (XVII) to nerylpyrophosphate (XVIII) has been investigated and a pathway for such an isomerization has been proposed by Banthorpe (1). The trans-cis isomerization in rose (14) and peppermint (19) has been reported although this type of biochemical reaction is uncommon (14) and not observed in other systems (18).

The exact pathway of nepetalactone (IX) biosynthesis is unknown. It has been reported that 2-^{14}C-mevalonate is incorporated into the proper positions of the nepetalactone (IX) skeleton in whole plants (16), and large doses of ^{14}C-dihydronepetalactone (X) fed to whole plants lead to incorporation of label into nepetalactone (IX) (20). The cyclization of geraniol (I) or geraniol derivatives to form a cyclopentane ring is proposed by Banthorpe (1) and Francis (2). Waller (4) proposes a biogenic scheme, shown in part in Figure 3, for the synthesis of nepetalactone (IX) from geranylpyrophosphate (XVII) to irodial (VII) and dihydronepetalactone (X) or nepetalactone (IX).

Since a monoterpene reductase has been observed in a plant system (21), it is possible that there is an enzyme or enzyme system available for the conversion of 5,9-dehydronepetalactone (XI) to nepetalactone (IX) and dihydronepetalactone (X).

The feeding of ^{14}C-mevalonate to whole plants has led to two major problems in the study of the biosynthesis of monoterpenes, 1) low levels of incorporation (0.01 to 0.1 per cent of available mevalonate) into monoterpene fractions (1, 8), and 2) the unequal distribution of label arising from the isopentenyl pyrophosphate (XV) and dimethylallyl pyrophosphate (XVI) portions of the monoterpene skeleton. Greater amounts of tracer ^{14}C were found in the isopentenyl pyrophosphate (XV) portion of the monoterpenes studied on peppermint

Figure 3. Biogenic Scheme of Nepetalactone


XVII
VII
IX
X

plants (1, 2, 8, 9, 11). The secretory structures found on peppermint and N. cataria plants have been proposed as the site of synthesis of essential oils (2, 9), such structures are surrounded by a thick cuticle and appear to originate from a single cell which changes morophology during development (16, 22). Segregation of oil glands from xylem and phloem elements by thick cuticles could produce two separate pools of monoterpene precursors, those synthesized inside the oil gland from unlabeled endogenous precursors (9) and those synthesized outside of the oil gland from labeled precursors. Condensation of these two pools to form monoterpens could lead to the unequal distribution of label observed in monoterpene skeletons (2, 8).

Rose flowers contain osmophors (scent glands) that are in direct contact with phloem elements, this direct association with transport fibers could lead to the equal labeling of both pools of precursors and thus to the equal labeling observed in monoterpene skeletons derived in the rose system (3, 9, 14).

Compartmentalization problems encountered in whole plant feeding studies can be overcome by the use of cell-free systems (1, 2). The development of cell-free systems from plants has, in the past, proven to be rather difficult (2). Recent advances in methodology and the introduction of polyvinylpyrrolidone (23) into homogenization buffers has greatly enhanced the isolation of plant enzymes and production of cell-free systems (2). A number of cell-free systems have been reported to incorporate ^{14}C-mevalonate into terpenes in peppermint (9), pine (13), and iris (15). Other systems for the incorporation of geranylpyrophosphate (XVII) or nerylpyrophosphate (XVIII) into cyclic monoterpenes in pine (17), orange (18) and peppermint (19) have been

developed. It is easier to prepare cell-free systems for the interconversion of monoterpenes than to establish systems for efficient de novo synthesis from simpler precursors (1). Regnier (16) has stated that he observed the synthesis of nepetalactone (IX) from mevalonate in a cell-free system from N. cataria leaves. This statement has been quoted in reviews (1, 2) although there are no experimental details given to support the claim of cell-free incorporation of mevalonate into any monoterpene.

Recent reviews by Banthorpe et al. (1), Francis (2) and Waller (4) cover the terpene literature up to 1971 very thoroughly. The reader is referred to the above reviews for further information on terpenes other than those covered in this review.

B. Enzymes of Mevalonate Metabolism

1. Mevalonate Kinase

Mevalonate kinase (ATP:mevalonate 5-phosphotransferase, EC 2.7.-1.36) is the first enzyme involved in the metabolism of mevalonate (XII) to monoterpenes, the reaction catalyzed is shown in Figure 2a. The first reported isolation and purification of mevalonate kinase was in 1958 by Tchen (24) from a yeast autolysate. Mevalonate kinase from a large number of plant sources has been studied. These include pumpkin seedlings (25), rubber plant latex (26), bean seedlings (27, 28, 29, 30), orange fruit (31) and pine seedlings (32). Sources other than higher plants that have provided mevalonate kinase for study include euglena (33), hog liver (34, 35), chicken liver and rabbit liver (36) and rat liver (37). The phosphorylation of mevalonate by hog liver mevalonate kinase has been shown to be an ordered sequential

reaction, mevalonate adding first followed by $Mg^{++}ATP$, 5-phosphomevalonate (XIII) is released first followed by ADP (34). ATP is the nucleotide that best supports phosphorylation of mevalonate; however, in different systems other nucleotides can also serve as phosphate donors (24, 25). Inhibition of enzymatic activity by high levels of ATP has been reported (24, 31, 33). Divalent cations are required for activity with Mg^{++} or Mn^{++} giving the best activity although other divalent cations will also support low levels of phosphorylation (24, 25, 26). An active -SH group is suggested in mevalonate kinase by its sensitivity to sulfydryl binding agents (24, 25, 26, 29, 30, 31, 33) and the requirement for sulfhydryls in isolation buffers (33). The pH optimum of mevalonate kinase varies with the source. The pumpkin enzyme has an optimum of pH 5.5 and rubber latex at 9.0 (25, 26).

It is generally accepted that there are two pools of mevalonate kinase in plants, one pool is the chloroplastic pool with a pH optimum of 7.5 and the other is the extra-chloroplastic pool with a pH optimum of 5.5 (27).

Tissue cultures have also served as sources of mevalonate kinase. The extra-chloroplastic enzyme has been studied from Pauls Scarlet Rose tissue (28) and the chloroplastic enzyme studied in greening tissues of Kalanchoe crenata (38).

Mevalonate kinase can be inhibited by a number of products including geranylpyrophosphate (XVII) (30, 34, 37, 40), farnesylpyrophosphate (30, 37, 39, 40) and higher prenylpyrophosphates (30, 40). ADP and 5-phosphomevalonate (XIII) have been shown to inhibit the enzyme (30, 34), but isopentenyl pyrophosphate (XV) and

dimethylallyl pyrophosphate (XVI) do not inhibit (37, 39). The molecular weight of mevalonate kinase from a number of sources has been estimated to be 100,000 daltons (36).

2. 5-Phosphomevalonate Kinase

5-phosphomevalonate kinase (ATP:5-phosphomevalonate phosphotransferase, EC 2.7.4.2) is the second enzyme in the mevalonate pathway, and the reaction catalyzed by this enzyme is illustrated in Figure 2b. The enzyme has been partially purified from a number of plant systems including rubber latex (41) and pine (42). Other sources of 5-phosphomevalonate kinase have been yeast (43), and hog liver (35). ATP is the only nucleotide reported to support phosphorylation with a Km of 10^{-3} to 10^{-4} molar (41, 43). A divalent cation is required with Mg^{++}, Fe^{++} and Zn^{++} activating best, and Mn^{++} and Ca^{++} supporting low levels of phosphorylation (41, 43). The pH optimum for the enzyme depends on the source since the yeast enzyme has a wide optimum range from pH 5.5 to 7.4 and the optimum for the rubber latex enzyme is 7.0 (41, 43). Sulfhydryl reagents tend to increase the activity of 5-phosphomevalonate kinase from pine (42) and rubber latex (41). Rat liver 5-phosphomevalonate kinase is reported to be inhibited by a mevalonate analogue, 3,5-dihydroxy-3,4,4-trimethylvaleric acid, in a competitive manner with mevalonate (XII) (44).

3. 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 in the mevalonate pathway of terpene

biosynthesis. The reaction catalyzed by this enzyme is shown in Figure 2c. This enzyme is the most difficult of all the mevalonate activating enzymes to be isolated from hog liver (35). Better yields have been reported from yeast autolysates (43, 45) and the decarboxylase, free of kinase and phosphatase activity, has been purified from rubber latex (41). 5-pyrophosphomevalonate decarboxylase from yeast has a pH optimum from 5 to 10 (43), in rubber latex a broad peak of activity with an optimum at pH 6.4 is observed (41). Mg^{++}, Mn^{++} and Co^{++} are the cations that best activate the decarboxylase (41). Sulfhydryl binding reagents have been shown to have no effect on decarboxylase activity from some systems (41); however, iodoacetamide has been shown to decrease isopentenyl pyrophosphate (XV) levels in other preparations of the decarboxylase from rubber latex (46). ATP concentrations of 10^{-4} molar are optimum for activity and concentrations higher than 10^{-2} molar are inhibitory (41, 43). ADP and isopentenyl pyrophosphate (XV) inhibit decarboxylase activity in the rubber latex system (41).

4. Isopentenyl Pyrophosphate Isomerase

Isopentenyl pyrophosphate isomerase (EC 5.3.3.2) catalyzes the isomerization of isopentenyl pyrophosphate (XV) to dimethylallyl pyrophosphate (XVI) as illustrated in Figure 2d. The enzyme has been purified and studied from a number of sources. Two studies on the enzyme from plant sources are those of Ogura (47) who used an enzyme preparation from pumpkin fruit and Jedlicki (48) who studied pine and citrus isopentenyl pyrophosphate isomerase. Isopentenyl pyrophosphate isomerase is active over a wide pH range, 4.0 to 8.0 (49, 50)

and requires Mg^{++} (49). The enzyme is a sulfhydryl enzyme in that a number of sulfhydryl binding reagents inhibit isomerase activity (47, 48, 49, 50, 51, 52, 53) and in some cases glutathione was found to reverse the inhibition of the sulfahydryl binding agent (49, 50). There is only one report of two isopentenyl pyrophosphate isomerase isoenzymes from the same tissue (47). Each isoenzyme, A and B is differentially inhibited by pyrophosphate and iodoacetamide and each has a different Km for isopentenyl pyrophosphate (XV).

Most studies on isopentenyl pyrophosphate isomerase have involved the study of the stereochemistry of the reaction, the nomenclature used being that of Hanson (54). The 4-pro-S proton of mevalonate (XII) (2-pro-R proton of isopentenyl pyrophosphate (XV)) is lost with the formation of dimethylallyl pyrophosphate (XVI), as illustrated in Figure 2d (48, 49). The proton added to isopentenyl pyrophosphate (XV) through the isomerization to dimethylallyl pyrophosphate (XVI) is added to the re face of the double bond (52, 55). Three mechanisms of isomerization were proposed by Shah (49), 1) a 1,2 addition; 2) a 1,2 abstraction, and 3) a concerted reaction. More recent experiments have shown that the isomerization catalyzed by isopentenyl pyrophosphate isomerase is a concerted reaction (52, 55).

5. Prenyltransferase

Prenyltransferase (EC 2.5.1.1) is the enzyme that catalyzes the condensation of two isoprene units to form the pyrophosphates of acyclic monoterpene alcohols, the reaction is illustrated in Figure 2e. Enzyme preparations from hog liver have been investigated and show the mechanism of prenyltransferase to be an ordered sequential

reaction, dimethylallyl pyrophosphate (XVI) adding first followed by isopentenyl pyrophosphate (XV), geranylpyrophosphate (XVII) is released first followed by pyrophosphate (56). Loss of the 2-pro-R proton of isopentenyl pyrophosphate (XV) is observed in the formation of both cis and trans monoterpene pyrophosphates (48). Because trans-cis isomerizations of monoterpene pyrophosphates are uncommon (14) and seen only in some plant systems (14, 19) but not in others (18) a proposed scheme involving elimination of the 2-pro-R proton of isopentenyl pyrophosphate (XV) in the biosynthesis of both cis and trans prenyl pyrophosphates has been proposed (48). Products of prenyltransferase inhibit the enzyme as do analogues of geranyl- and neryl-pyrophosphates (56, 57).

Plant cells contain phosphatases which are released on maceration of the cell. These phosphatases hydrolyze intermediates of monoterpene synthesis which are normally not rephosphorylated (1). There have been, however, reports of the phosphorylation of the acyclic monoterpene alcohols geraniol (I) (58) and linalool (III) (59) in cell-free systems of pea and orange.

Various reports of the chemical synthesis and stability of the intermediates of mevalonate metabolism are available and the interested reader is referred to these sources for further information (35, 60, 61, 62, 63).

C. Biological Activities of Nepetalactone

Methylcyclopentane monoterpenes, unlike many monoterpenes, are physiologically active in a number of organisms. Their unusual biological activities have been the impetus for the study of these

compounds. Nepetalactone (IX) is known to be a powerful feline attractant. It has been postulated (64) that nepetalactone (IX) is a feline-like pheremone which elicits courtship activity. Evidence supporting this theory includes: 1) courtship and catnip responses are very similar, 2) catnip-like displays occur at field sites marked with tomcat urine, 3) catnip-like activity has been found in solvent extracts of tomcat urine, and 4) cross fatigue of courtship and catnip response has been observed.

More recent work on feline catnip response has shown that catnip response and estrous behavior are similar but not the same (65). Feline behavior responses to catnip can be regarded to occur in four stages: 1) sniffing, 2) licking and chewing with head shaking, 3) chin and cheek rubbing, and 4) head overrolling and body rubbing. In general the entire sequence of events from time of introduction of catnip to the cat to cessation of response is from 10 to 15 minutes. Age, environment, stress and anger all affected the catnip response which is suspected to be a heritable autosomal dominant trait (65). Hatch (65) states that in his experience, cats appear to display effects of visual or auditory hallucinations while under the influence of catnip. The effect of a number of drugs on the catnip response was studied (65) and the response is proposed as a model of human reaction to marijuana or lysergic acid diethylamide (LSD).

Other biological activities of nepetalactone or catnip extracts include the repelling of insects and ants from their natural foods sprayed with nepetalactone (66) and juvenile hormone activity on yellow mealworm pupae (67). Catnip is also being smoked for its supposed psychodelic effects (68).

The metabolism of orally administered nepetalactone in the feline was studied by Waller et al. (69). Approximately 50 to 75 per cent of the nepetalactone fed to felines was recovered in the urine as nepetalinic acid and dihydronepetalactone (X).

D. Plant Cell Culture

The use of tissue culture in the study of terpenoid biosynthesis and metaolism is rather novel. Studies on the metabolism of mevalonate in Pauls Scarlet Rose tissue (28), greening Kalanchoe crenata tissue (38) and monoterpene biosynthesis in Tanacetum vulgare (unpublished but reviewed by Francis(1)) are the only reports, to date, dealing with mevalonate or monoterpene metabolism in tissue culture. Staba (70) reviewed the possibility of utilizing plant cell culture for the biosynthesis or transformation of secondary plant metabolites. Lin (71) has reported the establishment of tissue callus and suspension cultures of peppermint and spearmint plants. Various plant cultures are reported to produce odors similar to that of the intact plant, Staba (70) however, reported that cultures of mint smell somewhat like freshly cut watermelon.

Reviews by Staba (70), monographs edited by White (72) and Kruse and Patterson (73) as well as a series of publications on studies on the growth in culture or plant cells by Street et al. (74-90) should be consulted by the interested reader for a more detailed review of plant cell culture.

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. Seedlings from 18 to 25 days old were used for $^{14}CO_2$ time studies and mature three to four month old plants were used for all cell-free studies.

2. Radioactive Compounds

2-^{14}C-mevalonate was purchased from New England Nuclear, Boston, Mass., as the dibenzoylethylenediamine salt with a specific activity of 7.1 microcuries per micromole of acid.

1-^{14}C-isopentenyl pyrophosphate was purchased as the ammonium salt, in aqueous solution, from Amersham/Searle, Des Plaines, Ill., with a specific activity of 60 microcuries per micromole.

^{14}C-barium carbonate was purchased from either New England Nuclear, Boston, Mass., or Amersham/Searle, Des Plaines, Ill., with a specific activity varying from 5 to 51 microcuries per micromole.

3. Chemicals and Reagents

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

Thin layer supplies, silica gel GF, silica gel HF and commercially prepared silica gel HF plates were purchased from Brinkman Instruments, Westbury, N. Y. Gas chromatography supports and Apiezon L were purchased from Analabs Inc., Hamden, Conn.

Bio-Gels were purchased from Bio-Rad Laboratories, Richmond, Cal. Dowex ion exchange resins were purchased from Sigma Chemical Co., St. Louis, Mo. Diaflow ultrafilter apparatus and membranes were purchased from Amicon Corp., Lexington, Mass.

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

Polyclar AT (washed in acid) was the generous gift of GAF Corp.

GAF HR-1000 x-ray film and Kodak x-ray developer and fixer were purchased locally from a hospital supply wholesaler.

4. Enzymes, Substrates and Cofactors

Calf intestine alkaline phosphatase, potato apyrase, rabbit muscle pyruvate kinase and lactate dehydrogenase and yeast glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co.,

St. Louis, Mo.

ATP and ADP in the sodium salt form, phosphoenolpyruvate as the trisodium or tricyclohexylamine salt, DPN, DPNH, TPH and TPNH as sodium salts, glucose-6-phosphate as the disodium salt, UTP, CTP, GTP and TTP as sodium salts and RS-mevalonate as the lactone were purchased from Sigma Chemical Co., St. Louis, Mo.

B. Methods

1. Preparation of Nepetalactone, Dihydronepetalactone and Nepetadiol

Crude oil from N. cataria plants was obtained according to Regnier (16). Nepetalactone (IX) was isolated and purified by preparative gas chromatography.

Dihydronepetalactone (X) was prepared by hydrogenation of nepetalactone (XI) with palladium on charcoal as described by Johnson (20). Preparative gas chromatography was used to purify dihydronepetalactone (X) from the reaction mixture.

Lithium aluminum hydride reduction of dihydronepetalactone (X) to nepetadiol was performed under conditions described by Johnson (20) and preparative gas chromatography was used for further purification.

2. Purification of 2-^{14}C-Mevalonate

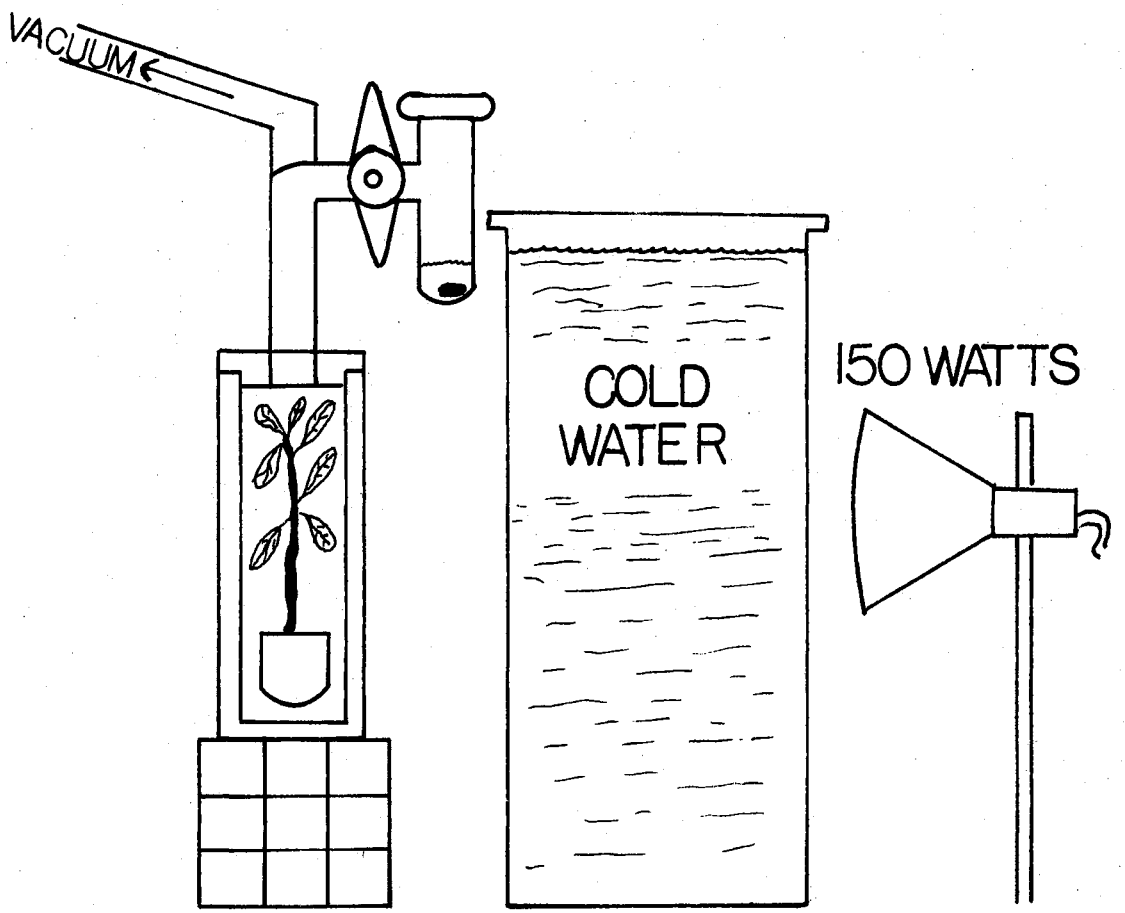
2-^{14}C-mevalonate was routinely analyzed for isotopic purity by thin layer autoradiochromatography (91). Those commercial lots less than 99 per cent pure were further purified by preparative thin layer chromatography in at least two solvent systems.

3. Incorporation of $^{14}CO_2$ into Nepetalactone

Large scale incorporation of $^{14}CO_2$ into nepetalactone (IX) was carried out as described by Regnier (16) using a modified photosynthetic chamber.

The incorporation of $^{14}CO_2$ into seedlings to study the time course of appearance of ^{14}C-nepetalactone was executed with N. cataria seedlings, 18 to 25 days old. Seedlings were removed from pots and washed. The seedlings were quickly weighed and the roots placed in water. The seedlings were stored in the dark for 10 minutes and then placed into a rectangular plexiglass chamber 147 x 110 x 23 mm. The lid had two openings, one leading to a vacuum line and the other equipped with a side arm tube with stopcock sealed with a rubber septum (Figure 4). The side arm contained a known amount of ^{14}C-$BaCO_3$. The light source was turned on, the chamber was evacuated and the side arm was injected with 1 ml 2 N H_2SO_4. The side arm was warmed by flame until effervescence was noted and simultaneously the stopcock was opened and the stopwatch was started. Five seconds before elapsed time the seedling was removed and placed into a mortar containing 4 to 5 ml of n-hexane. The seedling was quickly ground in n-hexane and centrifused to separate the hexane and aqueous phases. An aliquot of each fraction was taken for scintillation counting. The aqueous fraction was quantitatively applied to Whatman number one chromatography paper for two dimensional autoradiochromatography. The hexane fraction was quantitatively applied to two thin layer chromatography plates for autoradiochromatography in two solvent systems.

Figure 4. Photosynthetic Chamber


VACUUM
COLD
WATER
150 WATTS

4. Initiation and Maintenance of Callus Cultures

N. cataria leaf and stem tissue from mature plants was surface sterilized, cut into strips and placed on solid media in 125 ml flasks plugged with nonabsorbent cotton. After five weeks the callus growth was removed from the initial explant material and placed on new media. Subcultures were made at four to six week intervals. All transfers were made in a model HT-UB-L Tenney laminar flow hood using instruments dipped in methanol and flamed between each operation.

Three types of media were used for the initiation and propagation of callus cultures:

1) SM media as described by Brown and Lawrence (92) was modified by the addition of 100 mg per liter of aspartic acid and glutamine and by lowering the concentration of 2-4,dichlorophenoxy-acetic acid to 1.0 mg per liter in 0.75% agar.

2) SH media as described by Schenk and Hildebrandt (93) was modified by increasing the concentration of pyridoxine to 5.0 mg per liter in 0.75% agar.

3) STBA media as described by Lin and Staba (71) was modified by using the iron source described by Schenk and Hildebrandt (93), substituting pyridoxine for pyridoxal and using 2.0 mg per liter each of 2-4,dichlorophenoxy-acetic acid and αnapthaleneacetic acid in 0.75% agar.

All media was sterilized by autoclaving at 15 psi for 20 minutes.

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

The paste obtained on grinding of the tissue was pressed through six layers of cheesecloth and the exudate was subjected to a series of centrifugations as described in Figure 5. All procedures were carried out at $4^{o}C$ using precooled mortar and buffers.

6. Assays

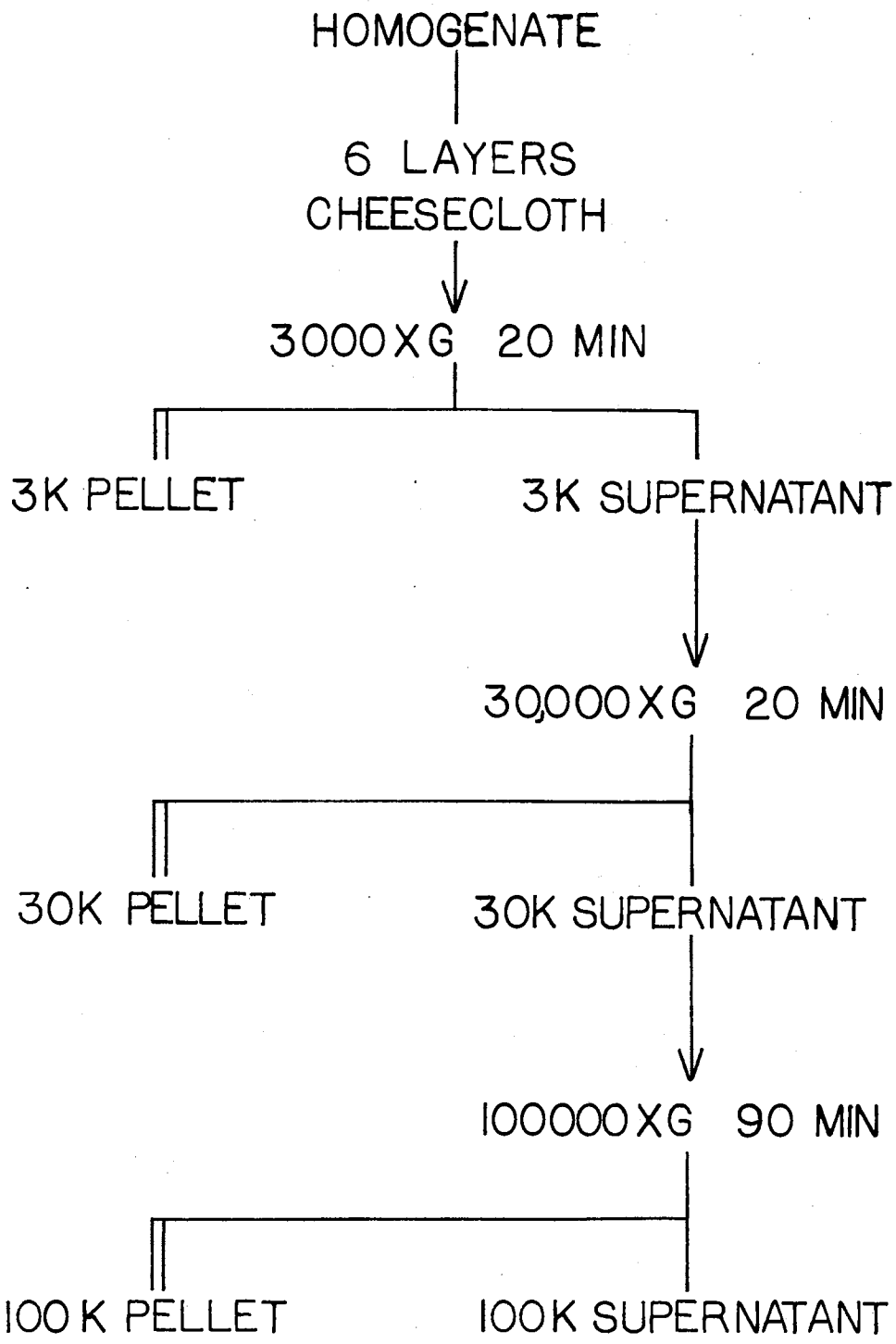
Protein concentrations were determined by a modification of the Lowry procedure as described by Hartree (94) using bovine serum albumen as a standard.

Alkaline phosphatase assays were performed as described for the *E. coli* enzyme (95).

Hydrolysis of phosphate and pyrophosphate esters from intermediate alcohols was performed as described by Beytía (13) using alkaline phosphatase and/or apyrase in incubations at $37^{o}C$.

Mevalonate kinase activity was measured by a coupled spectrophotometric assay (35) and a radioactive assay. The radioactive assay was based on that described by Green (96). Assays were run at $30^{o}C$ with shaking for the time periods specified in individual experiments. The assays included metal ions, cofactors, cofactor generating systems, sulfhydryls, inhibitors and enzyme sources as indicated for individual experiments.

Figure 5. Flow Chart for Preparation of Cell-Free Enzyme Systems


HOMOGENATE
6 LAYERS
CHEESECLOTH
3000XG 20 MIN
3K PELLET
3K SUPERNATANT
30,000XG 20 MIN
30K PELLET
30K SUPERNATANT
100000XG 90 MIN
100K PELLET
100K SUPERNATANT

Initially reactions were performed in a total volume of 0.5 to 2.0 ml, later assays were carried out on a micro scale in volumes of 0.05 to 0.2 ml. Reactions were killed by boiling for three to five minutes. The protein was removed by centrifugation and the supernatant extracted with n-hexane or aliquots were removed for scintillation counting and chromatography.

7. Affinity Chromatography

Mevalonate was coupled to Bio-Gel AF-102 (aminoalkyl agarose) by the procedure of Cuatrecasas (97). Affinity chromatography was carried out at room temperature over a mevalonate-agarose column (0.8 x 10 cm).

8. Chromatography

Thin layer chromatography of nepetalactone was carried out on silica gel GF or silica gel HF thin layer plates in two solvent systems (69). Solvent system 1 consisted of n-hexane:acetone:ethanol, 40:10:4 (v/v/v), nepetalactone has an R_f of 0.6 in this system. Solvent system 2 involved developing the plate in n-hexane until a solvent front of 15 cm was reached, removal and air drying of the plate and redeveloping in benzene:ethylacetate 9:1 (v/v) in the same direction to a 10 cm solvent front. In system 2 nepetalactone runs at the second solvent front.

Analytical gas chromatography of nepetalactone, dihydronepetalactone and nepetadiol was achieved with a modified model 5000 Barber-Coleman gas chromatograph equipped with a hydrogen flame detector.

A 12' x 1/4" glass column packed with 20 per cent Apiezon L on Anakrom ABS 100-110 mesh equipped for either solid or liquid injection was used for all analytical determinations. A typical profile is illustrated in Figure 6. Preparative gas chromatography on a model 711 Varian Aerograph Autoprep gas chromatograph equipped with a hydrogen flame detector with a 17' x 3/8" aluminum column packed with 20 per cent Apiezon L on 40-50 mesh Anakrom ABS was used to purify nepetalactone, dihydronepetalactone and nepetadiol. Relative retention times of nepetalactone, dihydronepetalactone and nepetadiol are given by Johnson (20).

Preparative thin layer chromatography on silica GF or silica gel HF in at least two solvent systems was used for the purification of mevalonate. System 1 is composed of 2-propanol:H_2O 85:15 (v/v), an R_f of 0.66 was observed for mevalonate in this system. System 2, n-propanol:NH_4OH;H_2O 6:3:1 (v/v/v), gives an R_f of 0.83 for mevalonate. A R_f of 0.31 for mevalonate is observed in system 3, composed of n-butanol:NH_4OH:H_2O 6:1:1 (v/v/v).

Two dimensional paper chromatography of the aqueous fractions obtained in $^{14}CO_2$ experiments were run first in n-butanol:propionic acid:H_2O 1246:620:874 (v/v/v) followed by phenol:H_2O 100:20 (v/v) in the second direction (98).

Paper chromatography for the identification of mevalonate metabolites was executed in a descending direction on Whatman number one paper. The seven solvent systems used are described (24, 26, 33, 35, 43, 45, 46, 49, 99-106) and the range of R_f values published in these references is given in Table I. Chromatography chambers were equilibrated with the eluting solvent for at least 24 hours prior to

Figure 6. Gas Liquid Chromatography Elution Profile

12' x 1/4" Glass Column
20% Apiezon L on Anakrom ABS
70°C Isothermal for 20 min. followed by a program of 10° C/min to 230°C
He 20 ml/min., Injector 100°C, Detector 240°C

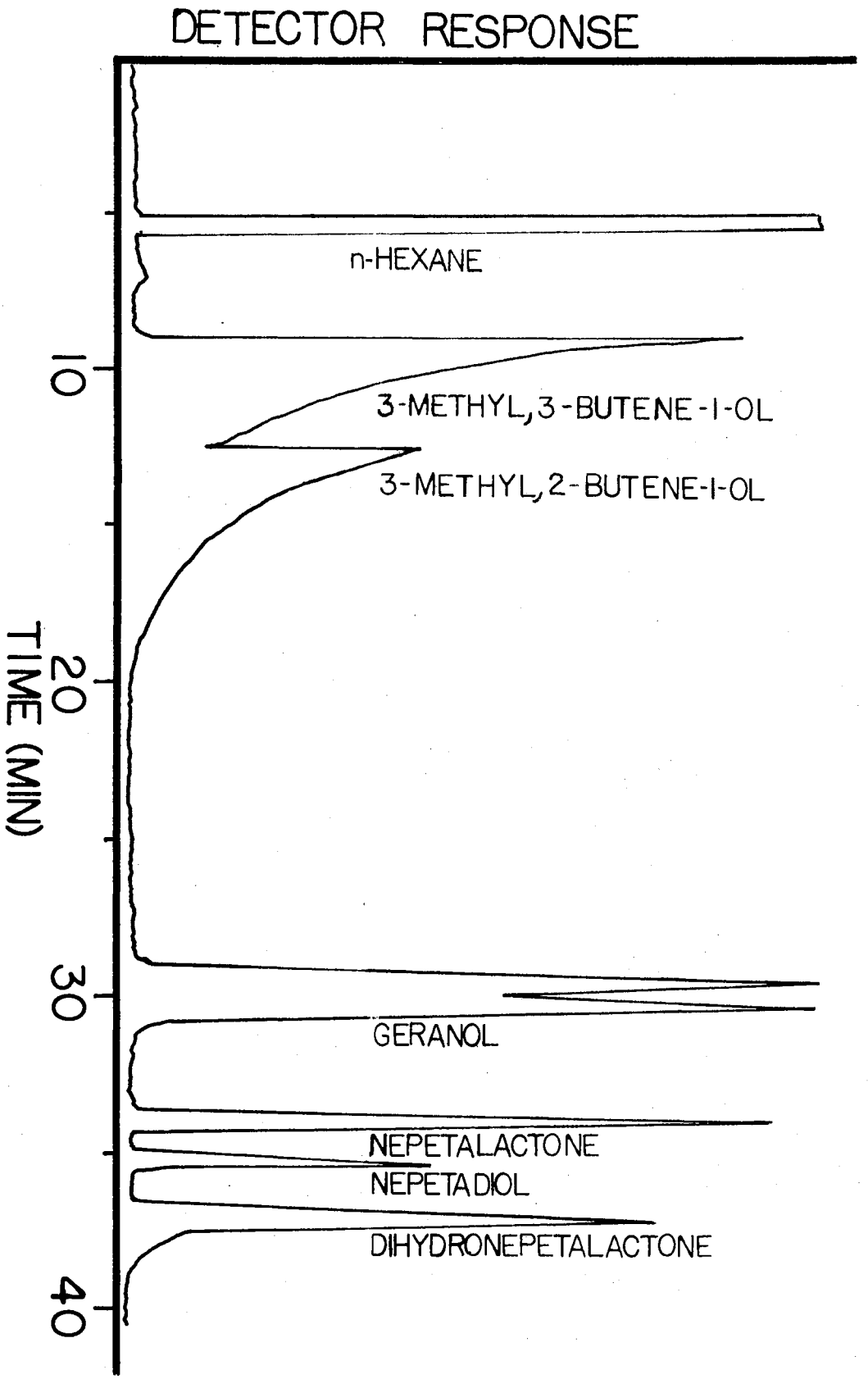

DETECTOR RESPONSE
n-HEXANE
3-METHYL, 3-BUTENE-1-OL
3-METHYL, 2-BUTENE-1-OL
GERANOL
NEPETALACTONE
NEPETADIOL
DIHYDRONEPETALACTONE
10
20
30
40
TIME (MIN)

TABLE I

PUBLISHED R_f RANGE FOR MEVALONATE METABOLITES

System	MVA	MVAP	MVAPP	IPP	Reference
A	.77-.95	.53-.71	.21-.38	.52-.74	26, 43, 45, 46, 99, 100, 101, 102
B	.65-.88	.09-.38	0.0-.10	0.0-.20	24, 26, 45, 46, 99, 101, 102
C	.74-.87	.12-.42	.09-.31	.25-.34	26, 46, 99, 103, 104
D	.66-.87	.27-.38	.09-.44	---	26, 35, 99, 100, 105
E	.56-.72	.18-.20	.36-.48	---	49, 102
F	.50-.64	.07-.25	.02-.10	0.0-.44	99, 100
G	.71	.50	---	0.0-.48	33, 106

System	Composition	
A.	t-butanol:formic acid:H_2O	20:5:8 v/v/v
B.	n-butanol:formic acid:H_2O	77:10:13 v/v/v
C.	t-amyl alcohol:acetic acid:H_2O	4:1:2 v/v/v
D.	i-butyric acid:NH_4OH:H_2O	66:34:30 v/v/v
E.	n-propanol:NH_4OH:H_2O	6:2:2 v/v/v
F.	n-propanol:NH_4OH:H_2O	6:3:1 v/v/v
G.	i-propanol:i-butanol:NH_4OH:H_2O	40:20:1:39 v/v/v/v

Abbreviations:

MVA: mevalonate (XII)
MVAP: 5-phosphomevalonate (XIII)
MVAPP: 5-pyrophosphomevalonate (XIV)
IPP: isopentenyl pyrophosphate (XV)

placing the chromatograms in the chamber. The chromatograms were placed in the chamber and the total 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 (51) over 0.5 x 5 cm columns collecting 3.3 ml fractions. Stepwise elution of Dowex-1 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 M ammonium formate in 4 N formic acid. Ammonium ion removal from samples prior to lyophilization was carried out by batchwise treatment with Dowex-50 (hydrogen form).

9. Detection and Measurement of Radioactivity

Radioactivity on chromatograms was detected either by auto-radiochromatography or by scanning the chromatograms with a model 1002 Nuclear Chicago Actigraph III chromatogram scanner. ^{14}C elution profiles from ion exchange chromatography columns were obtained by counting an aliquot of each fraction in a toluene-ethanol counting cocktail. A model 3320 Packard liquid scintillation spectrometer was used to measure radioactivity of samples in a toluene-ethanol scintillation counting cocktail. The toluene-ethanol scintillation cocktail was composed of four grams 2,5-diphenyloxazole (PPO) and 0.2 grams 1,4-bis (2-(5-phenyloxazole)) - Benzene; Phenyl-oxazolylphenyl - oxazolylphenyl (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 isotopic source with various amounts of reagent grade acetone used as a quencher. Samples counted in the toluene-ethanol cocktail were determined to be between 50 and 70 per cent efficient.

CHAPTER IV

RESULTS AND DISCUSSION

A. $^{14}CO_2$ Incorporation into Nepetalactone

The time course of appearance of ^{14}C labeled nepetalactone in N. cataria seedlings was studied by allowing the seedlings to fix $^{14}CO_2$.

N. cataria seeds were germinated on moist filter paper. When the first green leaves appeared and the root was 1 to 2 cm in length the seedlings were transplanted to a vermiculite-soil mixture and placed in a growth chamber. Emergence of the cotyledons was taken as day zero and seedlings between 18 and 25 days old were used in these experiments.

The procedure followed was that outlined previously. A weighed amount of ^{14}C-$BaCO_3$, which would produce between 300 and 340 millicuries of $^{14}CO_2$, was placed in the side arm of the photosynthetic chamber and sealed with a rubber septum. One ml of 2 N H_2SO_4 was injected through the sidearm septum onto the ^{14}C-$BaCO_3$ and warmed gently with a flame until effervescence was noted. This procedure was necessary to produce the maximum amount of $^{14}CO_2$ for fixation. The sidearm stopcock was opened and a stopwatch was started simultaneously. After the desired period of time had elapsed the seedling was quickly removed and ground in n-hexane until a smooth homogenate was obtained. The fastest time obtained between opening the chamber and grinding the seedling in hexane was three seconds.

The aqueous phase was quantitively applied to a sheet of Whatman

number one chromatography paper for two dimensional descending autoradiochromatography. Thin layer autoradiochromatography of the n-hexane phase was carried out in two solvent systems. Chromatography papers and thin layer plates were placed on X-ray film and exposed for four weeks.

Autoradiograms of the n-hexane phase show a high level of radioactivity remaining at the origin in all experiments. Nepetalactone is not seen until three minutes of photosynthesis has elapsed. Figure 7 is a negative photograph of a series of autoradiograms of the n-hexane phase. Figure 7A is a crude ^{14}C nepetalactone standard, 7B is the n-hexane phase after four minutes of photosynthesis and 7C, 7D and 7E are after three minutes, 45 seconds, and 35 seconds respectively. Table II illustrates the time course of $^{14}CO_2$ incorporation into the n-hexane fraction. Relatively high levels of ^{14}C are observed after one minute or longer photosynthesis. Nepetalactone is not observed until after three minutes. After 10 minutes, nepetalactone is the major product labeled in the n-hexane fraction. This is consistent with nepetalactone being the major constituent of N. cataria oils.

Autoradiograms of the aqueous fraction demonstrates that Calvin cycle enzymes are operative in that glyceric acid is the first product to appear with ^{14}C labeling. Heavy labeling of sugar phosphate is observed after one minute of photosynthesis. Figures 8, 9 and 10 are negative photographs of autoradiograms obtained after six, 60 and 180 seconds of photosynthesis respectively. Glyceric acid is labeled in each figure.

Figure 7. Thin Layer Autoradiograms of n-Hexane Extracts

A: Crude ^{14}C-Nepetalactone Standard
B: 4 Minute Photosynthesis
C: 3 Minute Photosynthesis
D: 45 Second Photosynthesis
E: 35 Second Photosynthesis

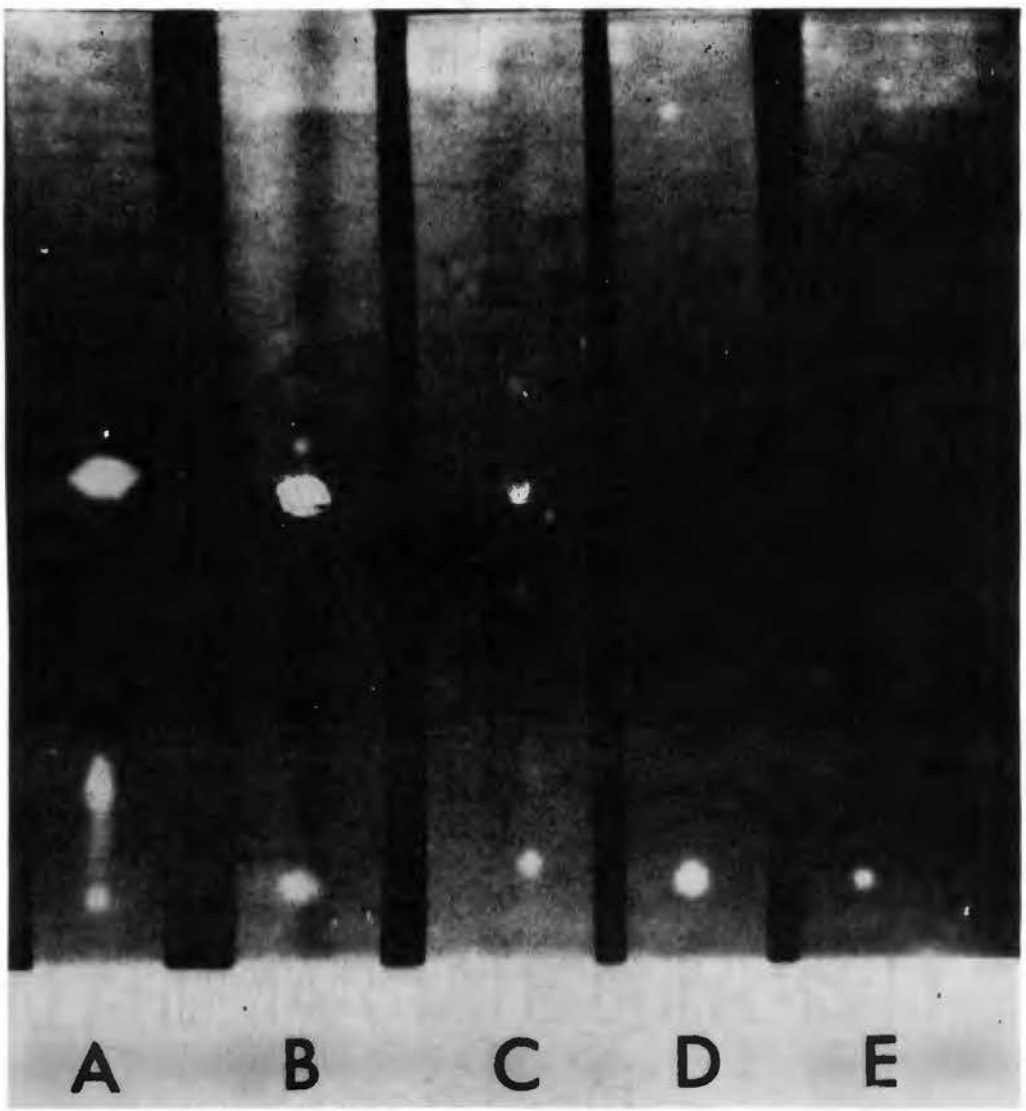

A
B
C
D
E

Figure 8. Autoradiogram of Aqueous Phase After Six Seconds of Photosynthesis

Figure 9. Autoradiogram of Aqueous Phase After One Minute of Photosynthesis

Figure 10. Autoradiogram of Aqueous Phase After Three Minutes of Photosynthesis

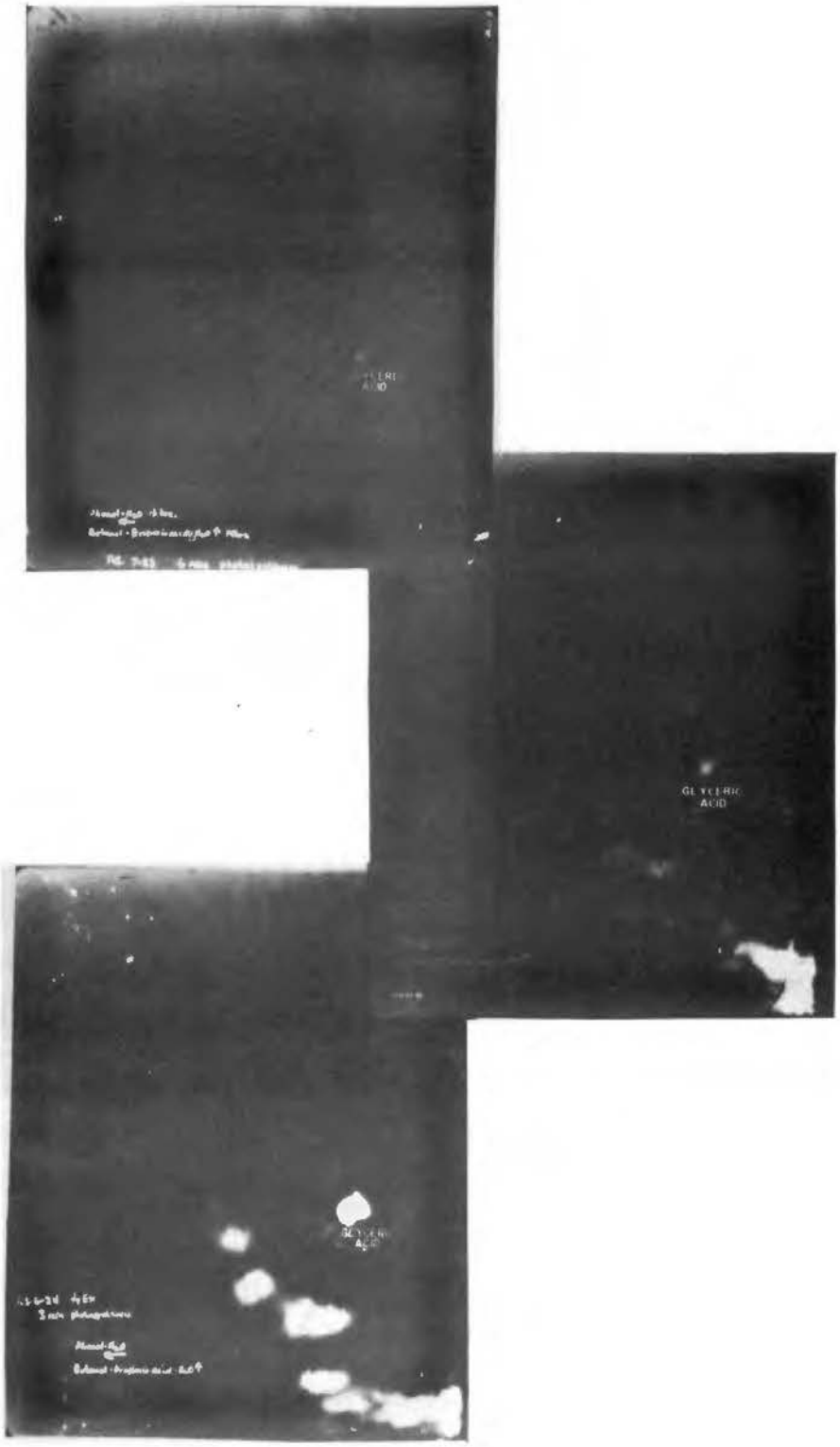

GLYCERIC ACID
GLYCERIC ACID

TABLE II

INCORPORATION OF RADIOACTIVITY INTO
HEXANE SOLUBLE FRACTION

Time (sec)	Wt. of Plant (g)	dmp/g Fresh Wt.
35	0.110	536
60	0.140	24,800
120	0.074	25,200
180	0.449	32,800
240	0.234	53,200
300	0.172	67,300
600	0.132	250,000

The observation that $^{14}CO_2$ is not incorporated into nepetalactone until a three minute period of photosynthesis had elapsed suggests a lag between $^{14}CO_2$ fixation in the chloroplast, transport of ^{14}C-labeled compounds out of the chloroplast and incorporation of the ^{14}C intermediate into the mevalonate pathway of terpenoid synthesis. This also suggests that the site of synthesis of nepetalactone is extrachloroplastic.

B. Callus Culture

1. Callus Initiation and Growth

Three types of media were used for the initiation of callus cultures. Tables III, IV and V show the components of SM, SH and STBA media respectively.

TABLE III

SM MEDIA COMPOSITION

Major Elements	mg/l For 4X Solution	Minor Elements	mg/l For 1000X Solution
NH_4NO_3	1,650	H_3BO_3	6,200
KNO_3	1,900	$MnSO_4.4H_2O$	22,300
$CaCl_2.2H_2O$	440	$ZnSO_4.4H_2O$	8,600
$MgSO_4.7H_2O$	370	KI	830
KH_2PO_4	170	$Na_2MoO_4.2H_2O$	250
		$CuSO_4.5H_2O$	25
		$CoCl_2.6H_2O$	25

Vitamins	mg/l For 1000X Solution		Iron	mg/l For 200 X Solution
Nicotinic acid	500)		Na_2EDTA	7,450
Pyridoxine HCl	100)	in 0.1%	$FeSO_4$	5,570
Thiamine HCl	100)	Toluene		

Kinetin

50 mg 6-furfurylaminopurine in 50 ml of 0.05 N HCl plus 0.1% Toluene for a 2000X Solution.

Gibberellic Acid

100 mg GA_3 in 100 ml 0.02 N NaOH plus 0.1% Toluene for a 1000X Solution.

Auxin

100 mg 2,4-dichlorophenoxy-acetic acid in 100 ml of 0.02 N NaOH plus 0.1% Toluene for a 1000X Solution.

Glutamine	100 mg/l	Aspartic acid	100 mg/l
Asparagine	100 mg/l	Inositol	100 mg/l
Sucrose	20 grams/l	Agar	7.5 grams/l

pH adjusted to 5.6 - 5.8

TABLE IV

SH MEDIA COMPOSITION

Major Elements	mg/l For 4X Solution	Minor Elements	mg/l For 100X Solution
KNO_3	10,000	$MnSO_4.H_2O$	1,000
$MgSO_4.7H_2O$	1,600	H_3BO_3	500
$NH_4H_2PO_4$	1,200	$ZnSO_4.7H_2O$	100
$CaCl_2$	604	KI	100
		$CuSO_4.5H_2O$	20
		$Na_2MoO_4.2H_2O$	10
		$CoCl_2.6H_2O$	10

Iron

1.5 grams $FeSO_4.7H_2O$ and 2.0 grams Na_2EDTA in 100 ml H_2O and heated to 80°C for a 1000X Solution.

Growth Regulating Substances

2,4-dichlorophenoxy-acetic acid	50)	
p-chlorophenoxy-acetic acid	200)	mg/l in 0.1% Toluene for a 100X Solution
6-furfurlyaminopurine	10)	

Vitamins

Thiamine HCl	500)	
Nicotinic Acid	500)	mg/100 ml 0.1% Toluene for a 1000X Solution
Pyridoxine HCl	500)	

Inositol 1 gram/l

Sucrose 30 grams/l

Agar 7.5 grams/l

pH adjusted to 5.6 - 5.8

TABLE V

STBA MEDIA COMPOSITION

Major Elements	mg/l For 4X Solution	Minor Elements	mg/l For 100X Solution
KNO_3	3,800	KI	37.5
NH_4NO_3	2,880	$(NH_4)_6Mo_7O_{24}.4H_2O$	9.25
$MgSO_4.7H_2O$	740	$CuSO_4.5H_2O$	1.0
$CaCl_2$	664		
KH_2PO_4	272		
$MnSO_4.H_2O$	28		
$ZnSO_4.7H_2O$	16.1		
H_3BO_3	9.6		

Iron

1.5 grams $FeSO_4.7H_2O$ and 2.0 grams Na_2EDTA in 100 ml H_2O and heated to 80°C for a 1000X Solution.

Vitamins

Biotin	10)	
Choline	10)	
Folic Acid	10)	
Nicotinamide	10)	mg/100 ml slightly basic 0.1%
Pantothenic Acid	10)	Toluene for 100X Solution
Pyridoxine HCl	10)	
Thiamine	10)	
Riboflavin	1.0)	

Growth Regulating Substances

20 mg 2,4-dichlorophenoxy-acetic acid and 20 mg α-napthalene-acetic acid per 10 ml of 0.1% Toluene for a 1000X Solution

Sucrose	20 grams/l
Inositol	5 grams/l

pH adjusted to 5.6 - 5.8

Deionized glass distilled water was used in the preparation of all stock solutions and media. In the preparation of all stock solutions as well as media, the order of addition of components must be that given in Tables III, IV and V or an insoluble complex may be formed. Toluene at a concentration of 0.1% was added to stock solutions of vitamins and growth regulating substances as a preservative when stored at $4^{o}C$.

Initial experiments on surface sterilization of plant tissue and transfer to media were carried out in a glass hood on a laboratory bench. This procedure was unsatisfactory in that contamination was observed in all flasks. Subsequent sterilizations and transfers were performed in a laminar flow hood with instruments dipped in methanol and flamed between each operation.

Leaf and stem tissue were surface sterilized by shaking tissue in 100 ml of 70% ethanol with 0.1% Tween-20 for various periods of time, decanting the ethanol wash, replacing it with 100 ml of 2.75% sodium hypochlorite (a 1:1 dilution of chlorox) with 0.1% Tween-20 for various time periods followed by decanting and rinsing six times in sterile glass distilled water. As Table VI illustrates, lengthy exposure to either ethanol or sodium hypochlorite wash resulted in no callus formation, presumably due to destruction of the meristmatic capability of the tissue. Exposure to either wash for short periods resulted in high levels of contamination. The final procedure indicated in Table VI resulted in 50% of the flasks becoming contaminated and 50% producing callus.

Following surface sterilization leaf material was cut into 8 to 10 mm strips, 10 to 15 mm stem sections were sliced longitudinally and

placed on 0.75% agar media. Callus growth was first noticed on the cut ends of stem sections and around veins and wounds (i.e., where forceps punctured the leaf) on leaf tissue.

Callus growth was removed from the original explant tissue after five weeks and subcultured onto new media. Subsequent subcultures were made at four to five week intervals. If the cultures were allowed to grow more than four to five weeks browning was detected. Subcultures made after browning had begun resulted in highly variable growth. All callus cultures were grown in continuous light or darkness at room temperature.

TABLE VI

EFFECT OF TIME IN STERILIZATION WASH ON CONTAMINATION AND CALLUS GROWTH

Ethanol (Min.)	NaOCl	Contamination*	Callus Growth*
1	1	10	0
1	5	10	0
1	10	6	0
5	1	10	0
5	5	4	2
5	10	0	0
10	1	7	2
10	5	0	0
10	10	0	0
	- - Final Protocol - -		
2	10	5	5

*Number of Flasks From a Set of 10

The growth of stem and leaf callus tissue was visually determined at the sixth subculture and is illustrated in Table VII. Although there is a difference in size of callus, other differences observed were minimal. A very compact and hard callus was obtained on SM and SH media, STBA media produced a soft friable callus.

No greening of callus was observed after the third subculture on any of the three media. At the 12th subculture tissue on SH media was allowed to continue growth, after 12 to 14 weeks the callus began to green and exude droplets of liquid that smelled like watermelon. The exudate did not have the odor of essential oils of N. cataria plants.

TABLE VII

GROWTH OF N. CATARIA TISSUE UNDER VARYING CONDITIONS

	Dark		Light	
Media	Stem	Leaf	Stem	Leaf
SH	2	2	4	3
SM	1	1	2	2
STBA	0	0	1	1
No Growth	0		Maximum Growth	4

Liquid suspension culture of callus material was attempted by placing a piece of callus into a 125 ml flask containing 50 to 80 ml of media without agar and shaking at 180 rpm on a gyroshaker. Cells from SM and SH media did not grow in suspension, presumably because

the callus tissue was so compact the single cells could not be removed by shaking. Suspension cultures in STBA media grew very well requiring subculture after three weeks of growth. Further work with the suspension cells in STBA media was terminated due to high levels of contamination and large diurnal temperature fluctuations.

In the first nine to ten months of growth, the tissue on SM and SH media grew very well while that on STBA media failed to grow until only seven tubes of STBA media supported growth. At the 10th subculture the tissue on STBA media began to grow rapidly with a clear friable callus. Tissue on SM media began to fall at the 10th and 11th subculture and at the 13th subculture the tissue line on SM media was discontinued.

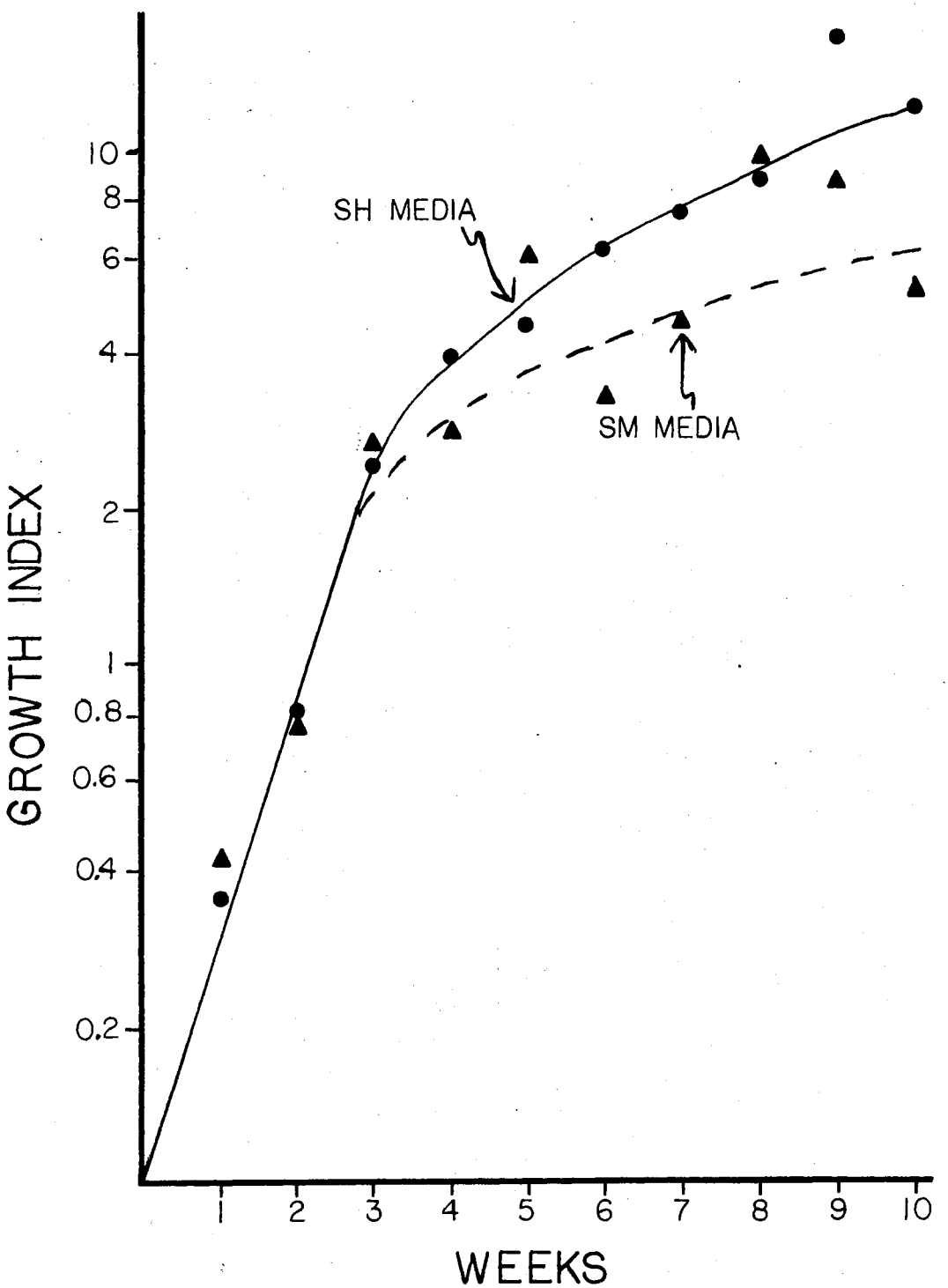
Growth of tissue on SH and SM media was measured at the 10th subculture under continuous light. Tubes of media were prepared and weighed, callus tissue was subcultured to the preweighed tubes and the weight was again recorded, the difference was taken as the initial weight (W_i) of the callus. At one week intervals the callus was removed from the tube and weighed, this weight was taken as the final weight (W_f). Determination of the growth index was determined by Formala 1. The growth curve is shown in Figure 11. Each point on the

$$\frac{W_f - W_i}{W_i} = \text{Growth Index} \qquad (1)$$

SH media curve is the average of 10 tubes, on the SM media curve each point is the average of four tubes. The growth curve is identical on both media for the first three weeks, after which the growth on SM media becomes highly variable and showed a general slowing of growth.

Figure 11. Growth Curve of *N. cataria* Tissue on SH and SM Media

Growth conditions of total light and room temperature on 0.75% agar, 10th subculture of tissue.


GROWTH INDEX
10
8
6
4
2
1
0.8
0.6
0.4
0.2
SH MEDIA
SM MEDIA
1
2
3
4
5
6
7
8
9
10
WEEKS

Tissue on both SM and SH media continued to grow through the 10 week study although browning was noted after four to five weeks.

2. Nepetalactone Synthesis in Callus Tissue

An attempt to isolate nepetalactone from callus tissue was made in the second subculture of leaf tissue on STBA media. A 1.63 gram sample of callus tissue was homogenized in 5.0 ml n-hexane and centrifuged to separate the aqueous and n-hexane phases. The aqueous phase was extracted two additional times with n-hexane and the n-hexane extracts pooled. The n-hexane was removed under a gentle stream of nitrogen at room temperature yielding 2.3 mg of crude oil. Thin layer chromatography of the crude oil, along with standard nepetalactone in two solvent systems, resulted in a band, visualized under UV light, similar to nepetalactone. Gas chromatography of the crude oil, however, did not confirm the presence of nepetalactone. The callus material grown on all three media smells like freshly cut watermelon.

N. cataria tissue can be propagated as masses of undifferentiated cells on the three types of media used in this study and as suspensions of single and small clumps of cells in liquid STBA media. The optimum size of tissue for subculture was found to be between 100 and 200 mg fresh weight. Although the tissue grown in static cytoculture does not produce nepetalactone, a number of n-hexane soluble oils are synthesized. The tissue was clear to yellow-white in color and did not show greening under normal culture conditions after the third subculture. It is assumed that the callus tissue does not contain protoplasts or chloroplasts although proplastids may be present.

C. Enzyme Assay and Preparation

1. Assay of Mevalonate Kinase

Mevalonate kinase activity can be easily measured by two methods. The first method is a spectrophotometric assay with mevalonate kinase being coupled to pyruvate kinase and lactate dehydrogenase, the phosphorylation of mevalonate can be measured as a change in absorbance at 340 nm. Tchen (45) and Popjak (35) have described the composition of spectrophotometric assay solutions. The second assay is a radioactive assay in which ^{14}C-mevalonate is incubated with ATP and mevalonate kinase, 5-phosphomevalonate is detected by paper or ion exchange chromatography. Green (96) and Tchen (45) describe the radioactive assay conditions.

In order to check both assays, mevalonate kinase was partially purified from hog liver (35) and yeast (45). Hog liver mevalonate kinase was purified and assayed spectrophotometrically as described by Popjak (35). A high level of mevalonate kinase is required in order to observe a significant change in absorbance in the spectrophotometric assay, thus spectrophotometric assays of yeast mevalonate kinase were not successful due to the apparent low specific activity of the enzyme. Radioactive assays were carried out as described by Tchen (45) on a partially purified mevalonate kinase preparation from yeast. Reactions were stopped by immersing in boiling water for three minutes. Protein was removed by centrifugation and the supernatant was applied to Whatman number one chromatography paper. Chromatograms were developed in n-butanol:formic acid:H_2O 77:10:13 (v/v/v). The chromatograms were scanned with a chromatogram scanner. Peaks of

radioactivity were detected near the origin indicating an active mevalonate kinase preparation.

Mevalonate kinase activity from enzyme preparations of N. cataria tissues was determined by both assays. The spectrophotometric assay was not utilized after initial experiments because crude enzyme preparations contained high levels of pigments which interfered with absorbance readings and subsequently presented low levels of mevalonate kinase activity.

Radioactive incubation assays initially were carried out in the presence of $MgCl_2$, $MnCl_2$, KCl, EDTA, dithiothreitol or dithioerythritol, an ATP and TPNH generating system. Later experiments indicated that TPN and TPNH were not required for synthesis of isopentenyl pyrophosphate and that by inhibiting phosphatases with fluoride the ATP generating system could be replaced with ATP.

The final radioactive incubation system, utilized throughout this study, was composed of ^{14}C-mevalonate at concentrations specified, ATP at concentrations specified, 5 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, 1 mM dithioerythritol and 5 mM KF in 100 mM phosphate or Tris pH 7.5 buffer.

The radioactive assay procedure referred to throughout the remainder of the text is as follows. Radioactive incubations were terminated by immersing in boiling water for five minutes. Protein was removed by centrifugation and aliquots of the supernatant were applied to Whatman number one chromatography paper. Chromatograms were developed in one of the systems described in Table I and scanned with a chromatogram scanner. Radioactive portions of the chromatograms were cut out, placed in toluene-ethanol counting cocktail and

radioactivity determined by liquid scintillation counting.

2. Homogenization Buffers

Buffers used for homogenizing tissue were composed of 5 mM $MgCl_2$, 10 mM KCl, 1mM EDTA, 5 mM dithioerythritol or dithiothreitol and 500 mM sucrose in pH 7.6 phosphate or Tris buffers of 3 to 500 mM. The concentrations of metal ions could be varied over 10 to 20 mM and not effect the final enzyme activity. Sulfhydryl reagents were definitely required for optimum activity. Initial homogenizations were performed with a 3 mM phosphate homogenization buffer. Use of low ionic strength buffers resulted in very low or no enzyme activity. A series of homogenizations with buffers of increasing ionic strength indicated that a 500 mM buffer was required to hold the pH of the crude homogenate at 7.2 to 7.3. Subsequent homogenizations were performed in 500 mM Tris or phosphate buffers at pH 7.6.

Triton X-100, at a concentration of 0.1%, was included in some homogenization buffers. It was thought that a detergent would aid in solubilizing membrane bound enzymes that may be required for nepetalactone synthesis.

D. Products of Mevalonate Metabolism

1. Order of Appearance of Metabolites

Paper chromatography of 30K supernatant incubations with ^{14}C-mevalonate indicated three radioactive products. Identity of the products could not be proven conclusively by paper chromatography alone because of the wide range of published R_f values for metabolites

of mevalonate (Table I). Supernatants of cell-free incubations with ^{14}C-mevalonate chromatographed over 0.5 x 5 cm Dowex-1 (formate form) columns, as described by Suzue (51), resulted in elution profiles with five peaks of radioactivity as seen in Figure 12. Stepwise elution of Dowex-1 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 M ammonium formate in 4 N formic acid.

A large scale incubation of a 30K supernatant, prepared as described previously with phosphate homogenization buffer including 0.1% Triton X-100, and ^{14}C-mevalonate was performed under standard radioactive incubation assay conditions. Aliquots were removed from the reaction at 15, 30, 60, 90, 120, 150, and 180 minutes. Reactions were terminated and protein removed by centrifugation. Supernatants remaining after removal of protein were chromatographed through Dowex-1 columns as described above. The radioactivity in each of peaks I through V was determined for each time interval. Figure 13 is a plot of the time course of appearance of each peak as a percentage of available R isomer of mevalonate. Disappearance of peaks I and II with the appearance of peaks III, IV and V indicate that peaks I and II are substrate and peaks III, IV and V are products of mevalonate metabolism.

Figure 12. Typical Elution Profile Following Assay of Enzyme System with ^{14}C-Mevalonate

Dower-1 (formate form) column (0.5 x 5 cm), 3.3 ml fractions collected. Stepwise elution performed as described in text. Under the 100K bar the dpm is times 100,000, under the 10K bar the dpm is times 10,000.

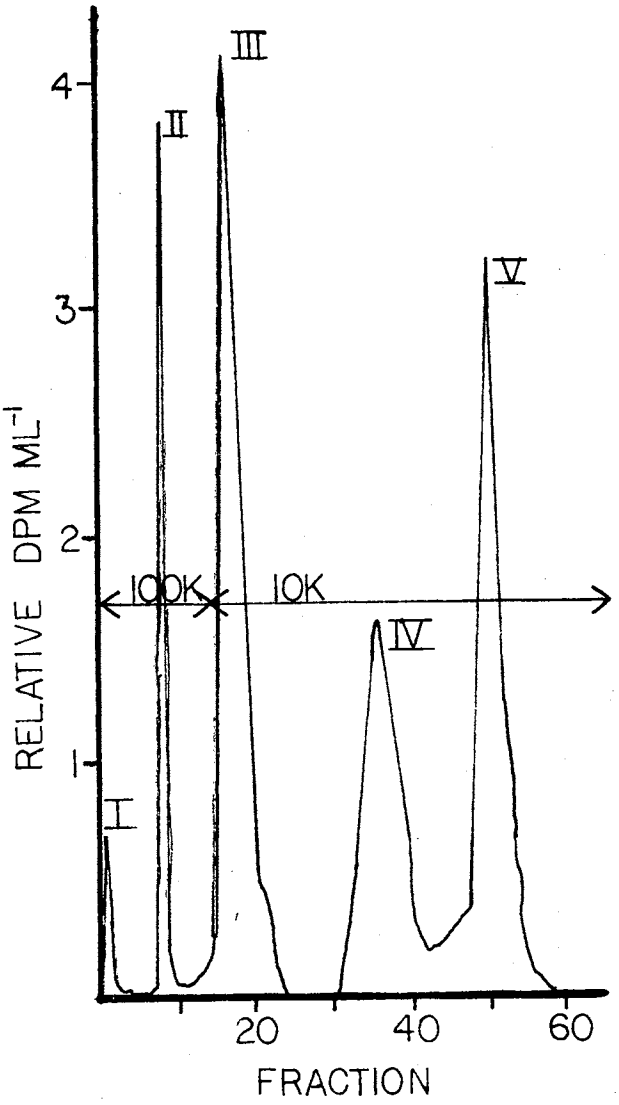
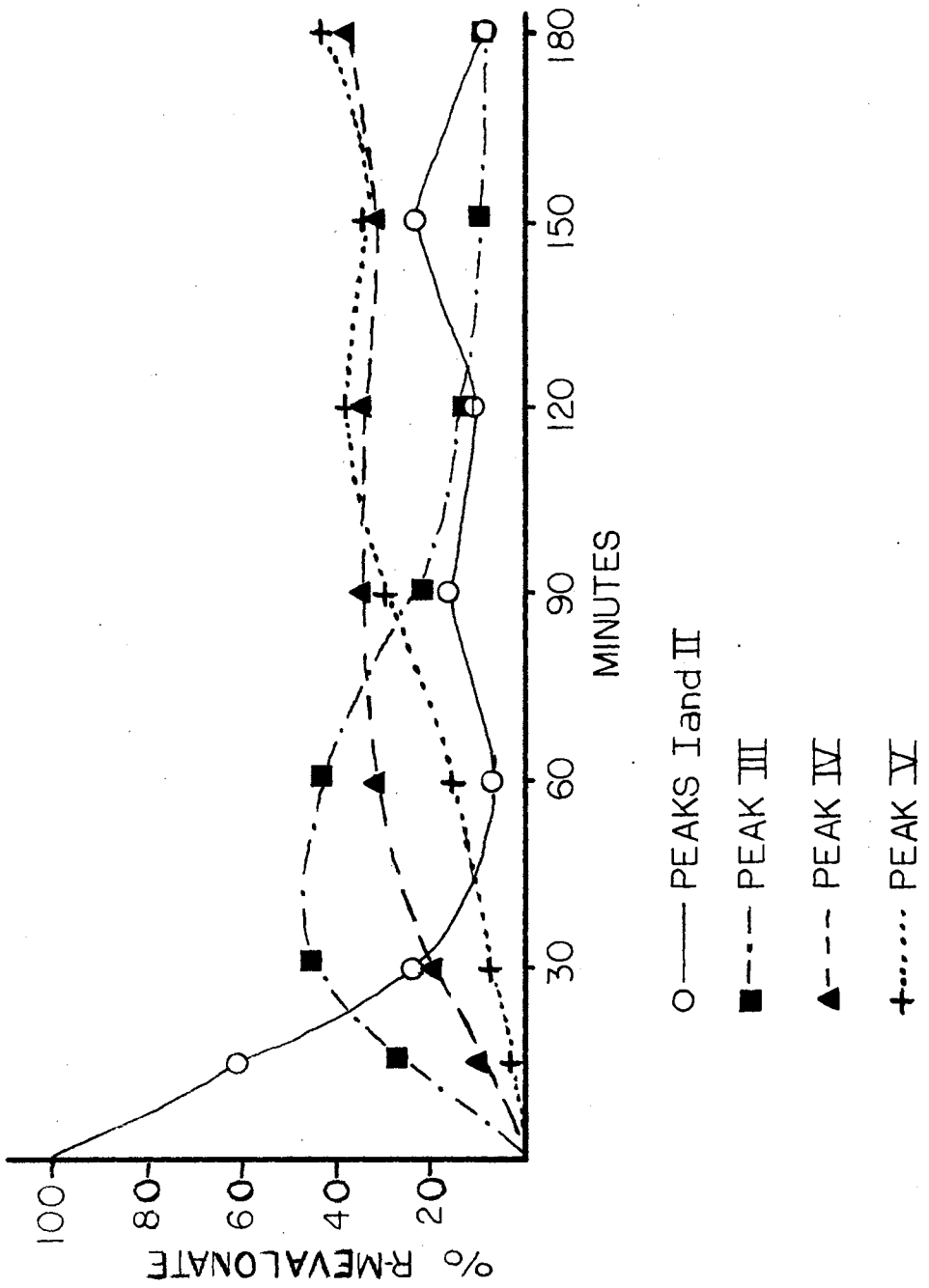

RELATIVE DPM ML⁻¹
4
3
2
1
I
II
III
IV
V
100K
10K
20
40
60
FRACTION

Figure 13. Time Course of Appearance of Peaks III Through V

Peaks I through V for each time period were collected and pooled. Following lyophilization, the material was dissolved in water and an aliquot counted. Fifty per cent of the total dpm for each time period was taken as the amount of R-mevalonate, from this value the per cent of R-mevalonate under each peak was determined.


% R-MEVALONATE
100
80
60
40
20
30
60
90
120
150
180
MINUTES
PEAKS I and II
PEAK III
PEAK IV
PEAK V

2. Identification of Products

Further product identification was attempted by paper chromatography of each peak in seven solvent systems. Table VIII shows the R_f values obtained on paper chromatography of peaks I through V. The first R_f value listed is for the peak containing the majority of radioactivity on the chromatogram. Multiple spots of radioactivity on each chromatogram are due, in part, to hydrolysis of phosphate and pyrophosphate esters of mevalonate and isopentenyl pyrophosphate.

Peaks I through V from Dowex-1 column chromatography were further purified by rechromatography over an identical Dowex-1 column, lyophilized and stored at -20°C.

For further identification of the phosphate esters, enzymatic hydrolysis was used. Hydrolysis of phosphate and pyrophosphate esters was achieved by incubating at 37°C for two hours in three enzyme systems. The first system was composed of five international units of apyrase per ml of 15 mM $MgCl_2$ in pH 6.5 100 mM phosphate buffer. System two was composed of 15 international units alkaline phosphatase per ml of 15 mM $MgCl_2$ in pH 9.0 100 mM Tris buffer. System three was a mixture of apyrase (0.1 international unit) and alkaline phosphatase (1.25 international units) per ml of 15 mM $MgCl_2$ in pH 7.5 100 mM Tris buffer. Peaks III, IV, V and standard 1-^{14}C-isopentenyl pyrophosphate were used as substrates in the enzyme systems. Reactions were terminated, protein was removed by centrifugation and the supernatants chromatographed over Dowex-1 columns as described above.

TABLE VIII

R_f VALUES OF PEAKS I - V FROM DOWEX-1 COLUMN

System*	Peak I	II	III	IV	V
A	.80-.87	.80-.89	.65-.68	.40-.42 .85-.87	.68-.79 .89
B	.73-.74	.69-.72	.36-.45 0.0	.74-.75 0.0-.04	.12-.24 0.0
C	.70-.77	.74-.80	0.0 .19-.22 .74-.79	.06-.08 .55-.60 .73-.74	.22-.29 .54-.62 0.0
D	.81-.85	.77-.82	.34-.39 .79-.82	.19-.29 .80-.81	.63-.73 .76-.80
E	.58-.61	.57-.60	0.0-.10 .59-.64	0.0-.10 .20-.21	.11-.23 .33-.35
F	.60-.61	.60-.61	.06-.07 .60-.68	.03-.06 .18-.35	.20-.32 .04-.10 .64-.65

*Systems described in Table I

Figure 14 shows the elution profiles of substrate peak III. Peak III is hydrolyzed to peaks I and II by all three enzyme systems.

Peak IV was unstable on storage as more than 50 per cent of the substrate was found in peaks I and III, Figure 15. Hydrolysis by alkaline phosphatase increased the level of peak III with a corresponding decrease in peak IV. Of the reaction products obtained on apyrase hydrolysis only 81 per cent of the total radioactivity was recovered, however, a disappearance of peak IV was observed and higher levels of radioactivity were observed in peaks I and II.

Figure 14. Elution Patterns Following Enzymatic Hydrolysis of Peak III

The substrate elution profile is of purified peak III. The elution profile is from a Dowex-1 column eluted as described in the text. Elution profiles after enzymatic hydrolysis of peak III with alkaline phosphatase and/or apyrase are as indicated.

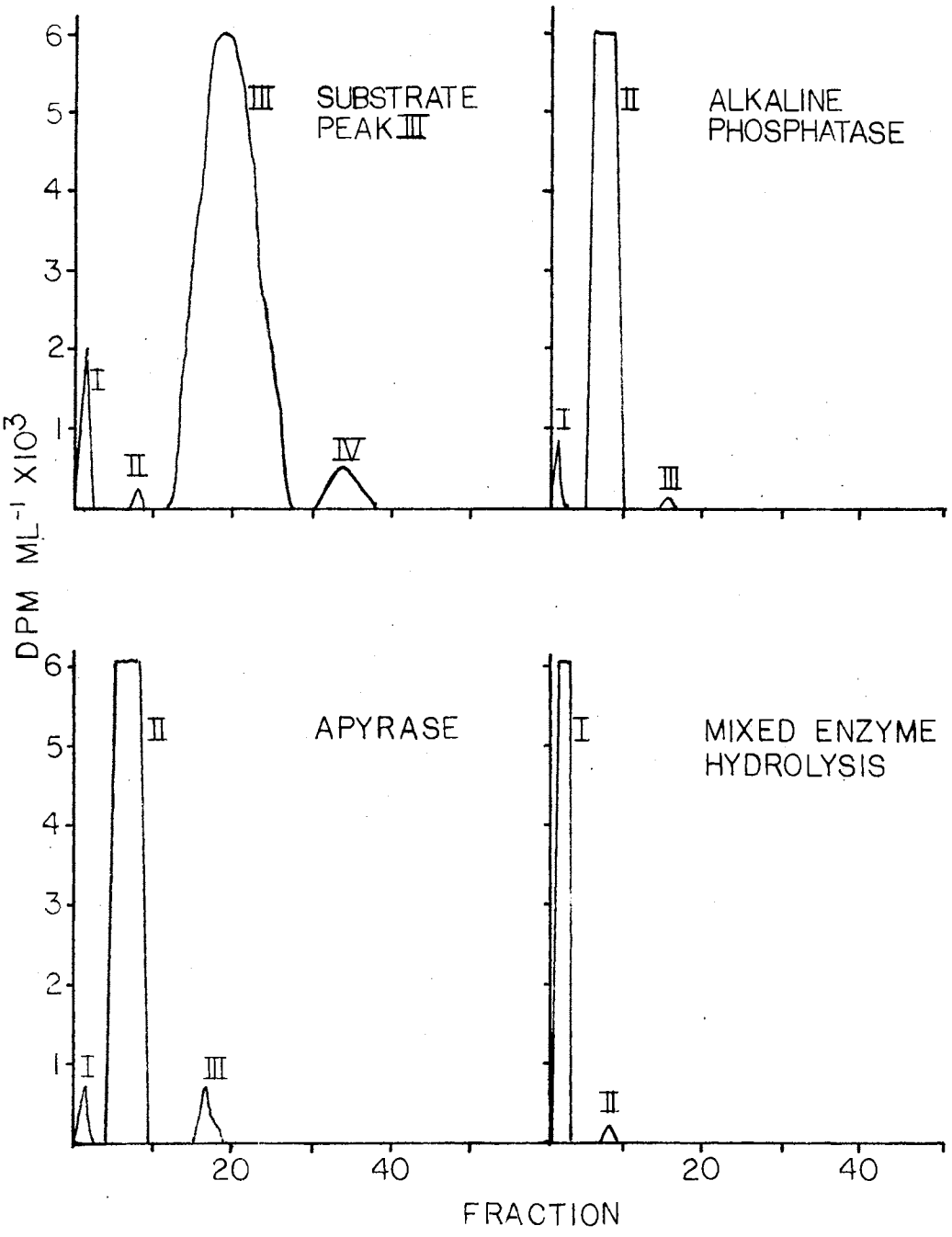
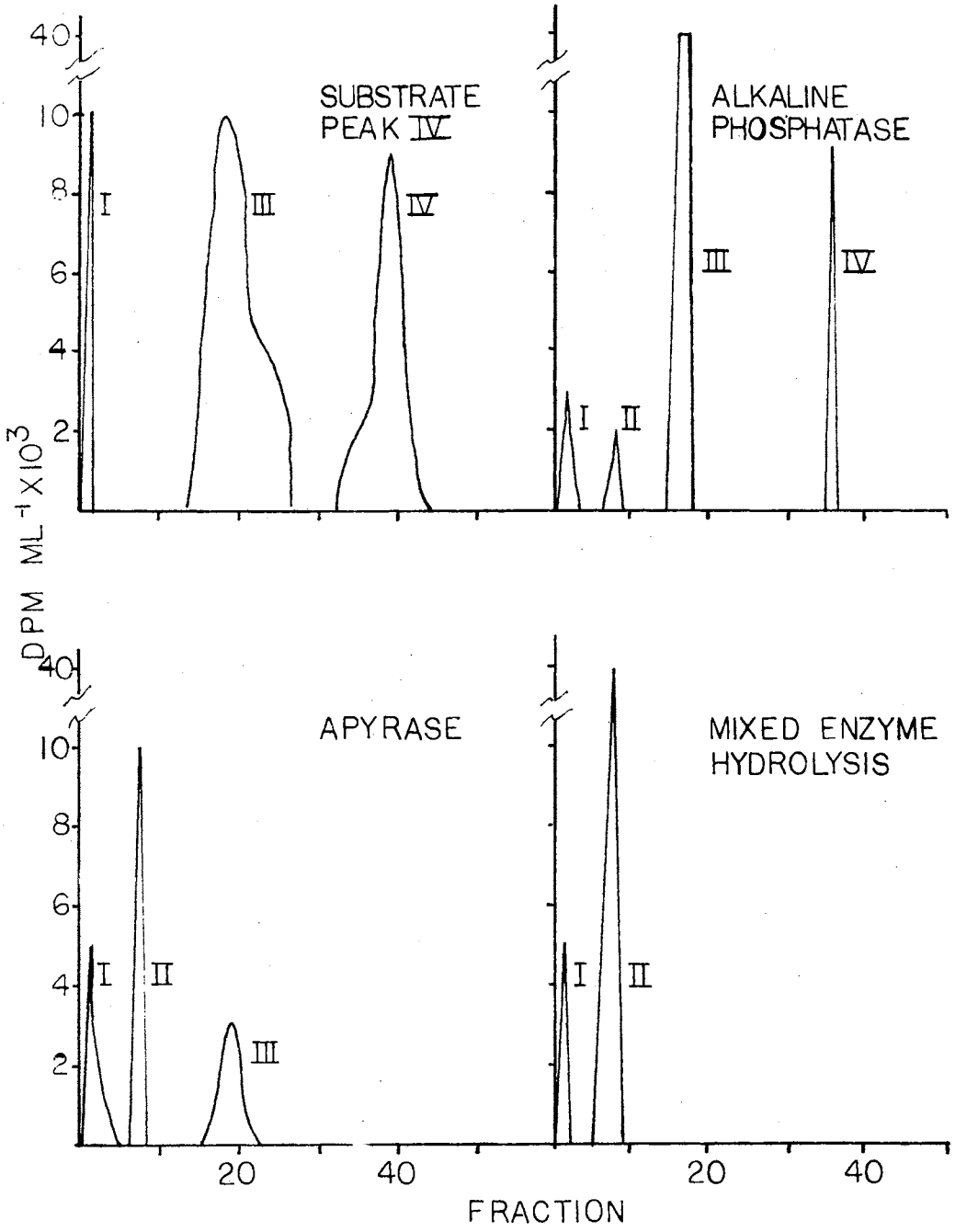

SUBSTRATE
PEAK III
ALKALINE
PHOSPHATASE
APYRASE
MIXED ENZYME
HYDROLYSIS
DPM ML⁻¹ X10³
FRACTION
I
II
III
IV
1
2
3
4
5
6
20
40

Figure 15. Elution Patterns Following Enzymatic Hydrolysis of Peak IV

The substrate elution profile is of purified peak IV after storage as a lyophilized powder at -20°C for seven weeks. The elution profile is from a Dowex-1 column eluted as described in the text. Elution profiles after enzymatic hydrolysis of peak IV with alkaline phosphatase and/or apyrase are as indicated.


SUBSTRATE
PEAK IV
ALKALINE
PHOSPHATASE
APYRASE
MIXED ENZYME
HYDROLYSIS
DPM ML⁻¹ X 10³
FRACTION
I
II
III
IV
2
4
6
8
10
40
20
40
20
40

Elution profiles of peak V after hydrolysis are shown in Figure 16. All three enzyme systems resulted in high levels of radioactivity upon elution with water (peak I). The radioactivity in peak I was dioxane and ether soluble and volatile as all radioactivity was lost when the ether and dioxane extracts were reduced in volume under a gentle stream of nitrogen at room temperature. An unidentified compound (peak U) was eluted with 4 N formic acid in the apyrase enzyme system. Peak U was not observed when alkaline phosphatase was used in the enzyme mixture.

Figure 17 shows the elution profiles of the enzyme reactions with standard 1-^{14}C-isopentenyl pyrophosphate as substrate. Alkaline phosphatase and the mixed enzyme action resulted in all the radioactivity being eluted with water in peak I. The material in peak I was dioxane and ether soluble and volatile as described for peak I in Figure 16. The reaction mixture from the apyrase incubation gave only peak IV and unknown peak U eluting as observed in Figure 16.

Peaks I and II from elutions shown in Figures 14 and 15 were pooled and found to co-chromatograph with standard ^{14}C-mevalonate in seven paper chromatography systems. Peak IV from the elution after apyrase hydrolysis (Figure 17) was treated with alkaline phosphatase and found to be converted to peak I (Figure 17) which was ether soluble and volatile. Peak IV (Figure 17) was chromatographed in seven solvent systems, the resulting R_f values obtained are given in Table IX along with the R_f values obtained for standard ^{14}C-mevalonate and ^{14}C-isopentenyl pyrophosphate.

Figure 16. Elution Patterns Following Enzymatic Hydrolysis of Peak V

The substrate elution profile is of purified peak V. The elution profile is from a Dowex-1 column eluted as described in the text. Elution profiles after enzymatic hydrolysis of peak V with alkaline phosphatase and/or apyrase are as indicated.

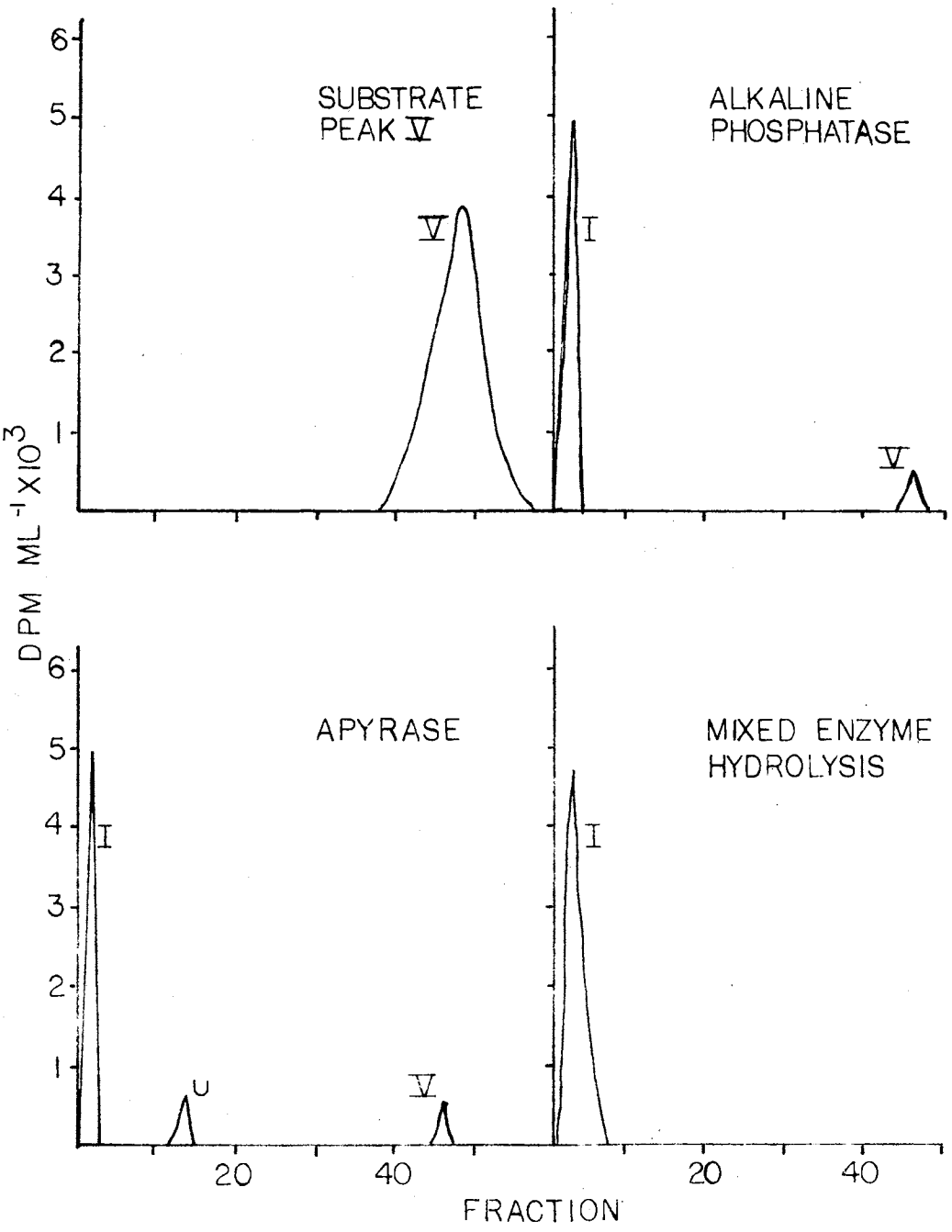

SUBSTRATE
PEAK V
ALKALINE
PHOSPHATASE
APYRASE
MIXED ENZYME
HYDROLYSIS
DPM ML⁻¹ X10³
FRACTION
I
U
V
1
2
3
4
5
6
20
40

Figure 17. Elution Patterns Following Enzymatic Hydrolysis of Standard 1-^{14}C-Isopentenyl pyrophosphate

The substrate elution profile is of standard 1-^{14}C-isopentenyl pyrophosphate. The elution profile is from a Dowex-1 column eluted as described in the text. Elution profiles after enzymatic hydrolysis of standard 1^{14}C-isopentenyl pyrophosphate with alkaline phosphatase and/or apyrase are as indicated.

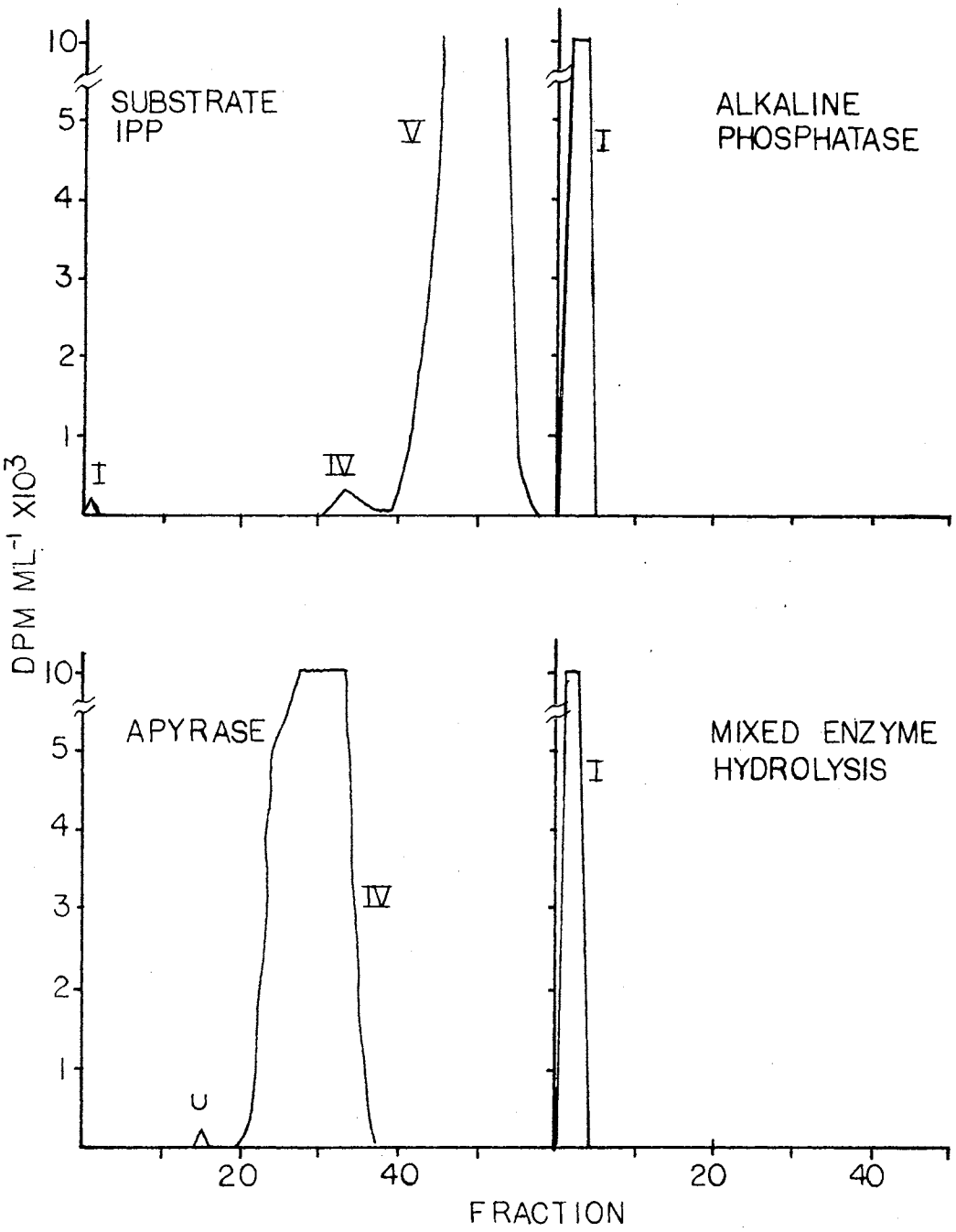

SUBSTRATE
IPP
ALKALINE
PHOSPHATASE
APYRASE
MIXED ENZYME
HYDROLYSIS
DPM ML-1 X10³
FRACTION
10
5
4
3
2
1
20
40
I
IV
V
U

TABLE IX

R_f VALUES OF PEAK IV, ISOPENTENYL PYROPHOSPHATE AND MEVALONATE

Compound	Solvent System* A	B	C	D	E	F
Mevalonic acid	.85	.72	.75	.80	.61	.61
Peak IV	.93	.72	.62	.80	.54	.55
Isopentenyl Pyrophosphate	.75	.20	.22	.70	.16	.20

*Solvent Systems as described in Table I.

Peaks I and II from an elution shown in Figure 12 are mevalonate lactone and mevalonic acid respectively. Peak III is a phosphate ester of mevalonate as shown by hydrolysis with apyrase and alkaline phosphatase to mevalonic acid. Peak IV, an unstable compound, is a pyrophosphate ester of mevalonate as indicated by hydrolysis with apyrase to peak III (5-phosphomevalonate) and breakdown on storage to peak III and mevalonate. Peak V elutes at a position identical to isopentenyl pyrophosphate. Both standard ^{14}C-isopentenyl pyrophosphate and peak V are hydrolyzed to an ether soluble volatile compound found in peak I (Figures 16 and 17). Hydrolysis of standard isopentenyl pyrophosphate and peak V with apyrase results in an unidentified compound, peak U. Peak IV obtained on apyrase hydrolysis of isopentenyl pyrophosphate is hydrolyzed to an ether soluble volatile peak I (Figure 17) by alkaline phosphotase.

The order of appearance, paper chromatography R_f values, Dowex-1

elution profiles and enzymatic hydrolysis data lead to identification of peaks III, IV and V as 5-phosphomevalonate, 5-pyrophosphomevalonate and isopentenyl pyrophosphate respectively. Isopentenyl monophosphate (peak IV, Figure 17) elutes from Dowex-1 columns in the same position as 5-pyrophosphomevalonate. Isopentenyl monophosphate co-chromatographs with mevalonate in paper chromatography systems B and D. It has R_f values so close to mevalonate in all other systems studied that it could easily be masked under the mevalonate peak on chromatograms (Table IX).

The identity of products eluting at peaks III, IV and V is confirmed by Suzue (51), Skilleter (104) and Dugan (102), all of whom report mevalonate metabolites eluting in the order of mevalanolactone, mevalonic acid, 5-phosphomevalonate, isopentenyl monophosphate, 5-pyrophosphomevalonate and isopentenyl pyrophosphate with increasing concentrations of formic acid and ionic strength. Popjak (35) reported that 5-pyrophosphomevalonate is unstable, observing 50 per cent decomposition in three months.

E. Metabolism of Mevalonate

Mevalonate metabolism in leaf and callus tissue cell-free extracts was studied to determine if there is a difference in the production of phosphorylated intermediates of mevalonate in these two enzyme systems. Because of the lack of chloroplasts and no observed nepetalactone synthesis in callus tissue, it was anticipated that mevalonate metabolism in the callus cell-free enzyme preparations would be altered when compared to that of leaf cell-free systems.

Leaf and callus tissue was homogenized in phosphate homogenization buffer including 0.1% Triton X-100. Supernatants from a

30,000 x g centrifugation, prepared as described previously, were used as one enzyme source. The pellet from the 30,000 x g centrifugation was resuspended in homogenization buffer, without Triton X-100, and washed three times in the same buffer. The washed 30K pellet preparations were suspended in 3.0 ml of homogenizing buffer and mixed with a Polytron homogenizer sonicator.

Enzyme activity measurements were performed under standard conditions with 20 mM ATP and 0.14 mM 2-^{14}C-mevalonate (1 microcure per ml) in 100 mM phosphate buffer pH 7.2. Iodoacetamide, which is an inhibitor of isopentenyl pyrophosphate isomerase (47, 48), was included at a concentration of 5 mM as noted in Table X. Protein concentrations in the 30K pellet preparations were very low and Lowry protein determinations were outside of the range of reliability. At the end of three hours of incubation the reactions were terminated, protein was removed and the supernatant was extracted with diethyl ether. The aqueous phase was chromatographed through Dowex-1 columns as previously described.

Less than 0.5 per cent of the total radioactivity in the assays was extracted into diethyl ether. Thin layer autoradiochromatography of the ether extracts along with standard ^{14}C-nepetalactone showed the radioactivity remaining at the origin indicating that the radioactivity extracted in the ether phase was of aqueous origin extracted as micelles in Triton X-100.

^{14}C-nepetalactone, synthesized from ^{14}C-mevalonate, was not detected in any cell-free enzyme preparations from N. cataria.

Radioactive peaks obtained from Dowex-1 column elutions were chromatographed in solvent systems A through D (described in Table I).

TABLE X

ENZYME SOURCES AND INHIBITOR INCLUSIONS IN ASSAY SYSTEMS

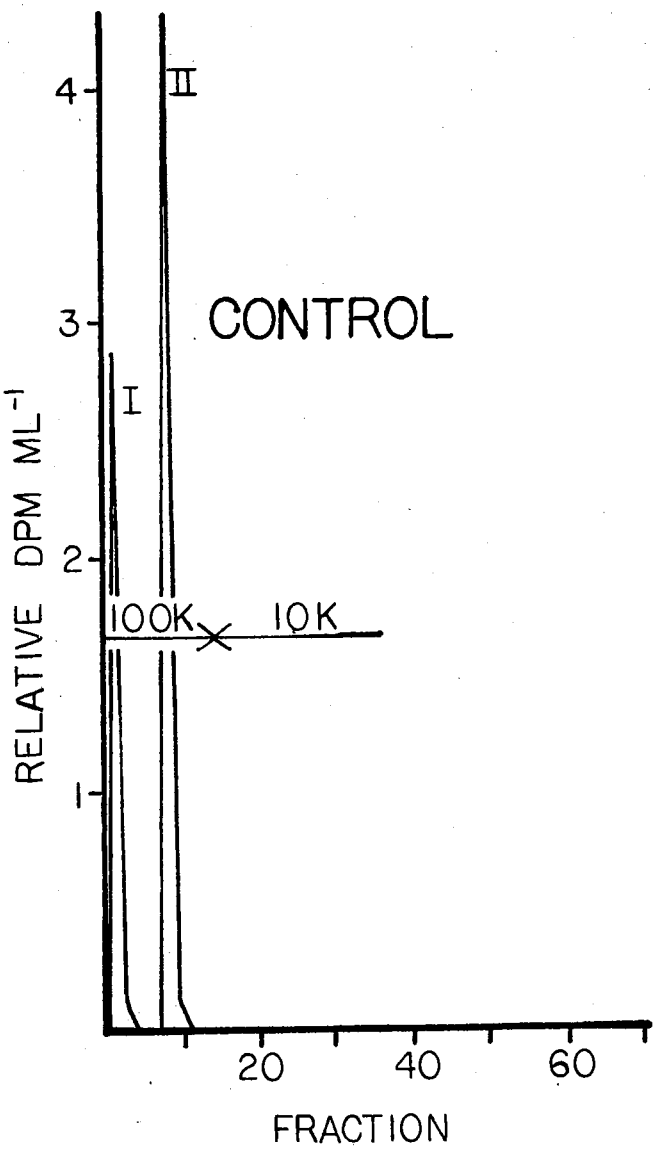
Enzyme Source	Protein mg/ml	Iodoacetamide	Assay
30K Leaf Supernatant	-- Boiled --		Control
30K Leaf Pellet	*	---	A
30K Callus Pellet	*	---	Ac
30K Leaf Supernatant	2.65	---	B
30K Callus Supernatant	0.69	---	Bc
30K Leaf Supernatant	2.65	5 mM	C
30K Callus Supernatant	0.69	5 mM*	Cc
30K Leaf Pellet and Supernatant	1.32	---	D
30K Callus Pellet and Supernatant	0.35	---	Dc

* Undeterminable

Figure 18 shows the elution pattern of the control assay. Mevalonolactone and mevalonic acid were the only radioactive compounds detected.

Figure 18. Dowex-1 Elution Pattern of Control

Elution of incubation supernatant following assay under standard conditions utilizing a boiled enzyme preparation as a control. Elution of Dowex-1 column was as described in the text collecting 3.3 ml fractions.


CONTROL
I
II
100K
10K
RELATIVE DPM ML⁻¹
1
2
3
4
20
40
60
FRACTION

Elution patterns of leaf 30K pellet (A) and callus 30K pellet (Ac) assays are shown in Figure 19. Besides substrate mevalonate, 5-phosphomevalonate (peak III) from the leaf assay was the only other radioactive compound observed.

Assay elution profiles of the 30K supernatant from leaf (B) and callus (Bc) enzyme preparations are shown in Figure 20. Peaks III, IV and V are observed in both systems. The leaf enzyme system produces a very high level of 5-phosphomevalonate (peak III) when compared to the callus enzyme system in which little of this compound is detected. Peak IV is found at low levels in both enzyme systems. Paper chromatography of peak IV from the leaf assay indicated a majority of the peak is composed of 5-pyrophosphomevalonate with some isopentenyl monophosphate also detected. The callus enzyme assay produced peak IV which was found to be composed of only isopentenyl monophosphate. Isopentenyl pyrophosphate (peak V) was observed at high levels in both leaf and callus assays.

Figure 21 shows the elution profiles of assays carried out in the presence of iodoacetamide. The leaf assay (C), when compared to the unhibited system (B, Figure 20), shows an increase in peak IV and a decrease in isopentenyl pyrophosphate (peak V). 5-pyrophosphomevalonate was detected as the major component of peak IV in the leaf system. Little difference was observed in elution profiles of the callus assays in the presence of iodoacetamide (Cc) and absence of the inhibitor (Bc, Figure 20). Peak IV in the callus enzyme assay was composed entirely of isopentenyl monophosphate.

Figure 19. Dowex-1 Elution Pattern of Leaf and Callus 30K Pellet Enzyme Assays

Elution profiles following assay of leaf 30K pellet (A) and callus 30K pellet (Ac) under standard assay conditions. Elution of Dowex-1 columns was as described in the text collecting 3.3 ml fractions.

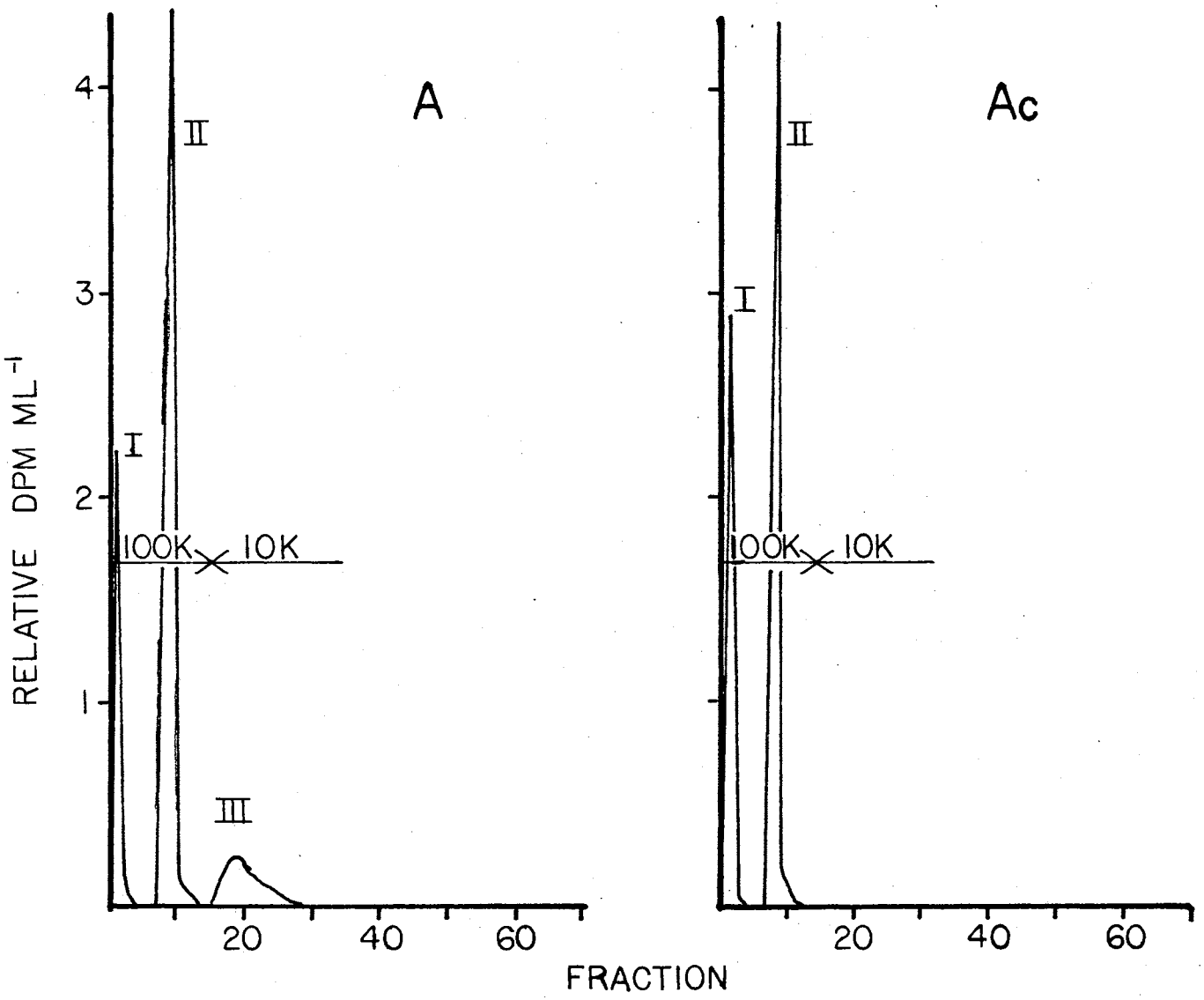

A
Ac
I
II
III
100K
10K
RELATIVE DPM ML⁻¹
FRACTION
1
2
3
4
20
40
60

Figure 20. Dowex-1 Elution Pattern of Leaf and Callus 30K Supernatant Enzyme Assays

Elution profiles following assay of leaf 30K supernatant (B) and callus 30K supernatant (Bc) under standard assay conditions. Elution of Dowex-1 columns was as described in the text collecting 3.3 ml fractions.

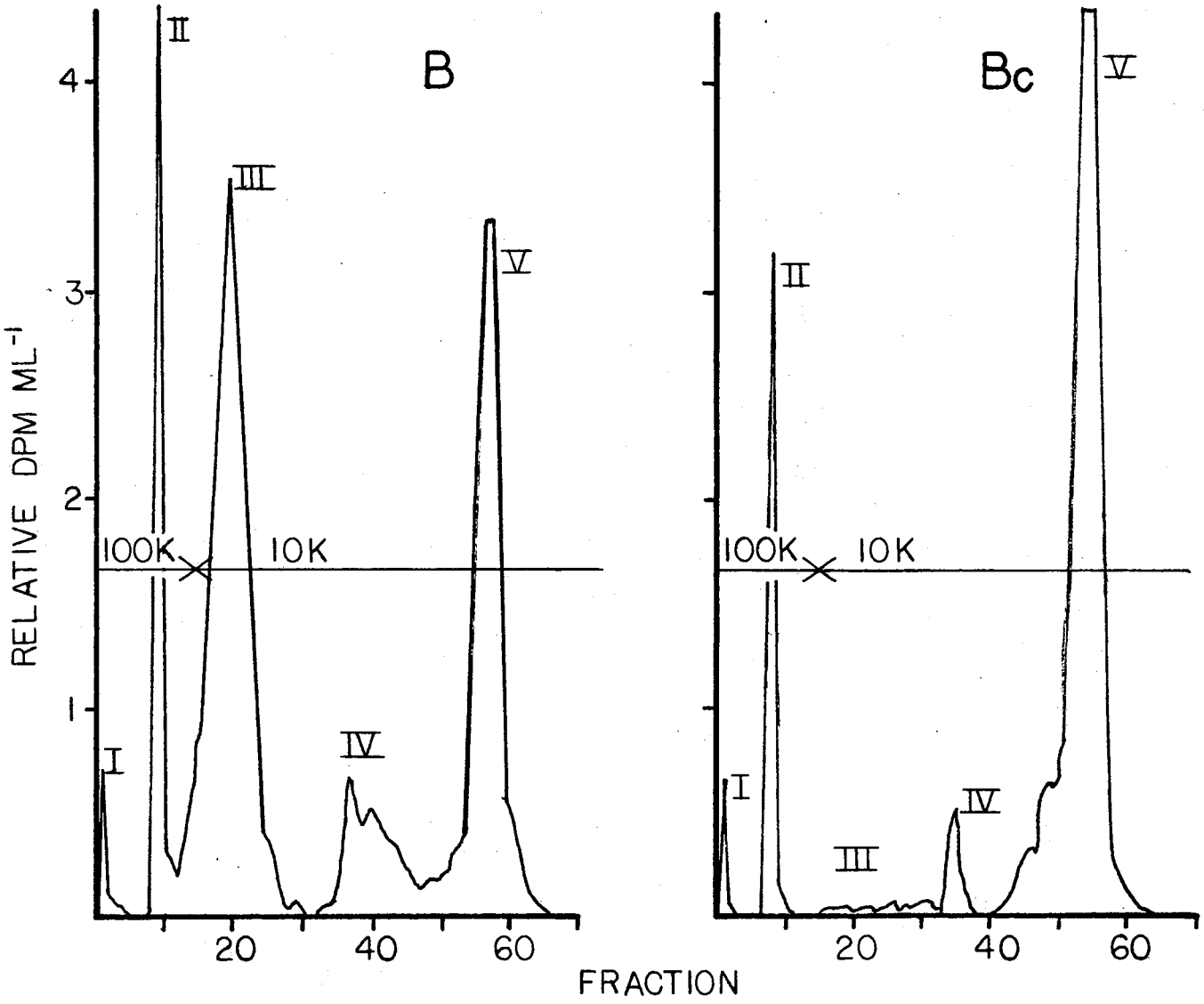
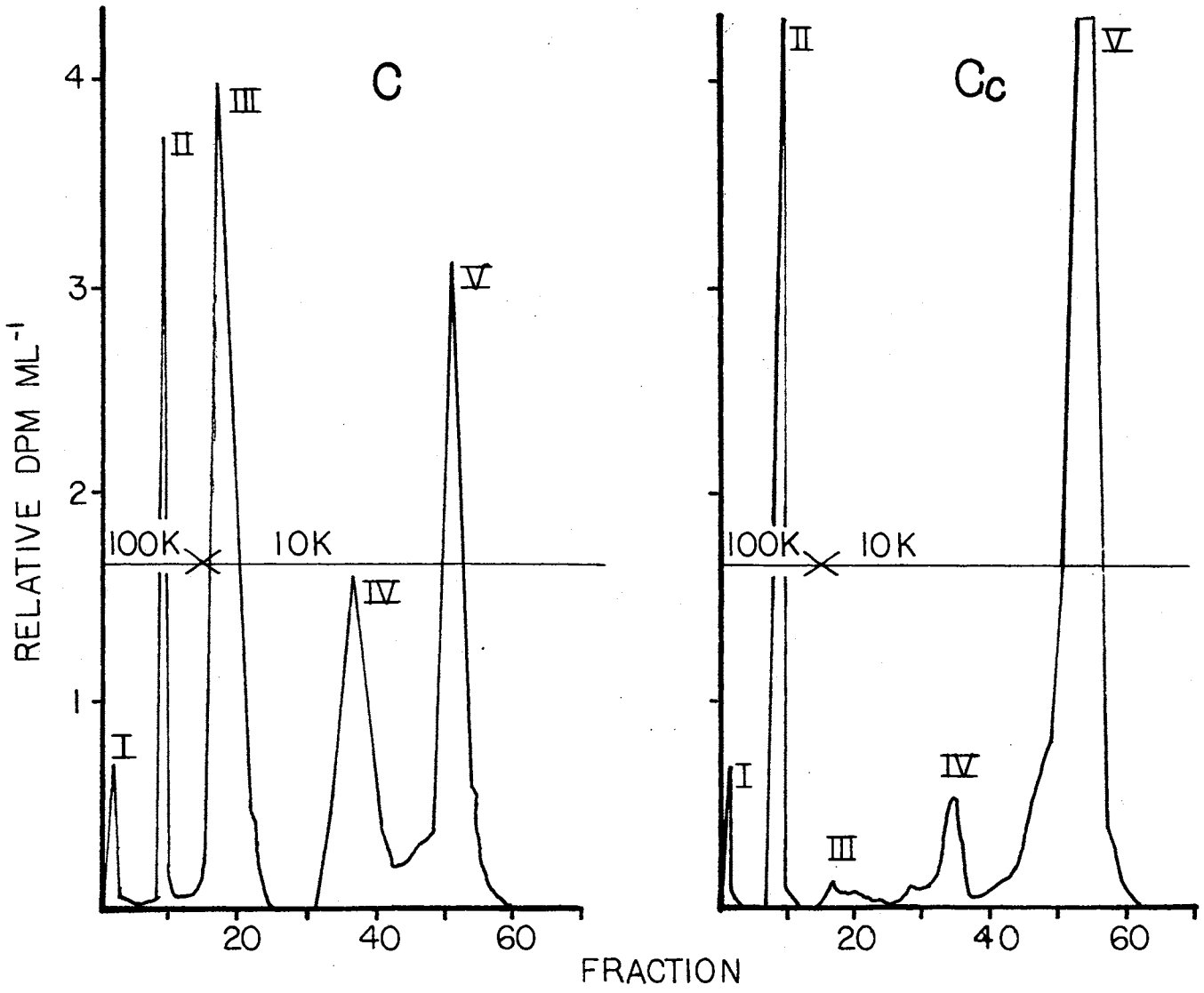

B
Bc
II
III
V
IV
I
100K
10K
RELATIVE DPM ML-1
4
3
2
1
20
40
60
FRACTION

Figure 21. Dowex-1 Elution Pattern of Leaf and Callus 30K Supernatant Enzyme Assays in the Presence of 5 mM Iodoacetamide

Elution profiles following assay of leaf 30K supernatant (C) and callus 30K supernatant (Cc) under standard assay conditions with the addition of 5 mM iodoacetamide. Elution of Dowex-1 columns was as described in the text collecting 3.3 ml fractions.


C
Cc
I
II
III
IV
V
100K
10K
RELATIVE DPM ML-1
FRACTION
1
2
3
4
20
40
60

Elution profiles from the mixed supernatant and pellet assays for the leaf (D) and callus (Dc) systems are shown in Figure 22. The leaf extract shows little difference in elution profile when compared to a 30K supernatant assay (B, Figure 20). Peak III in Figure 22 (Dc) is significantly larger than in Figure 20 (Bc). The shoulder on peak V is more prominent and trails into peak IV. Paper chromatography of peak IV showed approximately equal levels of 5-pyrophosphomevalonate and isopentenyl monophosphate.

Figure 23 shows the percentage of available R isomer of mevalonate found under each peak for each assay. Inhibition of isopentenyl pyrophosphate isomerase by iodoacetamide results in better utilization of mevalonate, lower levels of 5-phosphomevalonate and higher levels of 5-pyrophosphomevalonate, isopentenyl monophosphate and isopentenyl pyrophosphate. A mixed enzyme system composed of pellet and supernatant enzyme preparations results in higher levels of 5-phosphomevalonate and better utilization of substrate.

Marked differences in metabolism of mevalonate are observed when callus and leaf cell-free enzyme assays are compared. The most prominent difference is in the high level of isopentenyl pyrophosphate produced in callus cell-free assays. Sixty-six per cent to 88 per cent of the available R isomer of mevalonate is incorporated into isopentenyl pyrophosphate in callus assays compared to 24 per cent to 37 per cent in leaf cell-free assays. Very little isopentenyl monophosphate is produced in leaf cell-free assays. The majority of radioactivity under peak IV in callus assays was identified as isopentenyl monophosphate, little 5-pyrophosphomevalonate was observed in callus assays. Iodoacetamide, in addition to inhibiting

Figure 22. Dowex-1 Elution Pattern of Mixed 30K Pellet and 30K Supernatant Enzyme Assays

Elution profiles following assay of leaf mixed enzyme (D) and callus mixed enzyme (Dc) preparations under standard assay conditions. Elution of Dowex-1 columns was as described in the text collecting 3.3 ml fractions.

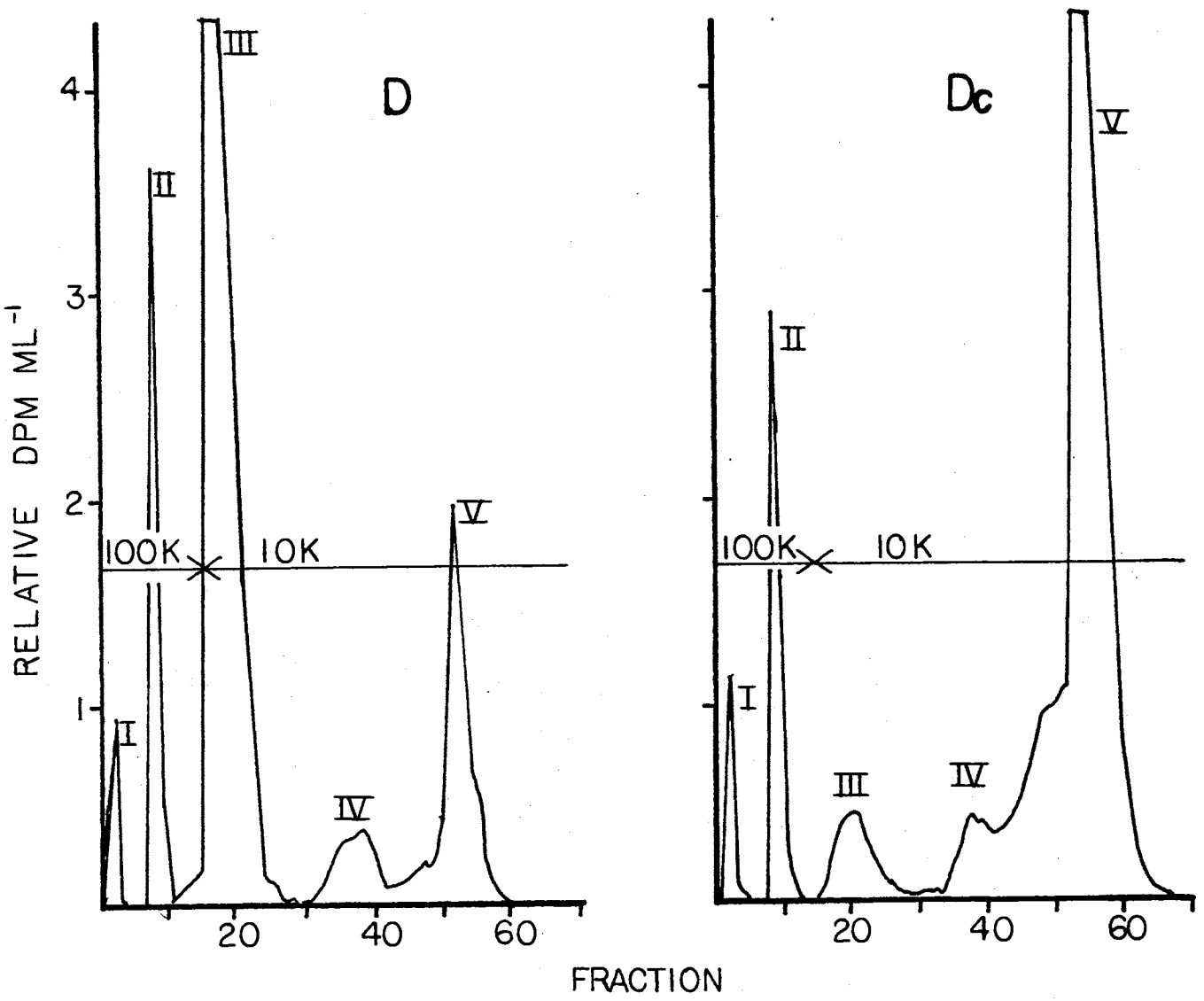
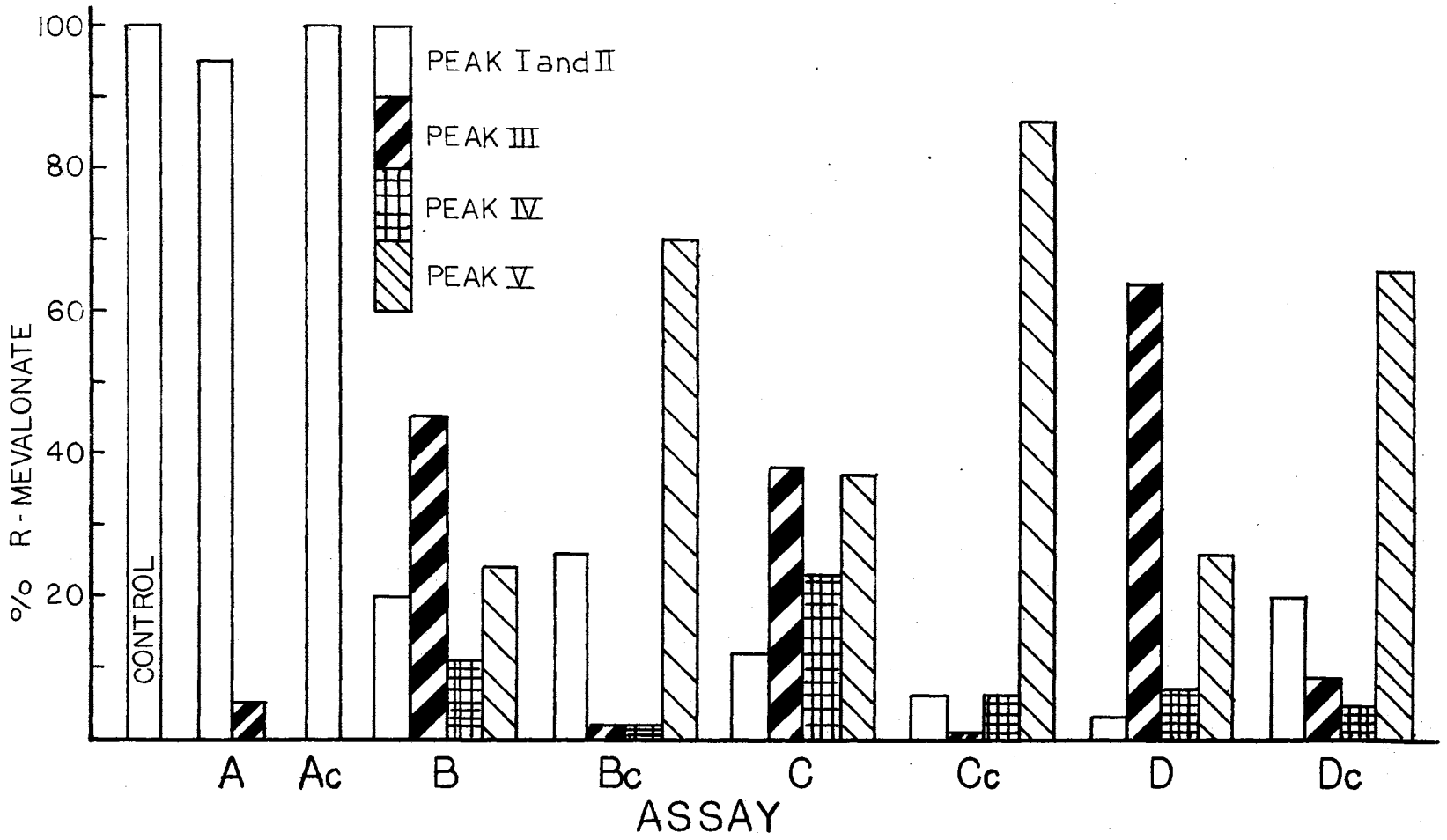

D
Dc
I
II
III
IV
V
100K
10K
RELATIVE DPM ML⁻¹
1
2
3
4
20
40
60
FRACTION

Figure 23. Percentage of R-Mevalonate Under Each of Peaks I Through V in Eash Assay System

Peaks I through V in each assay system were pooled, lyophilized, dissolved in water and an aliquot counted. Fifty per cent of the total dpm for each assay was taken as the amount of R-mevalonate, from this value the per cent of R-mevalonate under each peak was determined.


100
80
60
40
20
% R-MEVALONATE
CONTROL
PEAK I and II
PEAK III
PEAK IV
PEAK V
A
Ac
B
Bc
C
Cc
D
Dc
ASSAY

isopentenyl pyrophosphate isomerase, results in a more efficient utilization of substrate. One explanation for the better utilization of mevalonate in the presence of iodoacetamide is the lack of higher prenyl phosphates and pyrophosphates, which are inhibitors of mevalonate kinase, in the reaction mixture. A mixed enzyme assay composed of 30K pellet and 30K supernatant enzyme preparations yields higher levels of 5-phosphomevalonate and lower levels of radioactivity in peak IV, composed of isopentenyl monophosphate and 5-pyrophosphomevalonate.

Chesterton and Kekwick (46) reported high levels of isopentenyl monophosphate and low levels of 5-pyrophosphomevalonate synthesis in cell-free systems of rubber latex. It was proposed that the isopentenyl monophosphate observed in the latex system was the result of hydrolysis of isopentenyl pyrophophate by endogenous phosphatases (46). The action of phosphatases on isopentanyl pyrophosphate synthesized by N. cataria cell-free systems could produce the isopentenyl monophosphate observed in the above experiments. High levels of alkaline phosphatase activity are observed in cell-free systems from N. cataria tissue.

It appears that the enzyme preparations from leaf and callus tissue are not under the same type of control. Lack of chloroplasts and nepetalactone synthesis in callus tissue indicates that since the mevalonate pathway is active it is required for synthesis of secondary metabolites.

It remains to be determined if the isopentenyl monophosphate observed is synthesized by phosphatase action on isopentenyl pyrophosphate or by decarboxylation of 5-phosphomevalonate.

F. Purification of Mevalonate Kinase

The formation of monoterpenes via the mevalonate pathway requires the phosphate and pyrophosphate esters of mevalonate. Maceration of plant tissue breaks cells and cellular organelles releasing phosphatases which may hydrolyze the phosphate esters of mevalonate or of the substrate nucleotides required for synthesis of 5-phosphomevalonate. Sucrose density gradients were utilized in an attempt to separate mevalonate kinase from the phosphatases present in a cell-free system from leaf tissue. These experiments were conducted before the optimum assay conditions and homogenition procedures were elucidated and the results were not conclusive.

Leaf material was homogenized in 3 mM phosphate homogenization buffer. The 30K supernatant was layered onto sucrose gradients (5 to 20 per cent and 10 to 25 per cent) and centrifuged for three hours at 87,000 x g (avg.). The gradient was pushed from the tube such that fraction one is the top (light) end of the gradient and fraction 10 is the bottom (heavy) end of the gradient. Each fraction was assayed for alkaline phosphatase activity, protein concentration and mevalonate kinase activity.

The mevalonate kinase assay was carried out at 30°C with shaking in the presence of 0.175 mM mevalonate (1.25 microcuries per ml) 8 mM $MgCl_2$, 8 mM $MnCl_2$, 10 mM TPN, 10 mM glucose-6-phosphate, 36 mM KF, 30 mM ADP, 30 mM phosphoenolpyruvate and 10 units each of pyruvate kinase and glucose-6-phosphate dehydrogenase in a total volume of 3.0 ml, for three hours. Reactions were terminated, protein was removed and the supernatant lyophilized. The lyophilized material

was dissolved in 0.5 ml water and applied quantitatively to Whatman number one chromatography paper for development in n-butanol:formic acid:H_2O 77:10:13 (v/v/v). The chromatograms were placed on x-ray film and exposed for four weeks. Chromatograms with radioactive spots at R_f values less than 0.5 indicated mevalonate kinase activity, although no quantitation was possible.

Figure 24 shows the profile of protein concentration and alkaline phosphatase activity over a five to 20 per cent linear sucrose gradient. High levels of phosphatase activity were observed over the entire gradient with no separation of protein and alkaline phosphatase activity. Mevalonate kinase activity was detected at the bottom of the gradient, as indicated by the cross-hatched bar, at fractions 8, 9, and 10. Mevalonate kinase activity was detected at sucrose concentrations of 15 per cent and higher.

The profile of protein concentration and alkaline phosphatase activity is shown for a 10 to 25 per cent linear sucrose gradient in Figure 25. A significant fraction of protein was separated from phosphatase activity at the heavy end of the gradient. Mevalonate kinase activity was detected over the heavy half of the gradient, again beginning at about 15 per cent sucrose to the higher concentration as indicated by the cross-hatched bar. Use of a heavier gradient shifts the entire profile to the top of the gradient. Discontinuous sucrose gradients of five to 40 per cent and 15 to 45 per cent were also utilized for the separation of alkaline phosphatase and mevalonate kinase activity. The same general pattern of mevalonate kinase activity being detected at sucrose concentrations beginning at 15 per cent was observed.

Figure 24. Alkaline Phosphatase, Protein and Mevalonate Kinase Activity Profile From a Linear 5 to 20 Per Cent Sucrose Gradient

Mevalonate kinase activity was detected in fractions indicated by cross-hatched bar.

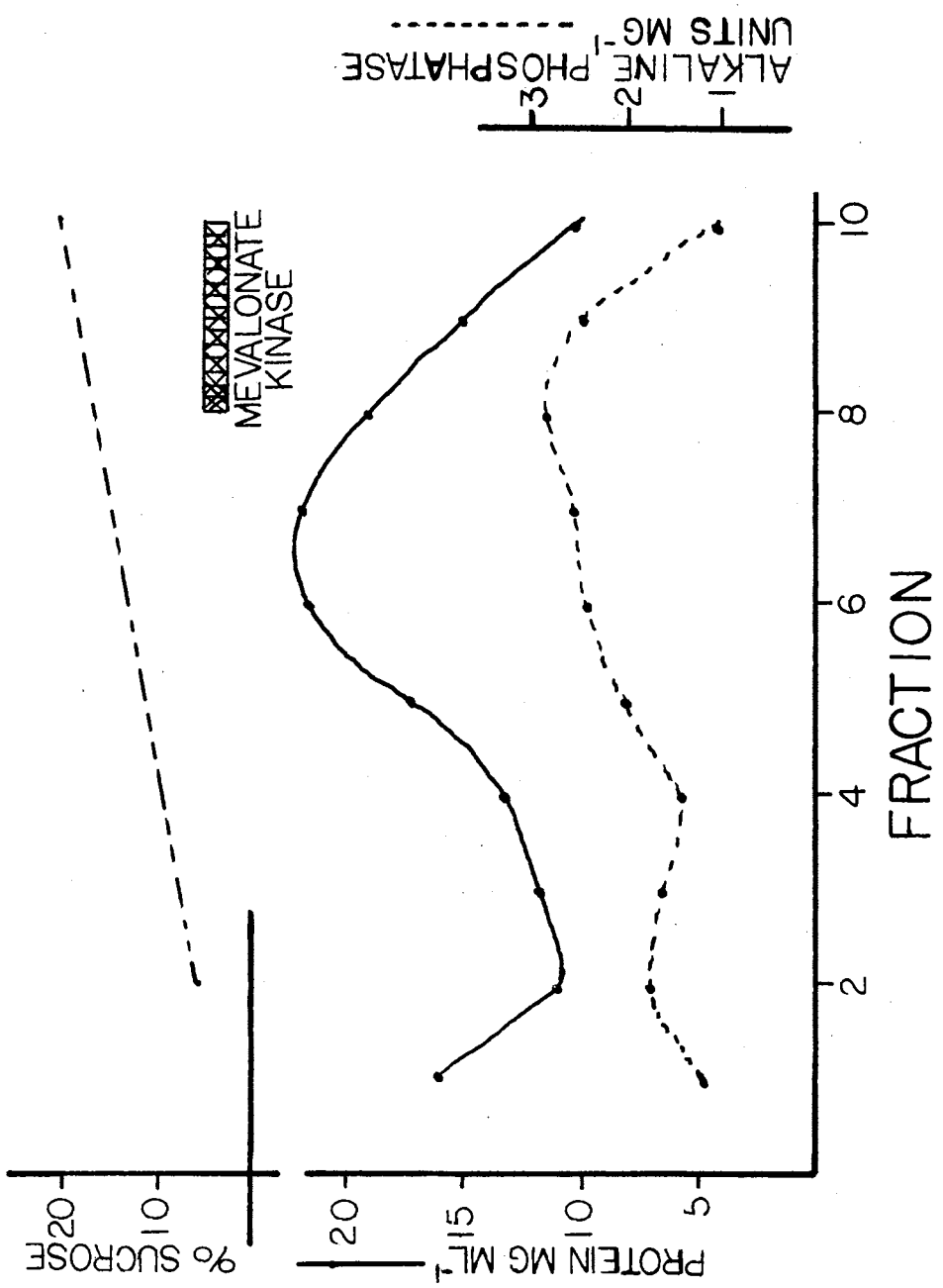
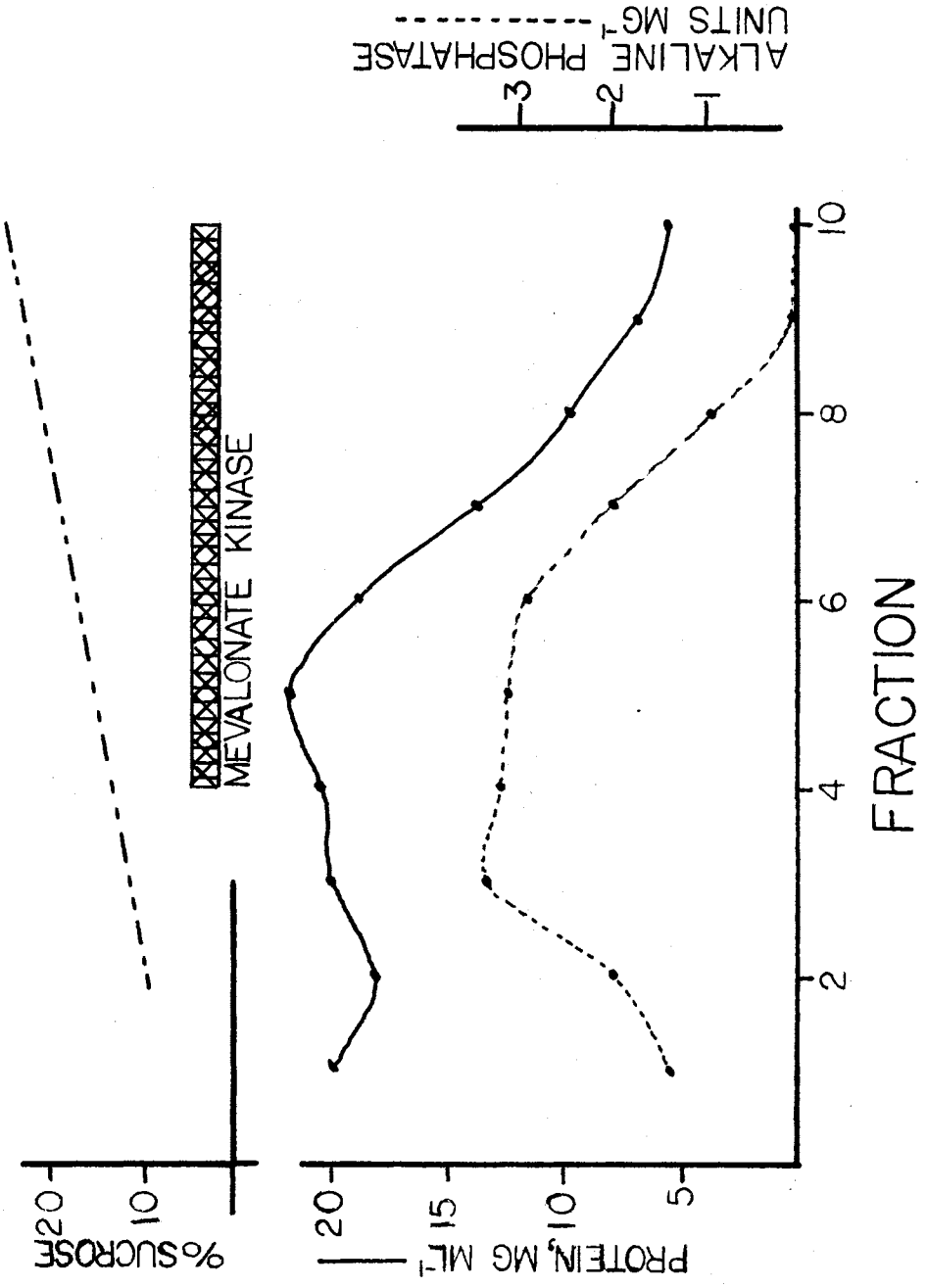

ALKALINE PHOSPHATASE UNITS MG⁻¹
3
2
1
MEVALONATE KINASE
% SUCROSE
20
10
PROTEIN MG ML⁻¹
20
15
10
5
2
4
6
8
10
FRACTION

Figure 25. Alkaline Phosphatase, Protein and Mevalonate Kinase Activity Profile From a Linear 10 to 25 Per Cent Sucrose Gradient

Mevalonate kinase activity was detected in fractions indicated by cross-hatched bar.


ALKALINE PHOSPHATASE UNITS MG⁻¹
3
2
1
PROTEIN, MG ML⁻¹
20
15
10
5
FRACTION
2
4
6
8
10
% SUCROSE
20
10
MEVALONATE KINASE

The pattern of mevalonate kinase activity at high concentrations of sucrose indicates the enzyme is of high molecular weight or perhaps bound to some cellular organelle or membrane.

Further purification of mevalonate kinase was attempted by fractionation with ammonium sulfate (107) and gel filtration chromatography. Ammonium sulfate was purified by recrystallization in 1 mM EDTA. Leaf tissue was homogenized in either phosphate or Tris homogenization buffer. Mevalonate kinase was purified 1.24 times with a yield of 64 per cent of total units from a phosphate homogenization system, as indicated in Table XI. The supernatant solution from a 25 per cent ammonium sulfate saturation contained the highest concentration of purified enzyme. The 50 per cent ammonium sulfate saturation pellet resulted in a loss of 84 per cent of total units and a 50 per cent decrease in specific activity. 5-phosphomevalonate kinase activity was also detected in this enzyme preparation as indicated by R_f values on paper chromatograms.

Use of a Tris homogenization buffer results in a different pattern of mevalonate metabolizing enzyme fractionation. Mevalonate kinase, 5-phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase activities were observed in the 30 to 70 per cent ammonium sulfate precipitate (see Figure 26 for a typical assay chromatogram scan). A yield of 21 per cent of the total units and a 1.5 fold purification was observed at the 30 to 70 per cent ammonium sulfate cut as seen in Table XII.

TABLE XI

MEVALONATE KINASE PURIFICATION FROM PHOSPHATE HOMOGENIZATION BUFFER

Step	Volume ml	Concentration Units/ml	Total Units	Protein Mg/ml	Specific Activity(a)	Yield	Purification
Crude Homogenate	63	241	15,215	11.8	20.5	100%	1.00
3K Supernatant	51	230	11,700	8.50	57.0	76%	1.31
30K Supernatant	46	225	10,350	10.0	42.5	68%	1.09
Ammonium Sulfate Fractionation - - - - - -							
25% Supernatant	47	209	9,830	8.20	25.5	64%	1.24
25% Pellet	5	0	0	4.90	0	0	--
50% Supernatant	44	0.023	1	4.70	0.005	6×10^{-3}	2×10^{-4}
50% Pellet	10	168	1,690	13.5	12.5	11%	0.60
75% Pellet	5	0	0	7.60	0	0	--

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

Figure 26. Typical Strip Scan of Assay Paper Chromatogram

Mevalonate kinase preparation from Bio-Gel P100 column assayed under standard conditions. The supernatant was developed in solvent system B as described in Table I.

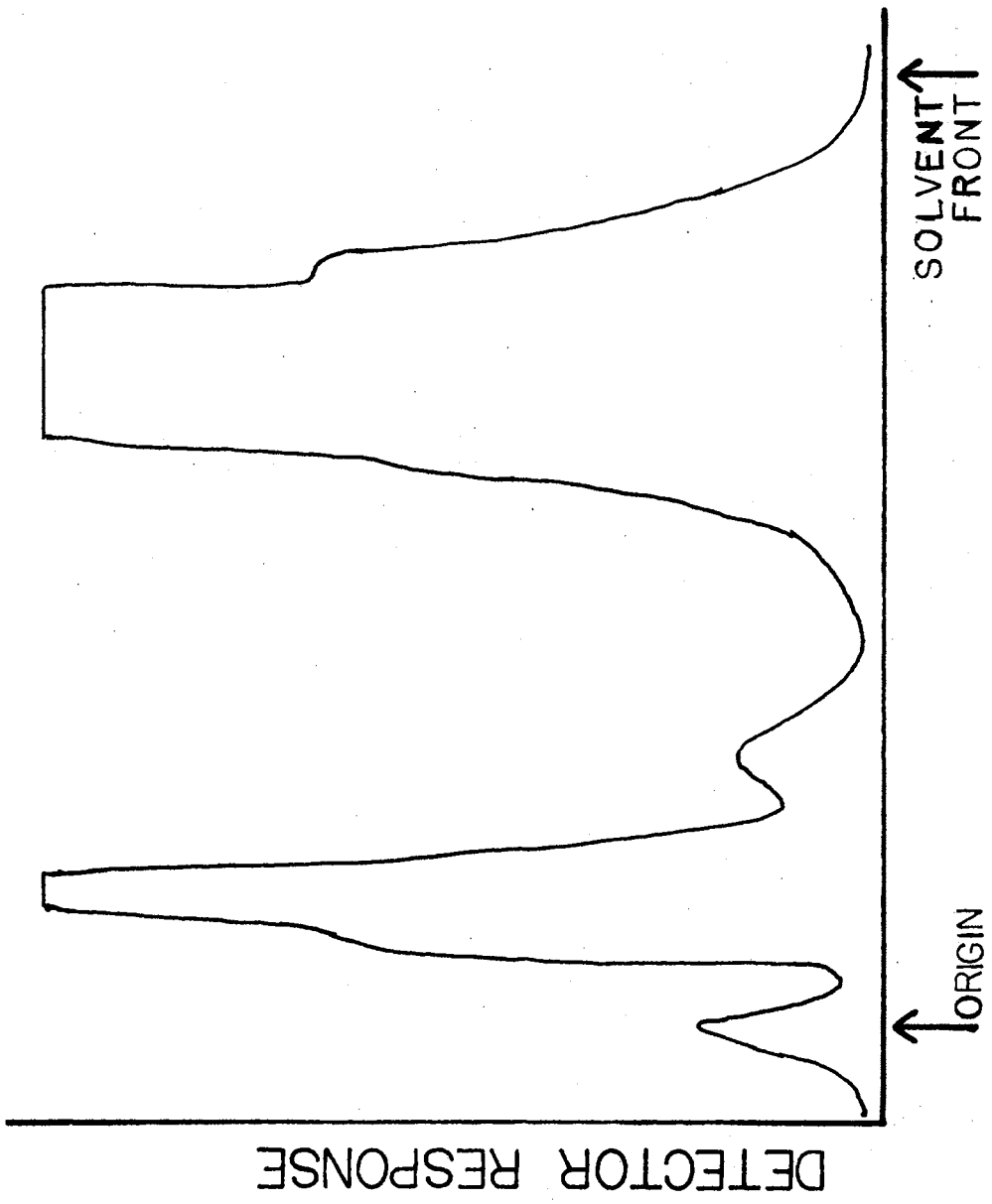

DETECTOR RESPONSE
ORIGIN
SOLVENT FRONT

TABLE XII

MEVALONATE KINASE PURIFICATION FROM TRIS HOMOGENATION BUFFER

Step	Volume ml	Concentration Units/ml	Total Units	Protein Mg/ml	Specific Activity(a)	Yield	Purification
Crude Homogenate	62	395	24,490	17.4	22.7	100%	1.00
3K Supernatant	55	439	24,160	15.8	27.8	98%	1.22
30K Supernatant	50	568	28,430	13.9	40.9	116%	1.80
100K Supernatant	46	469	21,580	9.40	49.9	88%	2.20
Ammonium Sulfate Fractionation - - - - - -							
30% Supernatant	44	380	16,730	9.70	39.2	68%	1.72
30% Pellet	6.5	0	0	2.16	0	0	--
70% Supernatant	43	21	900	5.70	3.7	3.6%	0.16
70% Pellet	11.3	471	5,330	13.4	35.2	21%	1.55
Bio-Gel P-100 column - - - - - - -							
Peak 1 Concentrated	11	409	4,615	6.90	59.4	18%	2.60
Peak 1 Concentrated and Dialized	15	255	3,830	5.20	49.2	15%	2.10

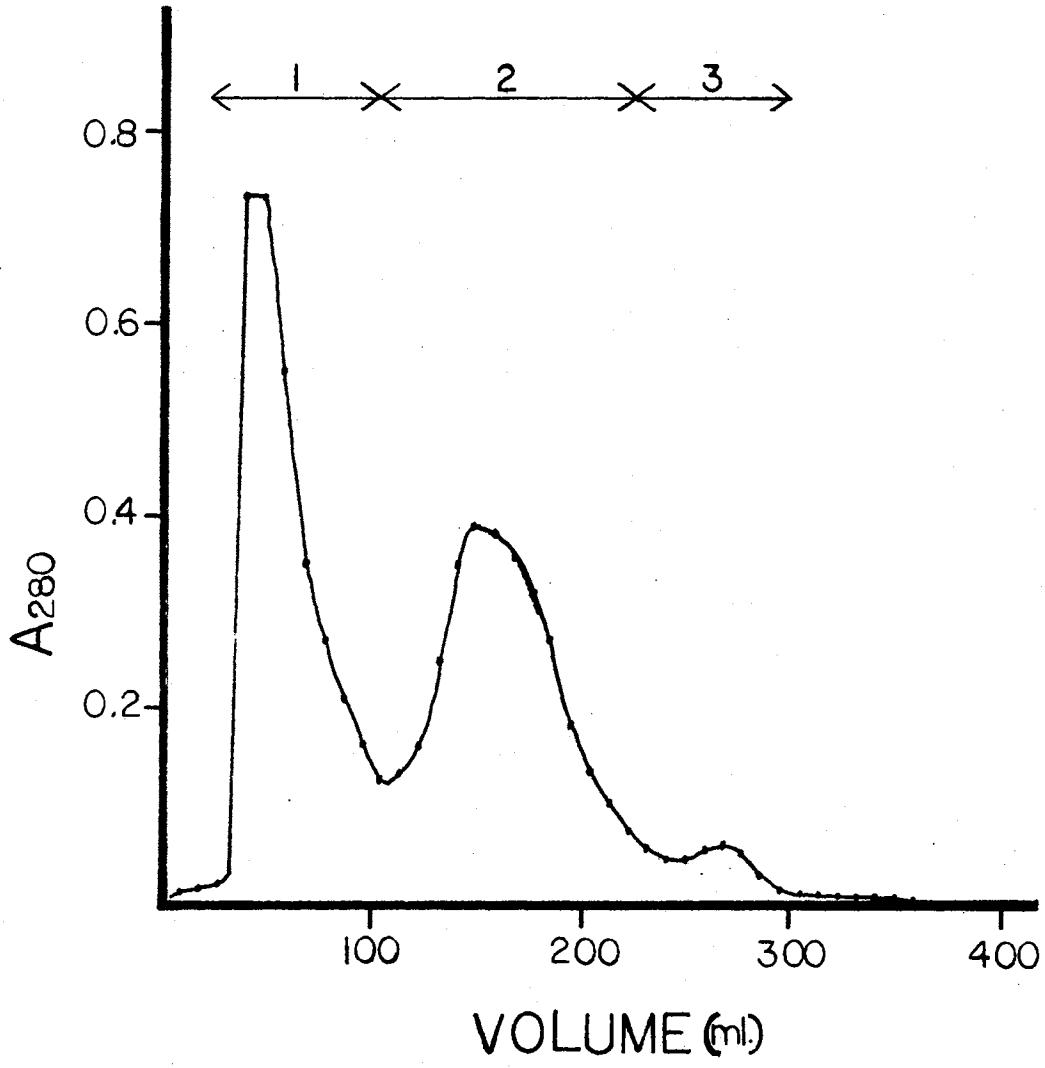
(a) Specific Activity Units are given in picomoles of mevalonate metabolized min^{-1} mg^{-1}

The 70 per cent ammonium sulfate pellet was dialyzed against 100 mM Tris pH 7.5, 5 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, 0.5 mM dithioerythritol and 0.1 mM mevalonate for 16 hours and applied to a Bio-Gel P100 column (2 x 40 cm) which was equilibrated and eluted with the same buffer. The elution profile is shown in Figure 27. Peaks 1 (void volume peak), 2 and 3 were pooled. Only peak 1 was found to have mevalonate kinase activity, it was concentrated over a XM-50 Diaflow Ultrafilter membrane. The above protocol resulted in an enzyme preparation which was purified 2.6 times as shown in Table XII.

Co-purification of mevalonate kinase, 5-phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase through ammonium sulfate fractionation and a Bio-Gel P100 column indicates that the enzymes are all physically similar and of high molecular weight or are associated in a multi-enzyme complex. The observation of high molecular weight is verified by detection of mevalonate kinase activity in the heavy fractions of sucrose density gradients. This suggestion of high molecular weight multi-enzyme complexes is consistent with the ideas expressed by Banthorpe (1) and Francis (2) that the enzymes involved in monoterpene biosynthesis may be associated in a multi-enzyme complex with a high degree of spatial organization for efficient substrate circulation. Gray and Kekwick (36) have reported the molecular weight of mevalonate kinase from a number of sources to be approximately 100,000 daltons. Since the mevalonate kinase activity was found to void a Bio-Gel P100 column, the molecular weight of the enzyme is in excess of 100,000 daltons.

Figure 27. Protein Elution Profile From Bio-Gel P100 Column

Bio-Gel P100 column (2 x 40 cm) was eluted as described in text. 5 ml fractions were collected and peaks 1, 2 and 3 were pooled as indicated by arrows.


1
2
3
0.8
0.6
0.4
0.2
A280
100
200
300
400
VOLUME (ml)

G. Attempted Affinity Chromatography

Mevalonate kinase in a crude enzyme preparation, when passed over an affinity column of mevalonate-agarose under appropriate conditions, should bind to the insolubilized mevalonate and be retarded in elution from the affinity column yielding a purified enzyme in one step.

Mevalonic acid -2-^{14}C was prepared from the dibenzylethylenediamine salt by the addition of 0.01 M Na_2CO_3, extraction of the base with ether and removal of the ether from the remaining aqueous phase under a gentle stream of nitrogen. Unlabeled mevalonate was added to give a specific activity of 0.05 microcuries per micromole.

R-S mevalonate, 200 micromoles (10 microcuries) was added to 10 ml (packed volume) of Bio-Gel AF102 and the pH was adjusted to 4.7 with HCl. 500 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl was dissolved in 5.0 ml water and added slowly to the mevalonate-agarose reaction mixture with gentle mixing.

The slurry was kept at room temperature, gently mixed, and the pH was kept at 4.5 by titration with NaOH at one, two, three, six and eight hours. After eight hours the pH remained constant. When the mixture had reacted for 24 hours the slurry was poured into a column and washed with 50 column volumes of water and 10 column volumes of 50 mM phosphate buffer, pH 7.2 containing 0.02% sodium azide. The column washes were pooled and brought to one liter with water, an aliquot was counted and found to contain 6×10^{-3} microcuries per ml. The last few drops of the phosphate buffer wash were collected and measured for radioactivity, none was detected. Fractions of the mevalonate-agarose gel were also counted and found to contain high levels of radioactivity. It was not possible to quantitate the amount

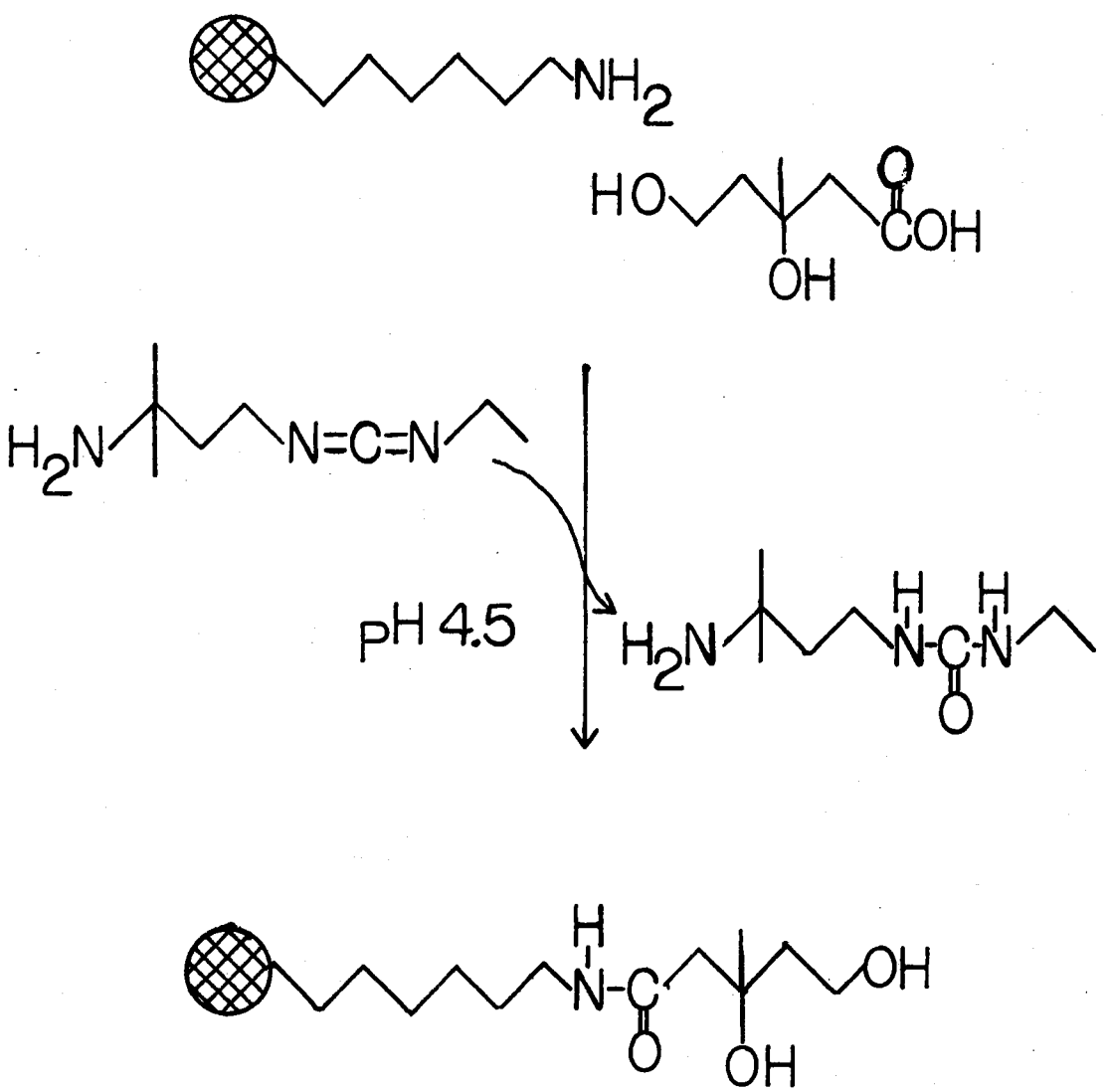
of ^{14}C coupled to the agarose by counting fractions of the gel due to difficulty of accurately determining the packed gel volume in the aliquots counted.

The original reaction mixture contained 10 microcuries of radioactivity. Six microcuries were recovered in the column washes which indicated that four microcuries of ^{14}C-mevalonate was coupled to the agarose by an amide bond as illustrated in Figure 28. Eight micromoles of R-S mevalonate was bound to each milliliter (packed volume) of agarose.

The mevalonate-agarose gel was divided into equal portions and two chromatography columns (0.8 x 10 cm) were prepared and stored in 50 mM phosphate buffer pH 7.2 containing 0.02% sodium azide at $4^{o}C$.

Two attempts at purification of mevalonate kinase over the affinity column were made, however, no success was achieved in the purification of the enzyme using the conditions employed. This technique may be a valuable tool for mevalonate kinase purification if the optimum conditions for enzyme binding and elution can be elucidated. No attempt was made, however, to follow this procedure for purification because of the limits of the scope of this dissertation.

Figure 28. Reaction of Aminoalkyl Agarose with Mevalonic Acid


NH2
HO
O
COH
OH
H2N
N=C=N
pH 4.5
H2N
H
N-C-N
H
O
H
N-C
O
OH
OH

H. Kinetic Studies

Kinetic studies of mevalonate kinase preparations were undertaken in order to determine the optimum concentration of substrates to be utilized in assays. An enzyme preparation from a leaf tissue homogenate was partially purified as described in Table XII. Protein eluting from a Bio-Gel P-100 column in the void volume was concentrated and dialyzed against 5 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA and 0.5 mM dithioerythritol in 100 mM pH 7.6 Tris buffer for 16 hours to remove mevalonate. The concentrated dialyzed protein fraction had a specific activity of 49.2 units/mg and was purified 2.1 times. Removal of mevalonate upon dialysis resulted in loss of specific activity as shown in Table XII, indicating a substrate stabilizing effect on the partially purified enzyme.

Stock solutions of mevalonate were mixed with 2-^{14}C-mevalonate to give specific activities of 0.138, 0.554, 1.08, 4.73 and 16.08 micromoles per microcurie. A 100 mM ATP solution was diluted to concentrations of 1.0, 5.0, 10 and 50 mM. Assays were incubated in a total volume of 0.1 ml at 30°C in the presence of 2-^{14}C-mevalonate and ATP at concentrations to give a 5 x 5 Lineweaver-Burk plot. Each assay included, in addition to ATP and mevalonate 0.26 mg of purified protein in the standard assay buffer. At the end of one hour the reactions were terminated, protein removed by centrifugation and the radioactive assay as described previously was performed. Velocity of the reaction was determined by calculating the number of picomoles mevalonate metabolized per minute. The results are presented as Lineweaver-Burk plots in Figures 29 and 30.

Figure 29. Lineweaver-Burk Plot of 1/Velocity vs. 1/Mevalonate Concentration

The assay system utilized was the standard system except for varying the concentrations of mevalonate and ATP.

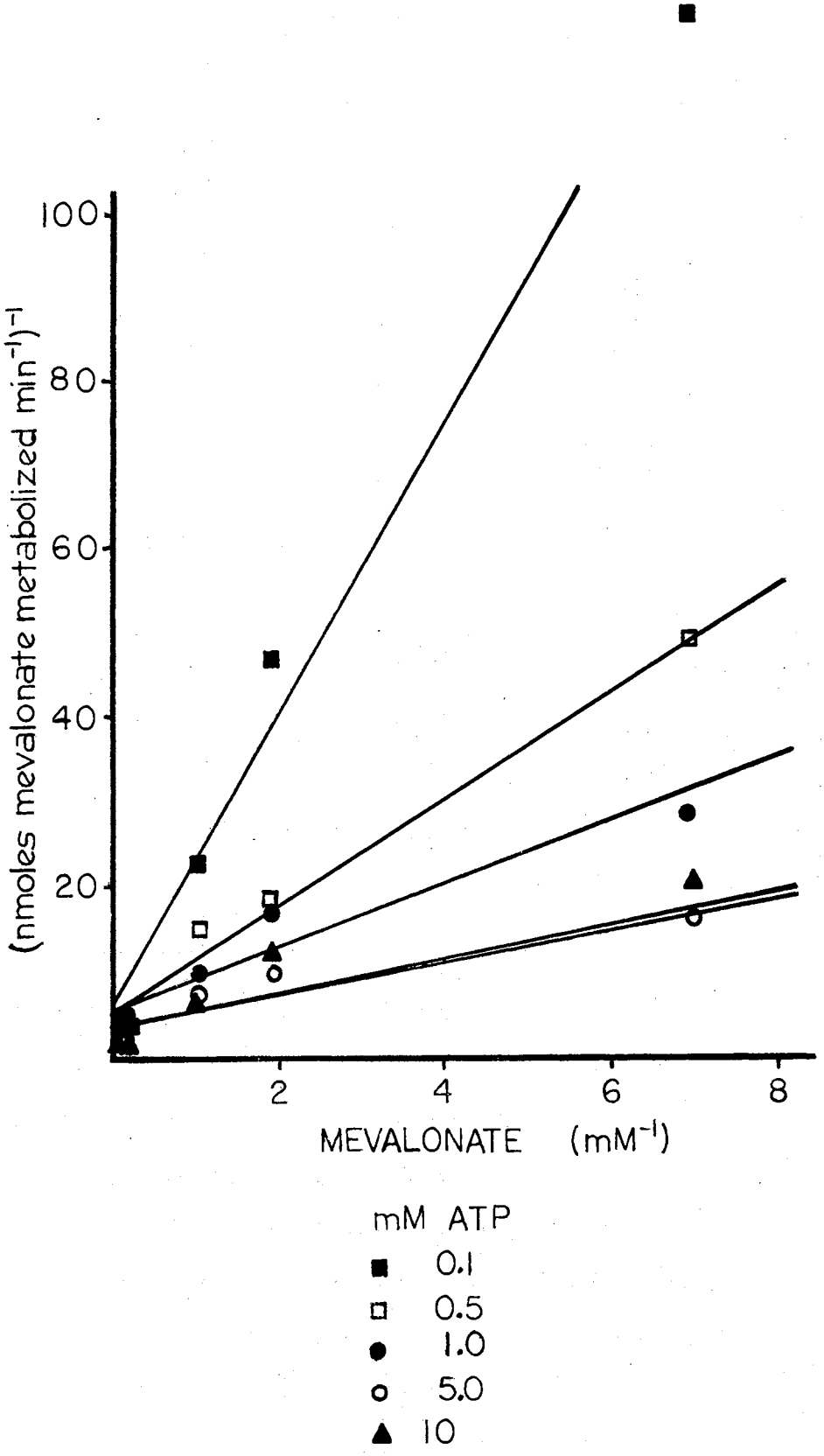
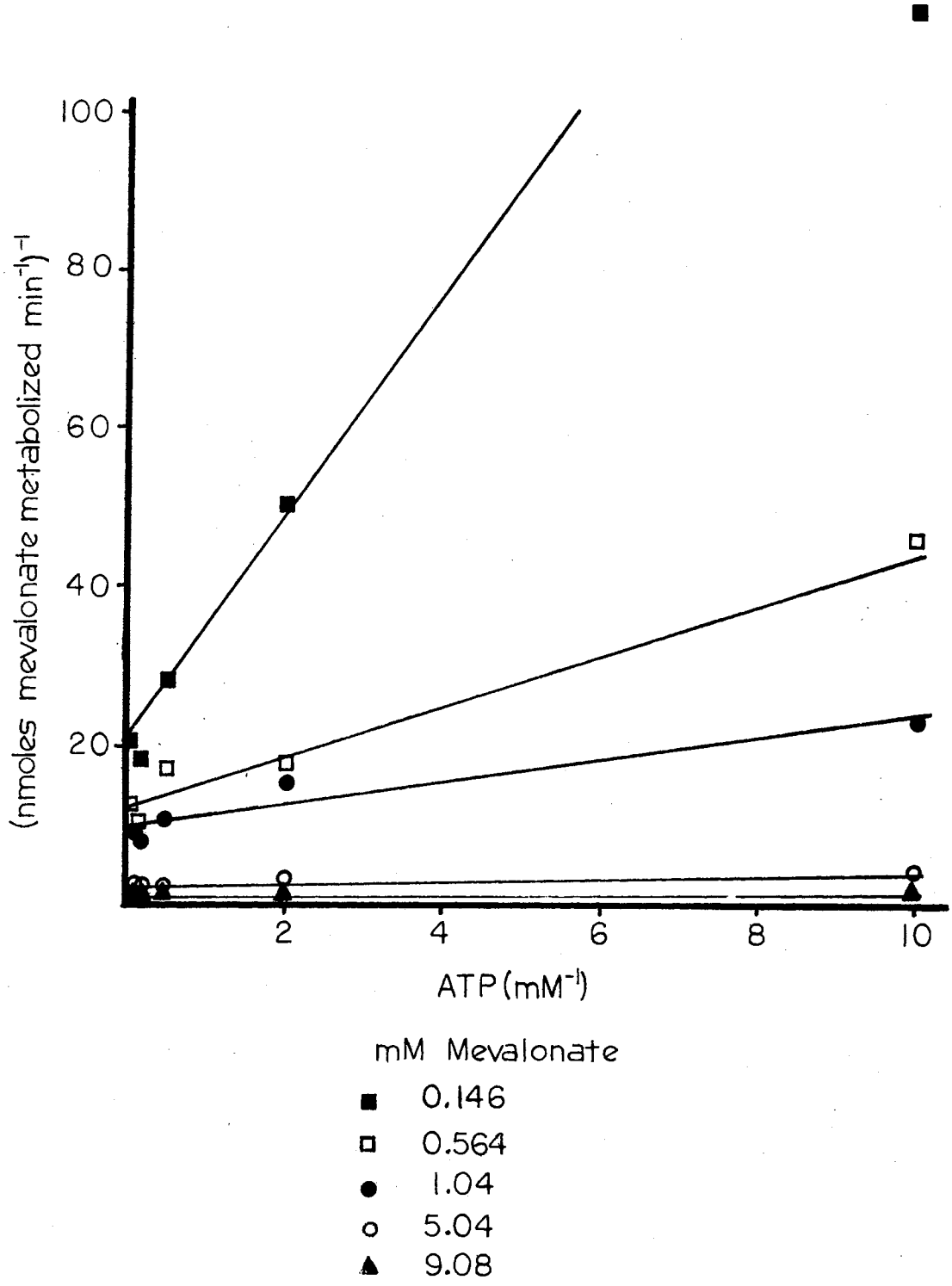

100
80
60
40
20
(nmoles mevalonate metabolized min⁻¹)⁻¹
2
4
6
8
MEVALONATE (mM⁻¹)
mM ATP
0.1
0.5
1.0
5.0
10

Figure 30. Lineweaver-Burk Plot of 1/Velocity vs. 1/ATP Concentration

The assay system utilized was the standard system except for varying the concentrations of mevalonate and ATP.


100
80
60
40
20
(nmoles mevalonate metabolized min⁻¹)⁻¹
2
4
6
8
10
ATP (mM⁻¹)
mM Mevalonate
0.146
0.564
1.04
5.04
9.08

Secondary plots of intercepts vs. the reciprocal of substrate concentrations are shown in Figures 31 and 32.

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

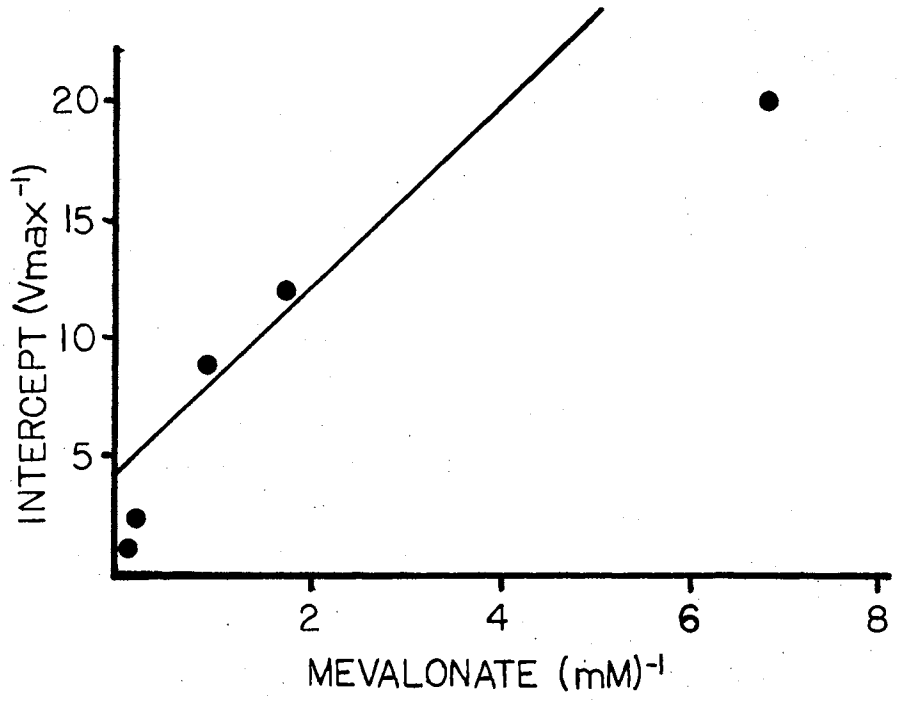
The apparent Km for ATP as calculated from the Lineweaver-Burk plot shown in Figure 30 is 1 to 2 mM. The secondary plot, Figure 32, however, indicates an apparent Km less than 1 mM. R-S Mevalonate concentrations of 1 to 2 mM were indicated in Figure 29 to be the apparent Km range. From Figure 31, a value of 1.42 mM is calculated as the apparent Km for R-S mevalonate.

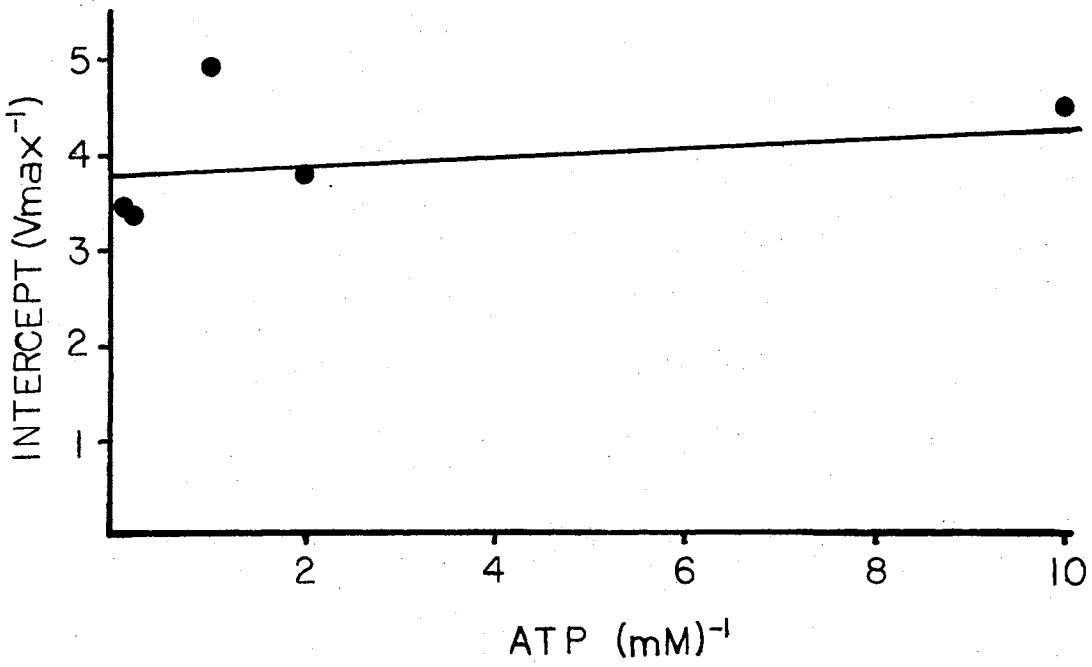
The enzyme preparation utilized was a mixture of mevalonate kinase, 5-phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase, each enzyme requiring ATP and each requiring the product of the preceeding enzyme as substrate. The crudeness of the enzyme preparation and the number of enzyme metabolizing mevalonate, products of mevalonate and ATP will result in the scattered data points observed in Figures 29 through 32. However, in this crude system, the data obtained is very important in that it gives apparent Km values that are useful in optimizing assay conditions.

Substrate inhibition of mevalonate kinase has been reported at high ATP concentrations (24, 31, 33). In this study, ATP substrate inhibition was observed at 10 mM ATP (Figure 30). This information led to a modification of assay conditions in the presence of ATP. The apparent Km value of 1.42 mM for R-S mevalonate falls within the range of reported values (i.e., from 0.13 mM in rubber latex (26) to 1.7 mM in the bean system (30)). The value of less than 1 mM as a Km for ATP is not unreasonable when compared to the range of Km values

Figure 31. Secondary Plot of Intercept vs. 1/Mevalonate Concentration

Figure 32. Secondary Plot of Intercept vs. 1/ATP Concentration


20
15
10
5
INTERCEPT (Vmax^{-1})
2
4
6
8
MEVALONATE (mM)$^{-1}$


5
4
3
2
1
INTERCEPT (Vmax^{-1})
2
4
6
8
10
ATP (mM)$^{-1}$

that have been reported; for example, 2 mM in rubber latex (26) to 0.042 mM in preparations from green bean (30).

I. pH Optimum

The pH optimum of mevalonate kinase from cell-free preparations is of interest because of reported differences in the pH optimum of chloroplastic and extra-chloroplastic enzymes (27).

Preparation of leaf and callus cell-free extracts was as described previously utilizing a Tris homogenization buffer including 0.1 mM mevalonate. The 100K supernatant was dialyzed against 10 mM KCl, 5 mM $MgCl_2$, 5 mM dithioerythritol, 1 mM EDTA and 0.1 mM mevalonate in 10 mM Tris buffer pH 7.0 overnight. The chloroplastic enzyme was prepared at $4^{\circ}C$ by homogenizing leaf tissue for three minutes in a Waring blender with the same homogenization buffer used for cell-free preparation and 10 per cent (w/w) Polyclar AT. The homogenate was filtered through six layers of cheesecloth and the exudate was centrifuged as described in Figure 33. The final washed pellet was suspended in 3.0 ml of a buffer identical to the dialyzing buffer, described above for cell-free preparations, and homogenized for two minutes at full setting in a Polytron homogenizer sonicator.

Fifty microliters of extract from leaf cell-free, callus cell-free and the chloroplastic preparation were incubated in a total volume of 0.2 ml at $30^{\circ}C$ with shaking. Each incubation contained 100 mM buffer as described in Table XIII, 5 mM ATP, 0.125 mM mevalonate (0.18 microcuries) in the usual radioactive incubation assay. At the end of one hour incubations were terminated, and the radioactive assay was carried out as described.

Figure 33. Flow Chart for Preparation of Chloroplastic Enzyme System

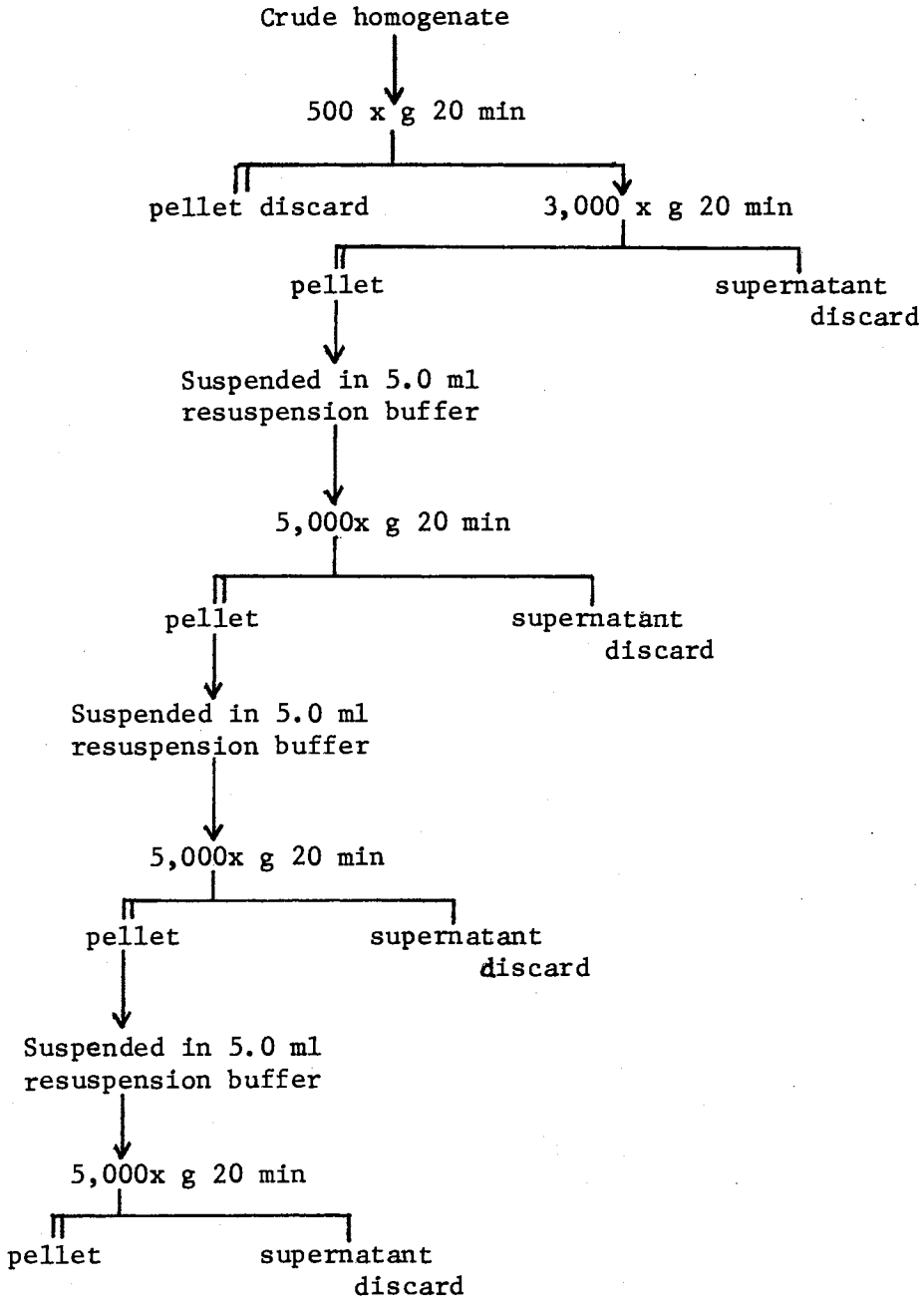

Crude homogenate
500 x g 20 min
pellet discard
3,000 x g 20 min
pellet
supernatant discard
Suspended in 5.0 ml resuspension buffer
5,000x g 20 min
pellet
supernatant discard
Suspended in 5.0 ml resuspension buffer
5,000x g 20 min
pellet
supernatant discard
Suspended in 5.0 ml resuspension buffer
5,000x g 20 min
pellet
supernatant discard

TABLE XIII

pH OF INCUBATION BUFFERS

Buffer	pH						
Acetate	3.35	4.0	4.5	4.75	5.1		
Malate	5.0	5.25	5.5	5.8	6.0	6.4	6.6
Phosphate	6.6	6.8	7.0	7.1	7.5	7.7	8.0
Tris	7.7	7.75	8.3	8.8	9.0		

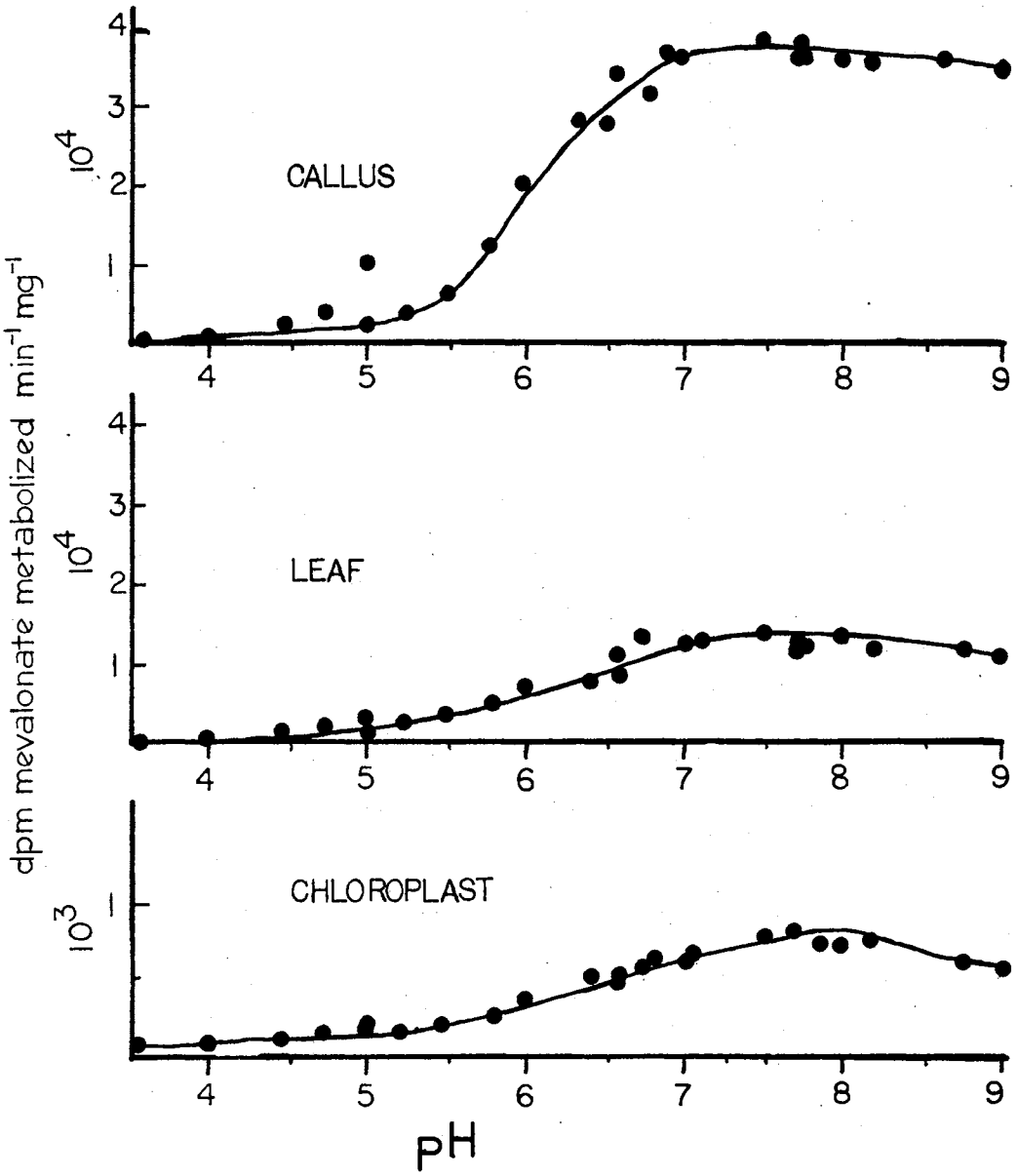
The results of the pH profile are shown in Figure 34. The callus cell-free system from the eleventh subculture on SH media has very high activity in that nearly 2.3 nanomoles of mevalonate are metabolized per min per mg of protein at pH 7.5. The leaf system is also active, but with only one-third the specific activity as the callus enzyme preparation. Chloroplastic activity is very low with a specific activity of less than ten per cent of the leaf system throughout the entire spectrum of pH.

All preparations show a wide pH optimum range from pH 6.5 to pH 9. The chloroplastic system has an apparent optimum at pH 8.0 where nearly constant activity is seen from pH 7 to 9 in leaf and callus cell-free systems.

There was no loss of radioactivity due to volatilization of free alcohols as determined by recovery of 96 per cent to 98 per cent of the total radioactivity.

Figure 34. pH Activity Profile for Callus, Leaf and Chloroplastic Enzyme Preparations

The assay conditions were standard conditions except for the incubation buffers which are described in Table XIII.


dpm mevalonate metabolized min⁻¹ mg⁻¹
CALLUS
LEAF
CHLOROPLAST
10⁴
10³
1
2
3
4
5
6
7
8
9
pH

It has been reported by Rogers et al. (27), that the chloroplastic pool of mevalonate activating enzymes has a pH optimum of 7.5 and the extra-chloroplastic pool has an optimum at pH 5.5. No sharp peak of optimum pH activity is observed in any of the three enzyme preparations studied from N. cataria tissue. A pH profile of a 30 per cent to 70 per cent ammonium sulfate precipitate from leaf tissue exhibited the same pH profile observed for the 100K supernatant enzyme system from leaf tissue shown in Figure 34.

It was anticipated that the chloroplastic enzyme preparation would have a single pH optimum activity, the callus preparation another peak and the leaf preparation would have both peaks of activity. The absence of two different pH optima of mevalonate activating activity would indicate that if there are two pools of mevalonate activating enzymes in N. cataria tissue both are optimally active at alkaline pH.

This is consistent with the study by Gray and Kekwick (30), who report etiolated and green leaf preparations of mevalonate kinase having identical pH profiles with an optimum at pH 7.0. This also suggests that perhaps the two enzymes are indistinguishable. The specific activity of the different enzyme sources is drastically different with approximately one nanomole of mevalonate being metabolized per min per mg of protein in the leaf cell-free preparation. This level of activity is one thousand fold higher than the specific activity reported by Rogers et al. (27). Differences in specific activities of the two mevalonate kinase enzyme preparations could be due to removal of the product 5-phosphomevalonate by 5-phosphomevalonate kinase which is also present in the crude N. cataria system. An alternative explanation for the relatively high specific activity

of N. cataria mevalonate activating enzymes could be due to the extra-chloroplastic enzyme being required for synthesis of essential oils such as nepetalactone.

N. cataria callus tissue does not produce nepetalactone, however, 0.13 per cent of the fresh weight of callus can be recovered as hexane soluble non-volatile oils, the yield of crude oil in steam distillates of N. cataria leaf tissue ranges from 0.06 to 0.1 per cent (20). It is probable that the high activity of mevalonate activating enzymes in N. cataria callus tissue is required for the production of essential oils some of which give the odor of freshly cut watermelon to the callus tissue.

J. Metal Requirement

Supernatant enzyme preparations from a 100K centrifugation of leaf and callus homogenizations were dialyzed overnight against 100 mM Tris buffer of pH 7.5 containing 5 mM dithioerythritol, 5 mM EDTA and 0.1 mM mevalonate. This was done to remove metal ions present from the homogenization buffer. The radioactive incubation assay was carried out in a 0.1 ml total volume containing 5 mM ATP and 0.125 mM 2-^{14}C-mevalonate (0.18 microcuries) in 100 mM Tris pH 7.5 buffer and 0.1, 1.0, 5.0 and 10.0 mM of the appropriate metal ion.

The results are shown in Figure 35 for the leaf tissue enzyme and in Figure 36 for the callus tissue assay. Mg^{++} is the divalent cation that best supports phosphorylation of mevalonate. At low concentrations Mn^{++} enhances activity, but above 5 mM is found to be inhibitory. Zn^{++} activates at a level of less than 7 per cent of the maximum observed for Mg^{++} and ferric ions supported phosphorylation

Figure 35. Metal Ion Activation Profile of the Leaf Tissue Enzyme Preparation

Standard assay conditions were utilized except for the substitution of the cation as indicated.

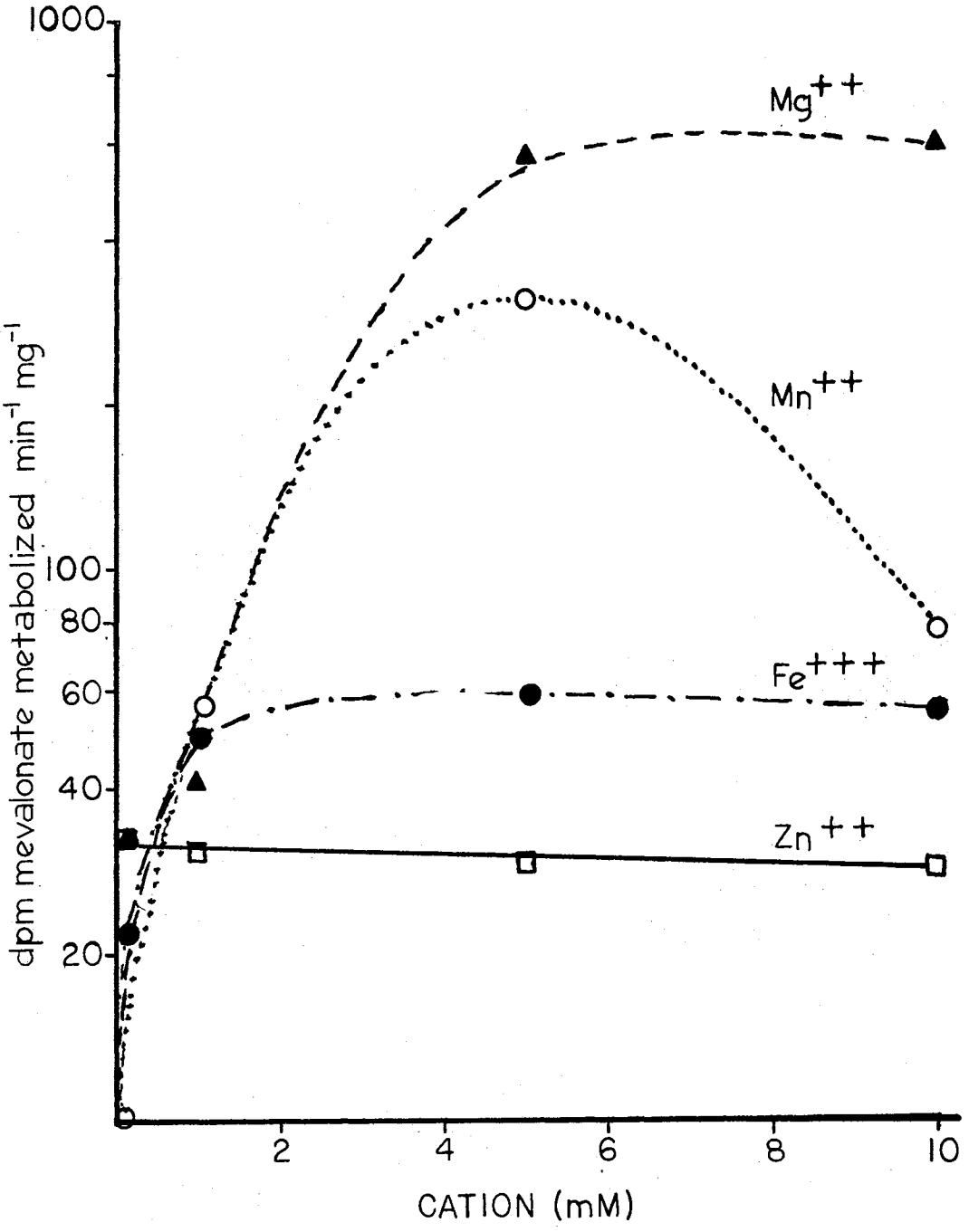
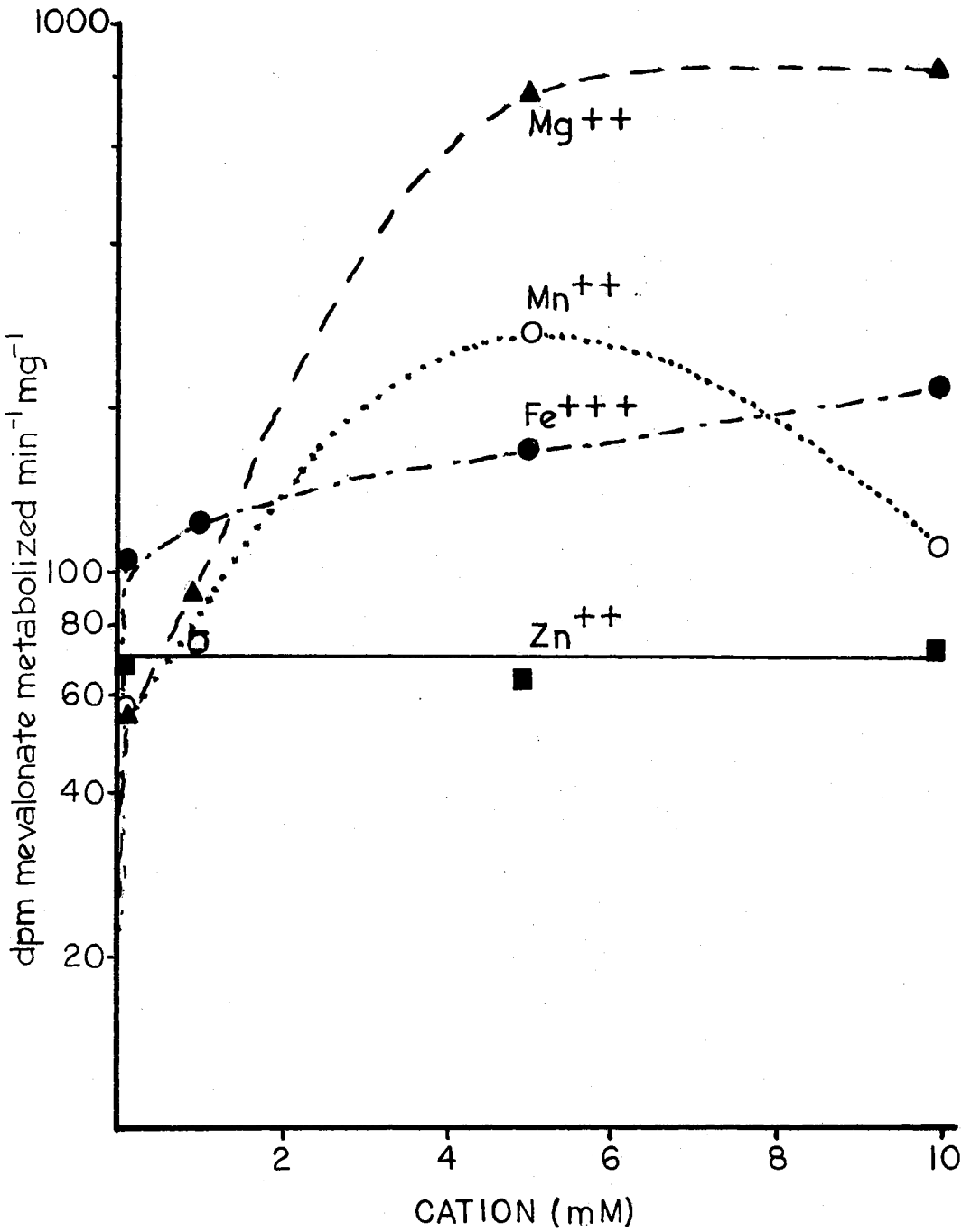

1000
100
80
60
40
20
dpm mevalonate metabolized min⁻¹ mg⁻¹
Mg++
Mn++
Fe+++
Zn++
2
4
6
8
10
CATION (mM)

Figure 36. Metal Ion Activation Profile of the Callus Tissue Enzyme Preparation

Standard assay conditions were utilized except for the substitution of the cation as indicated.


1000
100
80
60
40
20
dpm mevalonate metabolized min⁻¹ mg⁻¹
Mg++
Mn++
Fe+++
Zn++
2
4
6
8
10
CATION (mM)

less than six per cent of the maximum activity observed for Mg^{++} in the leaf assay. In the callus assay, ferric ion was observed to increase phosphorylation of mevalonate over the entire range of concentrations studied.

Low levels of phosphorylation at less than 5 mM cation may be due to insufficient cation to complex all of the ATP present in the assay solution.

Yeast mevalonate kinase, studied by Tchen (24), is activated by Mg^{++}, Mn^{++}, Co^{++}, Ca^{++}, Zn^{++}, and Fe^{++} and is inhibited at high concentrations of Mn^{++}. Loomis and Battaile (25) reported that mevalonate kinase from pumpkin fruit is activated by Mg^{++}, Mn^{++}, Fe^{++}, Co^{++}, Ca^{++} and Zn^{++}. High levels of Mn^{++} inhibited the pumpkin enzyme. Williamson and Kekwick (26) reported that the mevalonate kinase from rubber latex is activated at 1 mM Mg^{++} and 5 mM Mn^{++} and is inhibited by concentrations of either cation above 10 mM. N. cataria cell-free preparations of mevalonate kinase have the same metal requirement demonstrated for the enzyme from other plant sources, however, Mg^{++} was not found to be inhibitory at 10 mM as was observed for Mn^{++}.

K. Nucleotide Requirement

The nucleotide requirement of mevalonate activating enzyme in cell-free preparations of N. cataria tissue was investigated. A 30K supernatant enzyme preparation from leaf and callus tissue was assayed with 1, 5 and 10 mM concentrations of various nucleotides.

Table XIV illustrates the activity of the enzyme preparations with various nucleotides. A control experiment without nucleotide resulted in low levels of activity (152 dpm/mg/min in leaf assays and

654 dpm/mg/min in callus assays) which was subtracted from the specific activity obtained for each assay (Table XIV). High levels of mevalonate phosphorylation are observed using ATP and ADP as nucleotides in the leaf cell-free system, however, the callus cell-free systems show an apparent lack of specificity for nucleotide in that high levels of mevalonate phosphorylation were observed with all nucleotides studied. The lowest specific activity observed in the leaf extracts, in the presence of 5 mM TTP, is only two per cent of the maximal activity observed when 10 mM ATP is used. In the callus system the low value is seven per cent of the maximal activity.

TABLE XIV

NUCLEOTIDE REQUIREMENT

Nucleotide	mM Concentration	Leaf*	Callus*
ATP	1	1,222	15,925
	5	3,333	16,358
	10	3,720	18,271
ADP	1	462	5,839
	5	1,641	11,506
	10	2,047	11,691
CTP	1	210	1,493
	5	193	3,518
	10	310	3,617
UTP	1	145	1,271
	5	275	3,950
	10	170	3,888
GTP	1	120	1,753
	5	196	5,802
	10	233	6,728
TTP	1	96	1,790
	5	93	2,506
	10	173	2,444

*dpm mevalonate metabolized/mg/min

Tchen (24) and Battaile (25) reported that GTP, CTP, UTP, ATP and TTP act as phosphate donors for mevalonate kinase from yeast and pumpkin. N. cataria leaf enzyme preparations do utilize nucleotides other than ATP and ADP, but only at low levels. A possible explanation for the utilization of ADP could be the presence of adenylate kinase in the crude enzyme preparation which would produce ATP for the phosphorylation of mevalonate. Utilization of the other triphosphonucleotides by the mevalonate metabolizing enzymes could be result of phosphotransferases present in the crude enzyme system producing ATP, from endogenous ADP, for the phosphorylation of mevalonate. The callus enzyme preparations react more like those observed from pumpkin and yeast in that all nucleotides studied supported phosphorylation of mevalonate. Callus enzyme preparations were, in general, up to five times more active than leaf assays.

CHAPTER V

SUMMARY

^{14}C-nepetalactone from N. cataria seedlings was observed after three minutes of photosynthesis in the presence of $^{14}CO_2$, the lag time suggests an extra-chloroplastic site of essential oil synthesis. Radioactivity was observed in glyceric acid after six seconds of exposure of seedlings to $^{14}CO_2$ indicating that N. cataria plants are C_3 plants.

Leaf and stem tissue from N. cataria plants has been successfully propagated as masses of undifferentiated cells on defined media or as small clumps and single cells in liquid suspension culture. Callus tissue was not observed to produce nepetalactone, however, a n-hexane soluble oil was obtained. The odor of essential oils from whole N. cataria plants was not present in the callus tissue although the callus did smell like freshly cut watermelon.

High ionic strength buffers, including sulfhydryl reagents, were required for the preparation of an active cell-free enzyme system. Mevalonate kinase, 5-phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase activities were detected in all crude cell-free and partially purified enzyme preparations.

Mevalonate metabolism in leaf and callus enzyme preparations was observed to be basically similar; however, in activity measurements from callus tissue very high levels of isopentenyl pyrophosphate were

observed. Isopentenyl monophosphate was detected in enzyme assays from both leaf and callus tissue, whether it is a product of decarboxylation of 5-phosphomevalonate or nonspecific enzymatic hydrolysis of isopentenyl pyrophosphate remains to be determined.

Mevalonate kinase, 5-phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase activities were all observed in all partially purified enzyme preparations. Detection of mevalonate activating enzymes in the heavy end of linear sucrose gradients and in the void volume of Bio-Gel P100 columns suggests a molecular weight of the enzymes or enzyme complex in excess of 100,000 daltons.

Kinetic studies with a partially purified enzyme preparation indicated apparent Km values of 1.5 mM for R-S mevalonate and less than 1 mM for ATP. A broad pH optimum range from 6.5 to 9.0 was observed for mevalonate phosphorylation from callus, leaf and chloroplastic enzyme preparations indicating that all three preparations have the same enzyme(s).

The metal requirement of the enzyme preparation is satisfied by Mg^{++}. Mn^{++} activates at low concentrations but inhibits at levels above 5 mM. Zn^{++} and Fe^{+++} also support phosphorylation of mevalonate but at levels less than 13 per cent the maximum observed for Mg^{++}. Leaf enzyme preparations were observed to require ATP or ADP for phosphorylation of mevalonate. ATP, ADP, CTP, GTP, TTP and UTP were all observed to support phosphorylation of mevalonate in enzyme preparations from callus tissue.

Callus enzyme preparations were observed to have specific activities more than three times higher than leaf enzyme preparations and 30 times higher than chloroplastic enzyme preparations.

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