

AN ABSTRACT OF THE THESIS OF

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Title: CHANGES IN ENZYME ACTIVITIES OF THE ISOPRENOID

PATHWAY IN GERMINATING PISUM SATIVUM

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Dt/ Derek J. Baisted (D.J.B.)

The nature of the isoprenoid pathway and diversity of biologically significant products suggests that the pathway may be highly regulated, particularly under stress conditions, where the availability of high energy intermediates is limited. It was postulated that germinating seeds suffer from a limited supply of available energy and must therefore conserve energy wherever possible. This assumption should be reflected through enzymic changes in specific isoprenoid enzymes of the pathway, correlated with the developmental requirements for growth and differentiation.

An in vivo analysis of nonsaponifiable products formed from mevalonate-2-¹⁴C versus germination in Pisum sativum confirmed that β -amyrin (triterpene) and β -sitosterol (sterol) are sequentially synthesized at different rates, depending upon the germination stage. Kinetic transitions between squalene, squalene-triterpene, and

squalene-triterpene/squalene-sterol formation are discussed in relation to the metabolic steps required for either triterpene or sterol biosynthesis. Metabolically, either product must arise from 2,3-oxidosqualene. Changes in triterpene and sterol formation as a function of germination, however, suggest that the enzymes responsible for such synthesis do not compete for oxidosqualene, but for squalene. A regulatory model based on these observations is proposed. Squalene epoxidase is postulated to combine with specific cyclases as an enzyme aggregate. As an aggregate, the enzyme complex competes for squalene to make the appropriate cyclized product. Regulation and selection of product formation is controlled through association and dissociation of the aggregate.

Methods for the biosynthesis and isolation of phosphomevalonate-2-¹⁴C, isopentenyl-4-¹⁴C pyrophosphate and farnesyl-4, 8, 12-¹⁴C pyrophosphate from mevalonate-2-¹⁴C in yeast autolysates, prepared from commercial dry baker's yeast, are described in detail. Comparisons between various biosynthetic methods have been made. Methods of identification, isolation technics and criteria of purity are also discussed; those methods most suitable for the isolation and identification of each isoprenoid intermediate have been emphasized.

Individual assays of mevalonic kinase, phosphomevalonic kinase, pyrophosphomevalonic decarboxylase, isopentenyl

pyrophosphate isomerase, isopentenyl pyrophosphate/dimethylallyl pyrophosphate prenyltransferase and squalene synthetase in 40,000 g supernatants of Pisum sativum, prepared from germinating seeds at selected intervals after the onset of imbibition, suggest that the enzymes are regulated in such a manner as to always favor isopentenyl pyrophosphate and dimethylallyl pyrophosphate formation. The enzymes are activated in such proportions as to favor nonequilibrium conditions between mevalonic acid and isopentenyl pyrophosphate, and equilibrium conditions between isopentenyl pyrophosphate and dimethylallyl pyrophosphate, thereby insuring the most efficient synthesis of higher isoprenoid products at all times. A reversal of carbon flow from dimethylallyl pyrophosphate to isopentenyl pyrophosphate appears highly probable as a means of maintaining a sufficient supply of isopentenyl pyrophosphate to meet the new biosynthetic requirements induced by development. Dimethylallyl pyrophosphate may serve as a storage pool, to meet biosynthetic demands in times of stress, where the endogenous supply of mevalonic acid is limited.

Isopentenyl pyrophosphate/dimethylallyl pyrophosphate prenyltransferase was assayed indirectly in 40,000 g supernatants with mevalonate-2-¹⁴C and more directly with isopentenyl-4-¹⁴C pyrophosphate. Under identical conditions in the preparation of 40,000 g supernatants, the former assay yielded an estimated activity some 15-20 fold greater than by isopentenyl-4-¹⁴C pyrophosphate. These

results suggest that the prenyltransferase is allosterically activated by one, or more, of the first three isoprenoid intermediates (i. e. , mevalonate, phosphomevalonate, and/or pyrophosphomevalonate).

Indirect evidence was also obtained for the presence of two distinct isopentenyl pyrophosphate/dimethylallyl pyrophosphate prenyl transferases, tentatively identified as enzymes I and II. Detection of prenyltransferase I is independent of sucrose, while detection of prenyltransferase II is highly dependent upon the inclusion of sucrose (0.45 M) in the homogenizing buffer. The two prenyltransferases are activated sequentially during the course of germination. Prenyltransferase I is postulated to be associated with the cytoplasm, while prenyltransferase II may be associated with an organelle, possibly with immature chloroplasts (protoplasts) or mitochondria. Enzymic changes in the activities of prenyltransferases I and II appear closely correlated with developmental changes in the seed initiated with the breaking of dormancy.

A thermolabile, large molecular weight factor has been tentatively identified as a specific inhibitor of isopentenyl pyrophosphate/dimethylallyl pyrophosphate prenyltransferase. This factor is temporal, appearing at later stages of germination. The action of this factor as a means of diverting the flow of carbon between cytoplasmic and organelle associated pathways is discussed. Suppression of prenyltransferase I activity by this factor, coincident with the rise in

prenyltransferase II activity, may suggest that compartmentalized isoprenoid pathways are not completely autonomous, but are suppressed or activated to meet the needs of their respective environments, thereby conserving considerable energy and substrate.

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Changes in Enzyme Activities of the Isoprenoid
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CHANGES IN ENZYME ACTIVITIES OF THE ISOPRENOID PATHWAY IN GERMINATING PISUM SATIVUM

GENERAL INTRODUCTION

Isoprenoid Products Arising from Mevalonic Acid

Mevalonic acid (MVA) is the precursor of numerous isoprenoid type products in animals, plants and microorganisms (20). Implicated in the biosynthesis of these compounds are several phosphorylated intermediates arising from MVA: 5-phosphomevalonate (MVAP) (13, 159), 5-pyrophosphomevalonate (MVAPP) (13), Δ^3 -isopentenyl pyrophosphate (IPP) (13), γ, γ -dimethylallyl pyrophosphate (DMAPP) (1, 11), geranyl pyrophosphate (GPP) (111), farnesyl pyrophosphate (FPP) (111) and geranylgeranyl pyrophosphate (GGPP) (93), in the order that they are formed from MVA. Further isopentenylation can lead to higher acyclic terpenoids, while further condensations and cyclizations of these intermediates can lead to a great variety of terpenoids and also sterols (20). The biosynthesis of MVA, isoprenoid intermediates and higher products have been extensively reviewed (12, 45, 59, 62, 65, 99, 122, 134, 137, 177). With reference to higher plants, these products are frequently classified as follows: monoterpenes (C-10), sesquiterpenes (C-15), diterpenes (C-20), triterpenes

(C-30), tetraterpenes (C-40) and mixed terpenoids. The latter products are found primarily in the chloroplasts and mitochondria as isoprenoid adjuncts to preformed compounds of different origin. The three most common classes of mixed terpenoids are the chlorophylls (phytyl side chain), plastoquinones (C-45 side chain) and ubiquinones (C-50 side chains). Chemical structures for a number of common isoprenoid products of P. sativum are shown in Figure 1.

Biochemical Steps Between MVA and IPP

Phosphomevalonate was shown by Tchen (159) to arise through the action of a distinct enzyme on MVA and ATP in the presence of divalent metal ions. This enzyme, designated MVA kinase, was first isolated and purified by Tchen from yeast autolysates and was later identified in mammalian (103, 113) and plant (102, 107) tissues. Although the yeast kinase exhibited no specificity for ATP, functioning equally well in the presence of GTP, CTP or UTP, higher plant and, particularly, mammalian kinases exhibit a very high specificity for ATP. The pH optimum of MVA kinase from both yeast and mammalian sources lies between 6.5 and 7.5 (113, 124, 159). Loomis and Battaile (19) reported a lower pH optimum of 5.7 for MVA kinase derived from pumpkin seedlings. As the latter study was conducted on crude enzyme extracts, this value may be somewhat lower than the true optimum. Regardless of the source, MVAP is stereospecifically

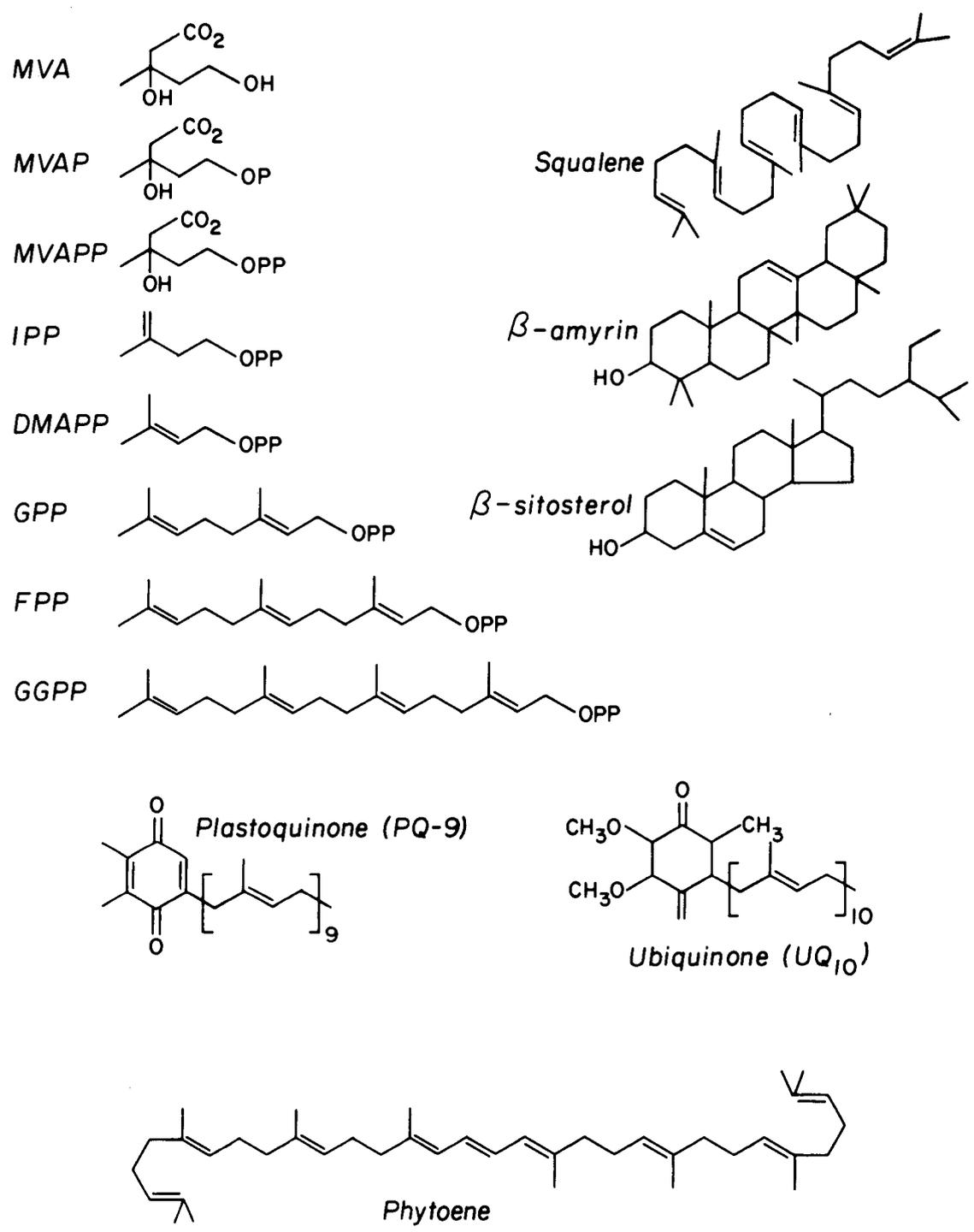


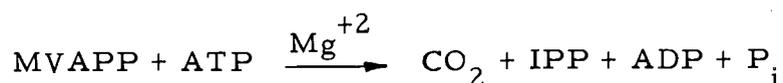
Figure 1. Chemical structures of some common isoprenoid intermediates of Pisum sativum.

produced as the levorotatory R-enantiomer (27, 32).

Chaykin et al. (18) demonstrated through a series of ingenious labelling experiments with MVA-2-¹⁴C and ³²P labelled ATP, that each of the phosphorous groups associated with MVAP and MVAPP arises from ATP in stoichiometric proportion to the appearance of product. The enzyme catalyzing the second phosphorylation step was first isolated by Bloch et al. (13) from yeast and designated MVAP kinase. The yeast MVAP kinase required ATP and either Mg⁺² or Mn⁺² ions for maximal activity. No pronounced pH optimum was reportedly observed between 5.5 and 10.0. Henning, Møsllein and Lynen (81) were able to show that yeast kinase catalyzed the reverse reaction to ATP and MVAP, when incubated with ADP and MVAPP in the presence of a divalent metal ion. At pH 7.3 and 37^o, the equilibrium constant was subsequently shown by Hellig and Popják (80) to lie between 0.7 and 1.1 in favor of MVAPP and ADP formation. MVAP kinase has since been identified in higher plants (133, 166) and in mammalian tissues (41, 80, 103). Unlike the yeast enzyme, the mammalian kinase is most active at pH 7.3 (124).

The work of several investigators (13, 40, 110, 139) clearly demonstrated the presence of a single enzyme, MVAPP decarboxylase, as the catalytic agent responsible for converting MVAPP into IPP. Lynen and co-workers (110) established that IPP-¹⁴C was an efficient precursor of squalene, while Bloch and several co-workers (13, 40,

139) established the stoichiometry, cofactor requirements and mechanism of decarboxylation. The reaction was shown to proceed as follows:



Bloch et al. (13, 139) showed that only the C-1 carbon of MVA was decarboxylated and that the decarboxylation was concerted with the elimination of the 3-hydroxy group of MVAPP. Bloch and co-workers (105) subsequently demonstrated that ^{18}O in synthetically prepared 3- ^{18}O -MVAPP was transferred to the orthophosphate released in the decarboxylation step. Since the latter phosphate was initially associated with ATP, Bloch et al. proposed that ATP phosphorylates the 3-hydroxy group of MVAPP, thereby activating MVAPP for a subsequent concerted decarboxylation. The pH optimum of the yeast decarboxylase was reportedly broad (5.5-7.4). More recently, Hellig and Popják (79) succeeded in isolating and partially purifying MVAPP decarboxylase from pork liver. In contrast to the yeast decarboxylase, however, the latter enzyme exhibits a sharp pH optimum at 5.1. At pH 4.0 and 7.4, the activity is 40% of its maximum (124).

The Biochemical Step Between IPP and DMAPP

Lynen and co-workers (1, 111) were the first to isolate an enzyme from yeast autolysates which isomerized IPP into a new product,

DMAPP. The enzyme, designated IPP isomerase, exhibited a broad pH optimum between 6.0 and 9.0 and required Mg^{+2} (or Mn^{+2}) ions for maximal activity. The yeast isomerase was strongly inhibited in the presence of iodoacetamide, p-chloromercuribenzoate (PCMB) and similar sulfhydryl inhibitors. With PCMB, 65% of the initial activity could be recovered by dialysis against glutathione. It was further shown that omission of iodoacetamide, and similar sulfhydryl inhibitors, allowed for the direct incorporation of IPP into FPP and squalene. Shaw, Cleland and Porter (158) isolated and partially purified IPP isomerase from pork liver, which exhibited very similar properties to the yeast isomerase. The latter isomerase showed a broad pH optimum between 4.0-8.3 and a preference for Mg^{+2} over Mn^{+2} ions for maximal activity. In addition, Shaw et al. demonstrated that the isomerization of IPP to DMAPP is reversible and that IPP passes through an enzyme bound transition state, possibly associated with a sulfhydryl group at the active site of the isomerase. Using tritiated water, the same group established that the isomerization involves stereospecific elimination of the C-2 hydrogen and a random incorporation of a proton from solution into either one of the dimethyl groups of DMAPP. Subsequent studies by Cornforth et al. (31) showed that the R-hydrogen from the C-2 carbon of IPP is eliminated during the isomerization. Randomization of label, initially associated with the methylene group of IPP, does not occur during isomerization.

Recently, Holloway and Popják (85) reported a 20 fold purification of IPP isomerase from pork liver. The latter group, in contrast to the findings of Shaw et al., reported that the isomerase exhibits a sharp pH optimum at 6.0 and prefers Mn^{+2} over Mg^{+2} ions for maximal activity. The reasons for these slight differences in enzymic properties are not presently clear.

The Prenyltransferases--Condensations Between IPP and GGPP

Biosynthetic steps beyond IPP and DMAPP are complex, leading to a wide variety of polyisoprenoid products. Much of the enzymology associated with these transformations is ill-defined and in need of further clarification. Lynen and co-workers (111) were the first to isolate and partially purify an enzyme, which they designated as geranyltransferase, capable of catalyzing the condensation of IPP (C-5) with DMAPP (C-5) to yield GPP (C-10). Geranyltransferase, in addition, was reportedly responsible for the condensation of IPP (C-5) with GPP (C-10) to yield FPP (C-15) (110, 125). Lynen and co-workers subsequently demonstrated a similar condensation between IPP (C-5) and FPP (C-15) to yield GGPP (C-20) (73). The yeast transferase exhibited a pH optimum of 6.0 and a preference for Mn^{+2} over Mg^{+2} ions. Recently, Porter and co-workers (119, 173) reported the isolation and purification of geranylgeranyl pyrophosphate synthetase from carrot plastids and pork liver. The enzyme reportedly

catalyzes only one condensation, that between IPP and FPP, to yield GGPP. The synthetases from carrot and pork liver exhibit pH optima of 6.7-6.8 and 6.9-7.0, respectively. Manganous ions are also required for maximal activity. Kandutsch et al. (90), however, reported the isolation and purification of an enzyme from M. lysodeikticus, which catalyzes all of the condensations with equal facility: IPP + DMAPP; IPP + GPP; and IPP + FPP. These results were interpreted as evidence for only one enzyme involved in catalyzing the above reactions. More recent studies on the pork liver prenyltransferase system, mainly by Benedict, Kett and Porter (9) and Holloway and Popják (83, 84) have not resolved whether one or several enzymes catalyze the above condensations.

Benedict et al. have reported the isolation of a specific transferase, farnesyl pyrophosphate synthetase, from pork liver which condenses IPP with GPP to make FPP. The enzyme has a pH optimum of 7.0 and prefers Mn^{+2} over Mg^{+2} ions. Iodoacetamide at 2 mM has no effect on enzymatic activity while p-hydroxymercuribenzoate (PHMB) completely inactivates the enzyme. Enzyme activity is restored in the latter case with the addition of glutathione. By contrast, Holloway and Popják have reported a 100 fold purification of pork liver transferase with no differential loss between IPP/DMAPP and IPP/GPP condensing activity. With either set of substrates, the product is FPP. Furthermore, the enzyme exhibits a sharp pH

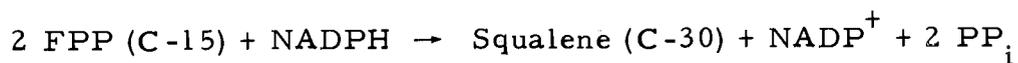
optimum at 7.9 and prefers Mg^{+2} over Mn^{+2} ions. Enzymatic activity is inhibited 80% with 2 mM iodoacetamide, 5 μ M N-ethylmaleimide and 0.5 μ M PHMB. The effect of the latter sulfhydryl inhibitor is reversible in the presence of 2-mercaptoethanol. Dorsey, Dorsey and Porter (42) recently reported a 200 fold purification of geranyl synthetase from pork liver, which appears to exhibit similar properties. The discrepancy between these findings and those previously reported by Benedict et al. are believed to be attributed to the former work with phosphate buffer which severely inhibits the transferase (124).

Presently, it appears that one enzyme catalyzes all condensations between IPP/DMAPP and FPP. A second enzyme may catalyze the condensation between IPP and FPP to yield GGPP. Several excellent studies on the chemical modifications, enzyme-substrate binding order and stereochemical changes accompanying each transformation have also been made (9, 32, 84).

The Biosynthesis of Squalene from FPP

An understanding of the enzymology and mechanism of squalene formation from FPP has proven less tenable than originally anticipated. The conversion of FPP (C-15) into squalene (C-30) in yeast (110) and pork liver (56) enzyme systems appears to be a reductive, tail to tail dimerization. The overall stoichiometry of squalene

formation may be represented as follows:



Several mechanisms to account for squalene formation have been proposed (28, 110, 123, 141). Among the more popular models has been the proposal that one molecule of FPP allylically rearranges to its isomer, nerolidyl pyrophosphate (NPP) and this then condenses with FPP as shown in Figure 2. This model is consistent with the result of the very elegant experiments of Popják and co-workers (128). Their findings may be summarized as follows: (i) with 5-²H-MVA, only 11 of the 12 theoretically possible deuterium atoms were incorporated into squalene; (ii) degradation of the deuterated squalene molecule by ozonolysis revealed an asymmetric succinate moiety, in which one of the deuterium atoms had been replaced by a hydrogen atom; (iii) when 1-³H-2-¹⁴C-FPP served as substrate, the ratio of ³H: ¹⁴C in squalene was found to be 0.76 of the ratio in FPP, indicating the loss of one ³H atom at C-1 of FPP during the condensation to squalene; and (iv) an incubation of unlabelled FPP with pork liver microsomes and NADP³H resulted in the incorporation of approximately one gram atom of ³H into the central region (succinate moiety) of squalene. The exchange of a single hydrogen atom arises solely from reduced pyridine nucleotide, since an analogous incubation in tritiated water and with NADPH gave no tritiated squalene. The

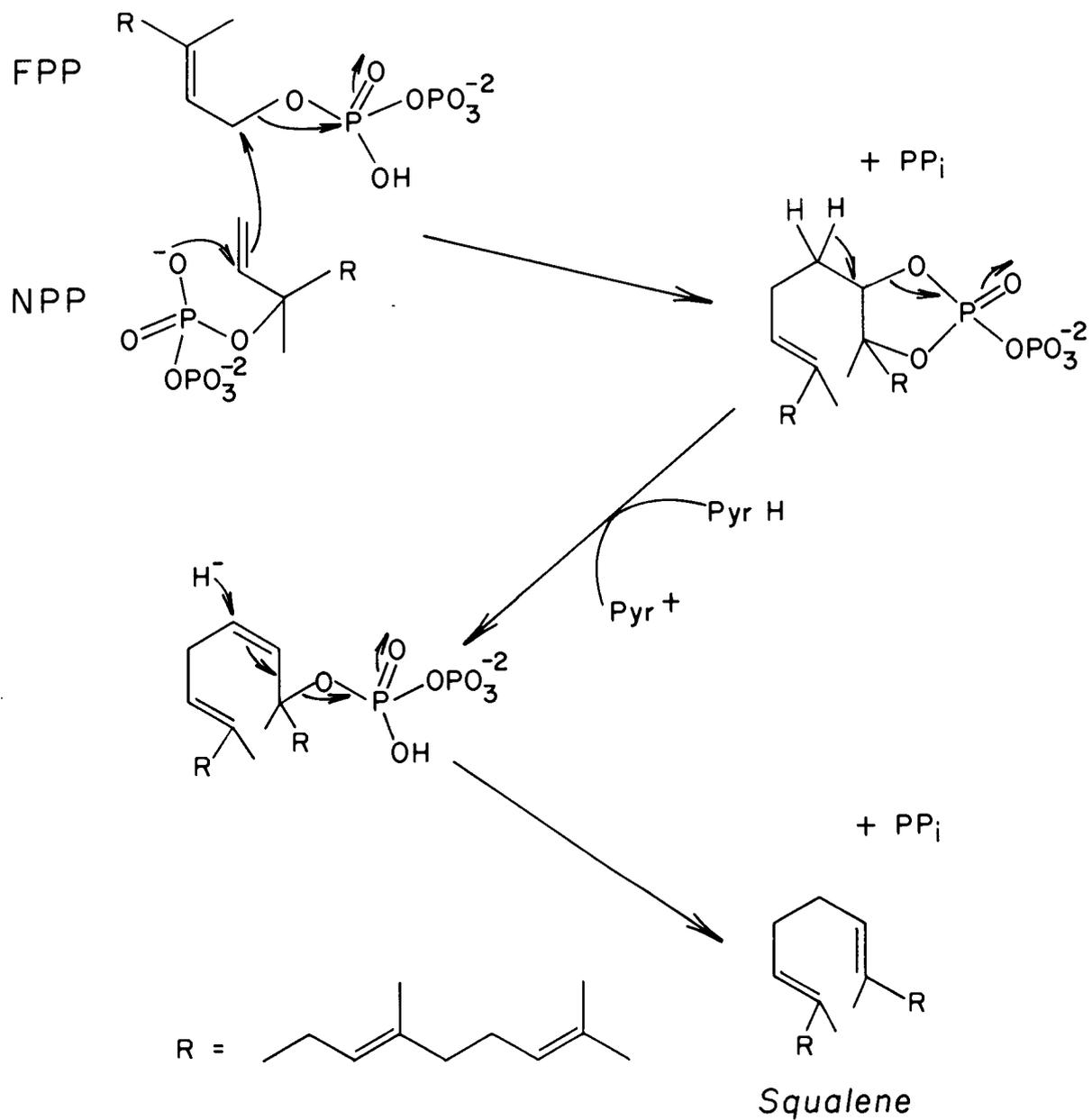


Figure 2. Schematic mechanism of squalene formation from farnesyl and nerolidyl pyrophosphate.

hydride transfer from reduced pyridine nucleotide was subsequently shown to be stereospecific (29, 148).

In recent years two new aspects in the biosynthesis of squalene from FPP have arisen: (a) The participation of NPP as a free intermediate in squalene formation has been questioned; and (b) a new precursor between FPP and squalene has been detected. Sofer and Rilling (64), using an enzyme system from yeast microsomes, recently presented convincing experiments which argue against the participation of free NPP in the tail to tail condensation with FPP to yield squalene. Their results may be summarized as follows: (i) tritium from 1-³H-FPP is not incorporated into NPP; (ii) tritium from 1-³H-NPP is not incorporated into squalene in the presence or absence of FPP; and (iii) unlabelled NPP does not affect the rate of α -H loss at C-1 of 1-³H-FPP during squalene formation. In the latter case, if NPP were involved in squalene formation, then the rate of α -H loss should be altered with the addition of unlabelled NPP via an isotopic dilution effect.

Krishna, Feldbreuegge and Porter (95), working with a pork liver microsomal enzyme system, and Rilling (68), working with yeast microsomes, recently reported the detection of a new pre-squalene intermediate between FPP and squalene, which accumulates in the absence of reduced pyridine nucleotide. Within the last year, Popják, Edmond and Clifford (126) have also reported the detection

of a presqualene intermediate in pork liver microsomes. Based mostly on mass spectral data, Popják et al. proposed that this new intermediate is the cyclic pyrophosphorylated ester of squalene-10, 11-glycol. In addition, the $^3\text{H}: ^{14}\text{C}$ ratio was shown to be identical to that of 1- ^3H -2- ^{14}C -FPP, indicating that the presqualene intermediate precedes the reductive step in squalene formation. Although Rilling (138) was the first to propose a structure for the presqualene intermediate, designated as compound X, Corey (21) has shown the latter structure to be incorrect. Rilling and Epstein (140) recently proposed still another structure, which differs from that proposed by Popják et al. While Popják's structure accounts for the tail to tail condensation between FPP and NPP, Rilling's structure arises through the tail to tail condensation of two units of FPP.

Regardless of the presqualene structure, an uncertainty presently exists regarding the status of squalene synthetase. Krishna et al. (95) proposed that squalene synthetase is a single enzyme and that the presqualene intermediate is normally enzyme-bound, being released prematurely in the absence of reduced pyridine nucleotide. Popják (124), on the other hand, has recently described squalene synthetase as a multienzyme system, composed of two or possibly more enzymes. The enzyme, or enzymes, catalyzing the conversion of FPP into squalene, appears to be associated with the microsomal fraction, exhibits a pH optimum near 7.4 and requires divalent metal

ions (Mg^{+2} , Mn^{+2} , or Co^{+2}) for maximal activity (124).

Transformations Between Squalene and Cyclic Products

Chemical transformations accompanying the oxidation and cyclization of squalene into triterpenes and sterols have been well characterized and extensively reviewed by many workers in the field (20, 45, 54, 137). The chemical structures of three very important products of squalene, 2,3-oxidosqualene, cycloartenol and lanosterol, are shown in Figure 3. In yeast (94, 152) and mammalian (163) sources, lanosterol appears to be the immediate product of oxidosqualene. However, the immediate product of oxidosqualene in higher plants is most frequently cycloartenol (54, 55). For either product, a series of hydrogen and 1,2 methyl shifts occur during the cyclization of oxidosqualene. In the case of lanosterol, a proton is lost from C-9, while cycloartenol appears to arise through migration of the C-9 hydrogen to C-8, followed by elimination of the C-19 proton and formation of a cyclopropane ring between C-9, -10 and -19 (54, 137). The cyclized C-30 product of oxidosqualene is normally demethylated at the 14α position. Frequently, particularly in animal systems, demethylation also occurs at the 4α and β positions. Gautschi and Bloch (52, 53) have given experimental evidence which suggests that demethylation of the 14α -group precedes that of the 4,4-dimethyl groups. Furthermore, the 14α -methyl loss is believed to be enhanced by the presence

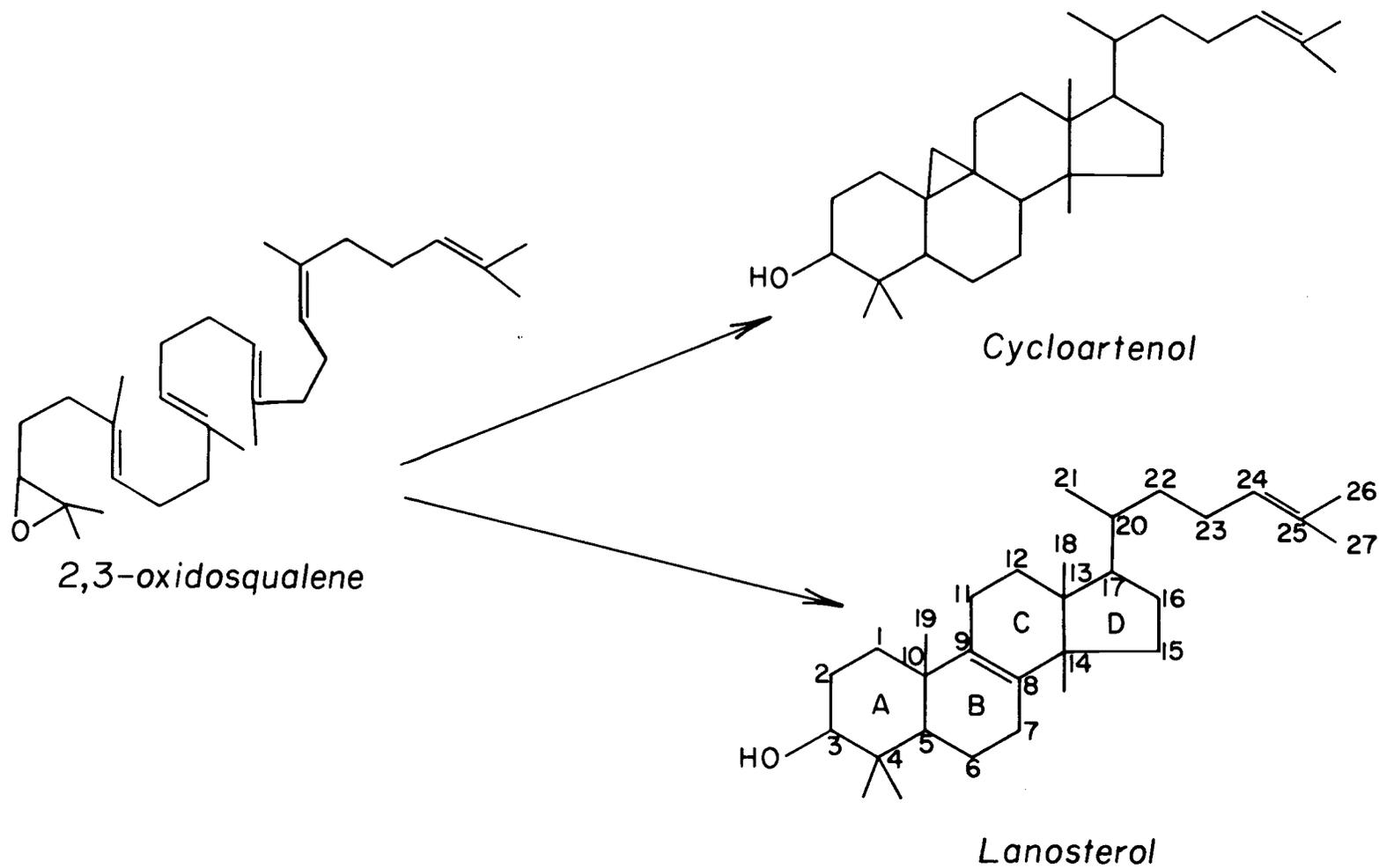


Figure 3. Chemical structures for 2,3-oxidosqualene, cycloartenol and lanosterol and numbering sequence (see lanosterol).

of the Δ^8 bond (137) (i. e. lanosterol is ideally structured for demethylation). With respect to these observations, Bernard and Reid (10) have recently suggested that triterpenes and sterols of N. tabacum may arise from divergent pathways: phytosterols (C-24 alkylated sterols) and $9\beta, 19$ -cyclopropane triterpenes are divided into separate classes because of their presumably separate biosynthetic origin. Experiments by Barton (7) and also by Baisted, Capstack and Nes (5), suggest that pentacyclic triterpenes may arise from still another biosynthetic route. The common intermediate (oxidosqualene?) from which these biosynthetic routes diverge has not been established.

The cyclization of squalene into lanosterol was shown by Tchen and Bloch (162) to require molecular oxygen and reduced pyridine nucleotide. In the presence of $^{18}\text{O}_2$ and NADPH, an enzyme fraction from pork liver microsomes, which Tchen and Bloch later designated as squalene oxidocyclase, was shown to catalyze the formation of 3- ^{18}OH -lanosterol. It was further shown that H_2^{18}O could not substitute for molecular oxygen as the precursor of the 3-hydroxy group and that $^2\text{H}_2\text{O}$ failed to incorporate into the lanosterol skeleton. The latter experiment led Tchen and Bloch to propose that the cyclization proceeded to lanosterol without the formation of a free intermediate. Richards and Hendrickson (137) have suggested that the requirement for molecular oxygen and reduced pyridine nucleotide by squalene

oxidocyclase is characteristic of a class of enzymes frequently referred to as mixed function oxidases (114, 115).

Squalene oxidocyclase now appears to be at least two enzymes, a mixed function oxidase and a cyclase, which act sequentially on squalene to produce a cyclized product. Corey and co-workers (24, 25) and Van Tamelen et al. (168) have given evidence that 2,3-oxidosqualene is a precursor of lanosterol in pork liver homogenates. It was further shown that the cyclization of oxidosqualene to lanosterol requires neither O₂ nor reduced pyridine nucleotide. Cyclase activity has been preferentially blocked by heat inactivation (179) and with oxidosqualene analogues (24, 124), resulting in the accumulation of 2,3-oxidosqualene. Since the work of Corey et al. and Van Tamelen et al., a large number of papers have appeared on the cyclization of 2,3-oxidosqualene into lanosterol by mammalian (31, 180) and plant (55, 117) enzyme systems and, more commonly in plants, into cycloartenol (44, 55, 135). Corey and co-workers (22, 23) have also recently established 2,3-oxidosqualene as the precursor of β-amyrin, a pentacyclic triterpenoid product, in an enzyme fraction prepared from germinating seeds of P. sativum.

The Biosynthesis of Carotenoids

Lynen and Henning (109) postulated that biosynthetic steps between IPP (C-5) and phytoene (C-40), a precursor of carotenes,

proceeded via IPP (C-5), GPP (C-10), FPP (C-15) and GGPP (C-20). Porter and co-workers (2, 8, 89, 173) have provided considerable indirect evidence that phytoene arises from IPP and FPP in carrot and tomato plastids and that the immediate product of the condensation is GGPP. The enzymatic synthesis of GGPP has been well documented (73, 90, 119). Lee and Chichester (101) have reported within the last year the first direct proof that GGPP is a precursor of phytoene. A cell-free enzyme system from P. blakesleeanus, exhibiting a pH optimum of 7.8 and requirement for Mg^{+2} ions, was incubated with GGPP- ^{14}C and shown to accumulate label among several carotenoid precursors, including phytoene, lycopene and ζ -carotene. Phytoene is presently believed to be the immediate product of GGPP and is subsequently transformed through a series of dehydrogenations, isomerizations, cyclizations and oxidative steps into several carotenoid derivatives (61).

Mixed Terpenoids

Very little is known regarding the isoprenoid enzymology of phytol (C-20) formation, nor of the side chains of plastoquinones (C-45) and ubiquinones (C-50). Goodwin and co-workers have demonstrated in vivo a preferential incorporation of $^{14}CO_2$ over MVA-2- ^{14}C into the phytol side chain of chlorophyll (116, 165) and plastoquinone side chains (163), but a preferential incorporation of MVA-2- ^{14}C

over $^{14}\text{CO}_2$ into the side chain of mitochondrial ubiquinones in maize seedlings (66). The difference between CO_2 and MVA incorporations was attributed to subcellular compartmentation of isoprenoid precursors between chloroplastidic and cytoplasmic environments by an impermeable membrane (63). In either compartment, the polyisoprenoid side chains are believed to arise from MVA, and more directly through GGPP and appropriate condensations with IPP. Excellent reviews on the chemistry of terpenoid quinones and the interrelationship of phytol with chlorophyll pigments may be found elsewhere (92, 100).

Additional Metabolic Products of the Isoprenoid Pathway

In addition to the isoprenoid products already discussed, the following compounds have also been implicated as metabolites of the isoprenoid pathway in higher plants: monoterpenes, diterpenoids (kaurene, gibberellins, etc.), and mixed terpenoids associated with a number of t-RNA's (i. e., IPA and derivatives of N^6 -(Δ^2 -isopentenyl) adenosine inserted into the nucleotide sequence of specific t-RNA's). Prenolic acids, which have recently been found in mammalian enzyme systems (124, 127), may also occur in higher plants. Several excellent papers and review articles on the biochemistry of monoterpenes (11, 15, 20, 26, 48-50, 68, 106, 149), gibberellins (36, 98, 143, 149,

174) and cytokinins (IPA, etc.) (75) in higher plants may be found elsewhere.

Developmental Changes and Regulation of the Isoprenoid Pathway

In order to survive, an organism must be capable of meeting certain obligatory requirements in cellular metabolism. Among the necessary biological functions are the processes of photosynthesis, respiration, energy accumulation and transfer, and biosynthetic transformations concerned with the synthesis and preservation of essential components for sustained life. Nonphotosynthetic organisms, of course, are ultimately dependent upon plants and similar living forms of life capable of converting carbon into a utilizable energy source. Each process is dependent upon the other. For growth and differentiation, a portion of the energy supply must be devoted toward functions which increase the energetic capacities of the organism. Hence, the biogenesis of chloroplasts and mitochondria is an expression of this interdependency between essential functions. During the early stages of development, subcellular membranes, pigments, enzymes, coenzymes, lipids, etc., which make up the mitochondrial and chloroplast machinery, must be synthesized to provide the necessary energy, once assembled, for new conditions encountered in later stages of development. Regulatory changes which accompany development and growth are frequently assumed to

reflect the adaptive strategy of the cell to meet its biological requirements for survival.

In microbial spores and vegetative cells, the breaking of dormancy initiates a great number of biochemical changes (157, 178) associated with striking physiological changes, which allow the developing organism to break away from the embryonic state. Changes in the protein synthesizing capacity, the concentration of high energy metabolites (ATP, NADH, NADPH, etc.), reserve protein and carbohydrates, and in the activity of specific enzymes, all indicate a well planned sequence of biochemical steps leading to the establishment of a self-sufficient organism. In germinating seeds, it is clearly necessary for the developing embryo to prepare all of the photosynthetic and mitochondrial components and assemble them in such a manner that they may provide energy for subsequent growth, following the depletion of energy reserves initially stored in the seed.

A developing organism may regulate the flow of essential metabolites through a number of mechanisms: compartmentation of metabolites; derivative formation; enzyme aggregation; isozymic compartmentation; and, more directly, through enzyme activation or inhibition, or alternately, through changes in the rate of synthesis and degradation of key enzymes in appropriate metabolic pathways. Several excellent review articles on the compartmentation of metabolites by membrane barriers, chemical derivatives, enzyme aggregates and isozymes

(121), by cofactors (4) and on the regulation of enzyme activities through synthesis and degradation (46, 76, 82, 151) have recently appeared. Filner, Wray and Varner (46) have given a particularly valuable review on enzyme activation in higher plants and the multitude of environmental factors which may influence enzyme levels during the early stages of development.

There are a number of salient features of the isoprenoid pathway which suggest that the flow of metabolites must be highly regulated and, furthermore, modulated to meet the developmental demands of growth and differentiation. Many of the isoprenoid products discussed in the previous sections appear to serve important biological functions in cell metabolism. The carotenoids, phytol side chain of chlorophyll, and polyisoprenoid side chain of plastoquinone (PQ-9) have been implicated as electron carriers in photosynthesis (64, 87, 132, 158); ubiquinones (coenzyme Q) play a very important role in oxidative phosphorylation (64, 104, 135); triterpenes and sterols appear to play an important role in membrane formation (64); while the cytokinins and gibberellins are particularly potent plant growth hormones (64, 175). Figure 4 depicts the metabolic pathways leading from MVA into a number of isoprenoid products, as previously discussed. The following features of the pathway should be noted: (i) It is energetically expensive to make any one of the biologically significant products. In addition to ATP requirements, reduced pyridine nucleotide is required

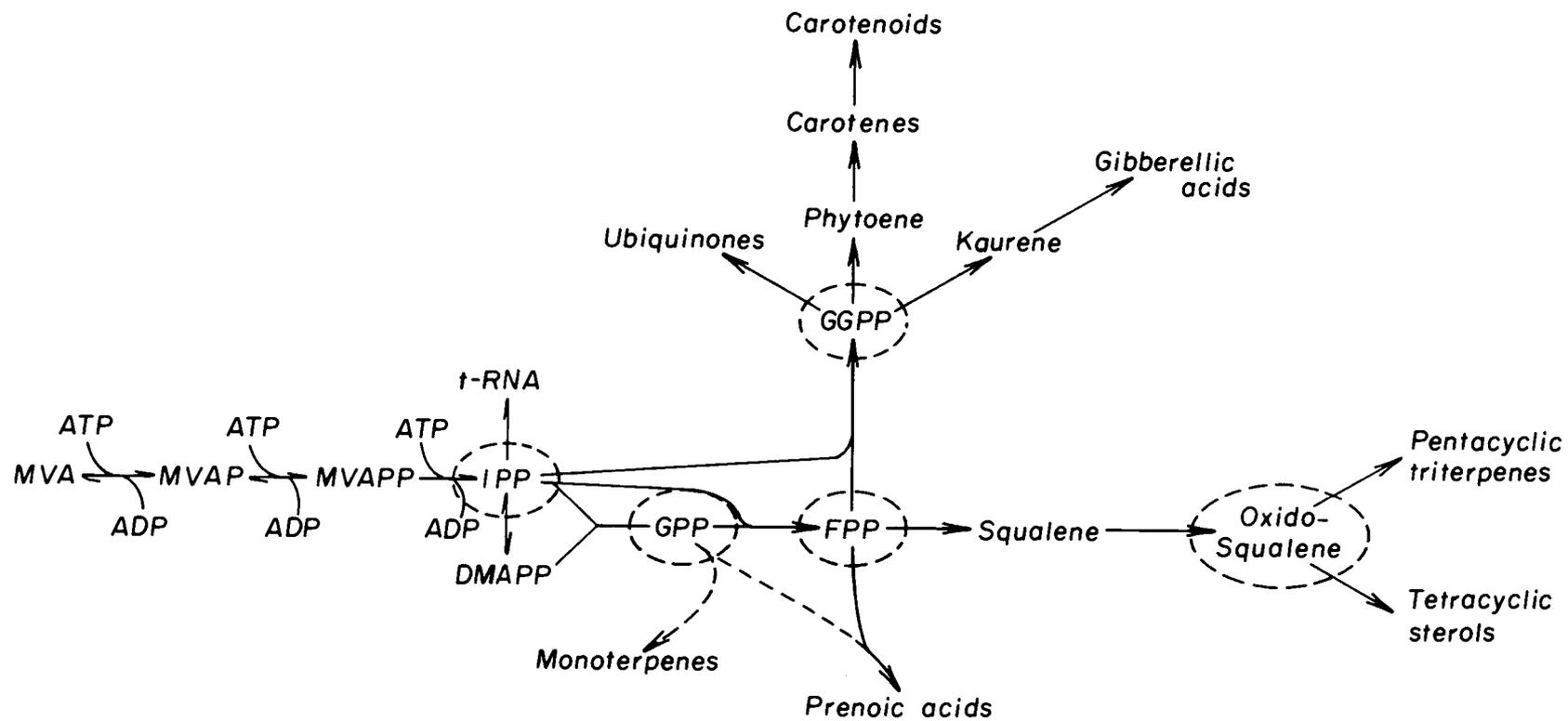


Figure 4. Metabolic pathways leading from MVA into higher isoprenoid products. Common intermediates of divergent pathways enclosed by dashed lines emphasize the anticipated sites of regulation.

as a cofactor in several steps (i. e., for the synthesis of squalene, oxidosqualene, kaurenol, etc.). If the oxidation of one unit of NADPH is equated with the loss of three units of ATP, then the energy required for the biosynthesis of one unit of isoprenoid product may be briefly summarized in the following form: IPP (3ATP), GPP (6ATP), FPP (9ATP), GGPP (12ATP), squalene (18ATP), triterpene (21ATP), phytoene (24ATP), ubiquinones and plastoquinones (27-30ATP), etc.;

(ii) From Figure 4 it is evident that several of the intermediates serve as common precursors for two or more diverging pathways (viz., IPP, GPP, FPP, GGPP, and oxidosqualene). Because of the high energy requirements for biosynthesizing many of the biologically significant products and the divergence of these pathways, it is to the benefit of a developing organism to regulate the flow of carbon among the pathways at maximum efficiency, thereby conserving energy;

(iii) A third and related feature of this pathway may be anticipated. Since the biologically significant products serve quite different roles in maintaining cellular metabolism, it is likely that the appropriate enzymes leading to such products may be modulated in activity to meet the needs of the organism at different states of development. It is also evident, however, that two or more divergent products may be needed simultaneously during development and that such conditions may not be satisfied through enzyme modulation alone.

Goodwin (63) has recently proposed that regulation is achieved by

two mechanisms: enzyme segregation and specific membrane permeability. It was shown by Griffiths (72) that in illuminated maize seedlings, the synthesis of plastoquinones and chlorophyll increases in parallel while ubiquinones and sterols show little change. Treharne (164) previously demonstrated that $^{14}\text{CO}_2$ fixation into nonsaponifiable products and β -carotene in illuminated maize seedlings rises in parallel with chlorophyll formation until the chloroplasts reach maturity. Thereafter, the capacity to make such products from $^{14}\text{CO}_2$ steadily declines to insignificant levels. Over the same developmental period, the capacity of MVA-2- ^{14}C to incorporate into β -carotene and the phytol side chain of chlorophyll was shown to be insignificant (57, 58). These and similar experiments (60, 66) led Goodwin and Mercer (66) to propose a scheme of enzyme segregation between the chloroplast and cytoplasmic isoprenoid enzymes. Goodwin (63) has recently proposed that each compartment contains the common isoprenoid enzyme responsible for biosynthetic steps through FPP and, thereafter, specific isoprenoid enzymes appropriate for each compartment. This proposal includes the assumption that the chloroplast membrane is impermeable to MVA and subsequent phosphorylated products of the pathway. Thus the capacity to make chloroplastidic pigments (viz., carotenoids, plastoquinones, phytol side chain of chlorophyll, etc.) or cytoplasmic products (viz., squalene, triterpenes, sterols, etc.) is independent of the other, sharing

no common intermediates by competition. Each pathway may be modulated independently to meet its biochemical requirements at any state of development.

Rogers, Shaw and Goodwin (144, 145) have recently given some support to the model by demonstrating the presence of MVA and MVAP kinase in the chloroplasts of P. vulgaris. The chloroplasts were isolated by two methods: discontinuous density gradients of sucrose in phosphate buffer and of carbon tetrachloride in hexane. Intact chloroplasts were first incubated with MVA-2-¹⁴C, from which no products were detected. Similar incubations were carried out on ruptured chloroplasts, treated to osmotic shock or sonication. In the latter case, both MVAP-2-¹⁴C and MVAPP-2-¹⁴C were detected. These results were interpreted as evidence of (i) an impermeable membrane to MVA in the chloroplast and (ii) direct evidence for the existence of MVA and MVAP kinase within the chloroplast. The incorporation of FPP into phytoene by a carrot plastid enzyme fraction (119, 173) may suggest the presence of geranylgeranyl pyrophosphate and phytoene synthetases within the chloroplasts. However, these results must be viewed with caution, as the plastid fraction was not shown to be homogenous nor was it isolated by satisfactory methods to insure uncontaminated fractions. Aside from the experiments of Rogers et al., no other direct evidence for the compartmentalization of isoprenoid enzymes has yet been given. Recent reviews on this

subject have been given by Kirk (91) and Porter and Anderson (131).

Purpose of the Study

It was shown by Nes and co-workers (5, 6, 16) that MVA is an efficient precursor in vivo of squalene, β -amyrin, and β -sitosterol in germinating seeds of P. sativum. It was subsequently demonstrated in cell-free preparations of P. sativum (17) that squalene is a precursor of β -amyrin. Thus germinating pea seeds appeared to contain all of the necessary isoprenoid enzymes for higher terpenoid synthesis. More recently, Nes et al. (120) demonstrated in vivo changes in the metabolic capacity of germinating pea seeds to biosynthesize triterpenes and sterols from MVA-2-¹⁴C. Graebe (68) has recently reported on the preparation of a cell-free enzyme extract from developing seeds of P. sativum in which several enzymes, including those responsible for squalene, kaurene and phytoene biosynthesis, were identified. Graebe has suggested that an understanding of the mechanism by which these products are formed in developing seeds of P. sativum may lead to a better understanding of plant development and regulatory relations of the isoprenoid pathway.

The purpose of this study was to examine the regulatory features of the isoprenoid pathway in relation to developmental changes, initiated by the imbibition of water in seeds of P. sativum. As was pointed out in the previous paragraphs, the nature of the isoprenoid

pathway and diversity of biologically significant products suggests that the pathway may be highly regulated, particularly under stress conditions, where the availability of high energy intermediates is limited. It was postulated that germinating seeds suffer from a limited supply of available energy and must therefore conserve energy wherever possible. This assumption should be reflected through enzymic changes in specific isoprenoid enzymes of the pathway, correlated with the developmental requirements for growth and differentiation.

This study also constituted, in part, an effort to evaluate suitable techniques for the biosynthesis and purification of appropriate substrates and analytical methods for assaying several of the isoprenoid enzymes in cell-free preparations from P. sativum.

MATERIALS AND METHODS

Materials

R, S-MVA-2-¹⁴C was obtained from New England Nuclear Corporation as the dibenzylethylenediamine (DBED) salt. The salt was converted to the sodium salt in aqueous solution, adjusted to pH 7-8, and diluted to a convenient activity with water. Free DBED was extracted from solution with diethyl ether and discarded.

R, S-MVA-1-¹⁴C was obtained from Amersham/Searle Corporation as the lactone dissolved in benzene. The benzene was evaporated off under a stream of nitrogen and the lactone allowed to stand in dilute NaOH for approximately 30 minutes at 37°. The solution was then brought to pH 7 with dilute HCl to yield the free sodium salt and diluted to a convenient activity with water.

Unlabelled MVA was purchased from Sigma Chemical Company as the lactone and converted to the free sodium salt by the same method as described above.

ATP, NADPH, glutathione and p-nitrophenylphosphate were all purchased from Sigma Chemical Company as Sigma Grade products. Potamine sulfate, Grade II, was also purchased from Sigma Chemical Corporation.

Bovine serum albumin, Grade B, was obtained from Cal-Biochem. Glucose-6-phosphate dehydrogenase was purchased from P-L

Biochemicals, Inc.

Standard samples of geraniol and linalool were obtained from Aldrich Chemical Company. Farnesol was obtained from International Chemical and Nuclear Corporation and nerolidol was purchased from Frinton Laboratories.

Chromosorb W (AW-DMCS), 60/80 mesh, and butanediol succinate polyester were both purchased from Perco Supplies.

All other supplies and chemical reagents were those commonly found in the laboratory.

Methods

Radioactivity Measurements

Quantitation of radioactivity was achieved by liquid scintillation counting on a Packard Tricarb scintillation counter, Model 574. Two fluids, either Bray's (14) or a non-aqueous mixture were used. The composition of each scintillation fluid was as follows:

Bray's fluid -	60	g naphthalene
	4	g PPO
	0.2	g POPOP
	100	ml absolute methanol
	20	ml ethylene glycol
diluted to	1	l in p-dioxane
nonaqueous fluid -	4	g PPO
	30	mg POPOP
diluted to	1	l in toluene

The former was counted with a window setting of 50-1000 divisions

and a gain of 18%; the latter was counted with the same window settings but a gain of 8%. Samples were corrected for quenching and instrument inefficiency by either internal standardization with a toluene- ^{14}C spike or by external standardization with ^{137}Cs . Several excellent reviews on the theory and practical methods of scintillation counting may be found elsewhere (38, 170).

The distribution of label on paper or thin layer chromatography was determined by scanning on a Packard strip scanner, Model 7301. A Packard Disc Chart integrator, Model 215, coupled with the tracing pen allowed the area of each radioactive peak to be accurately evaluated. The distribution of label between the components of a radioactive mixture was expressed as a percentage of the total radioactivity observed on the paper strip. Chromatograms were marked above the solvent front and below the origin with a trace of radioactivity to allow correlation with the recorder tracing.

Gas radiochromatographic detection was achieved with a Nuclear Chicago ionization detector, Model 4998. The Geiger-Mueller type detector was calibrated for optimal efficiency with a ^{60}Co source. Counting gas (propane) was adjusted to flow through the detector at a rate of 40-60 ml/min. Further details on the detection and quantitation of radioactivity by gas chromatography have been cited under individual assays and methods.

Paper Chromatography of Isoprenoid Intermediates

Five solvent systems were used to identify and resolve the common phosphorylated intermediates and MVA on the basis of R_f values in each system. Strips of ordinary Whatman #1 filter paper approximately 5 x 40 cm were subjected to descending chromatography, dried at room temperature and then scanned to locate the migration patterns of the various labelled intermediates. Table 1 is a summary of solvent systems and corresponding R_f values for the common isoprenoid intermediates compiled together in a recent paper by Dugan et al. (42). Hereafter, a paper chromatographic solvent system will be referred to as solvent system I, II, III, ... etc. in accord with the listing of Table 1.

Thin Layer Chromatography (TLC)

Analysis of the Nonsaponifiable Fraction (NSF)

NSF products were identified primarily by TLC on silica gel G. Aliquots of the NSF were run on Eastman Chromagram silica gel sheets 6061 which were 100 μ thick. Glass plates, either 20 x 20 cm or occasionally 4.5 x 20 cm, were also prepared by mixing silica gel G with distilled water in a ratio of 1:2.25, respectively, and spreading the slurry to a thickness of 250 μ . Prior to chromatography, the plates were activated for 15-30 minutes at 110^o. Three percent

Table 1. R_f Values for Isoprenoid Intermediates - Paper Chromatographic Systems.

Compound	Solvent Systems ¹				
	I	II	III	IV	V
MVA	0.72-.77	0.77-.80	0.80-.82	0.68-.72	-
MVAP	0.28-.33	0.53-.61	0.52	-	-
MVAPP	0.00-.05	0.29-.35	-	-	-
IPP	0.15-.17	0.52-.61	0.57	0.42-.48	-
IP	0.79	0.80-.82	0.69-.70	-	-
DMAPP	-	-	-	0.33-.36	-
FPP	-	-	-	-	0.85-.90

¹Solvent systems were as follows:

- I. n-butanol:formic acid:water (77:10:13) (159, 176)
- II. t-butanol:formic acid:water (40:10:16) (13)
- III. methanol:ammonia:water (60:10:30) (130)
- IV. n-propanol:ammonia:water (60:20:20) (153)
- V. i-propanol:i-butanol;ammonia;water (40:21:1:39) (3)

impregnated AgNO_3 plates were prepared in the same manner by mixing the gel with 3% AgNO_3 aqueous solution instead of the usual distilled water. The latter plates were not heat activated.

The major components of the NSF, squalene, β -amyrin and β -sitosterol, were identified by their migration with authentic carrier. Common solvent systems were the following: benzene, chloroform, 1-3% ethanol in chloroform, 30% ethyl acetate in benzene, 5-10% acetone in dichloromethane and 12% ethyl acetate in hexane. Triterpenes and sterols were routinely identified by spraying with one of the following color reagents: 20% SbCl_3 in chloroform, concentrated $\text{H}_2\text{SO}_4/\text{H}_3\text{PO}_4$ (1:9), or 10% H_2SO_4 and heating the plate in an oven at 110° for 5-10 minutes.

Silver nitrate impregnated plates worked best in a benzene solvent. Spots were detected by spraying the plates with 10% H_2SO_4 and charring.

Analysis of Water Soluble Intermediates

Water soluble intermediates were chromatographed in n-propanol:ammonia:water (6:3:1) on silica gel H. Plates were prepared in the same manner as described above, except the gel to water ratio was 1:3, respectively. The plates were activated to 110° prior to use. Intermediates were identified by their radioactive R_f values, as compared to previously reported values (33). Further

characterizations of presumptive products isolated by TLC were also carried out (see page 36).

Dowex-1 Formate Column Chromatography of Acid Stable Intermediates

Acid stable intermediates were resolved and identified by Dowex-formate chromatography (13, 87). A 1.5 x 10 cm column of Dowex-1-formate (100-200 mesh) was prewashed in five to ten bed volumes of 6 N formic acid and 1 M sodium formate; washed with several bed volumes of 88% formic acid; then with glass distilled water until the eluant was no longer acidic. Isolation of the various acid stable intermediates was accomplished by passing three step-wise gradients through the column in the following order: (i) 4 M formic acid, (ii) 0.4 M ammonium formate in 4 M formic acid, and (iii) 0.8 M ammonium formate in 4 M formic acid. Solution eluted from the column was automatically collected on a fraction collector in eight milliliter fractions with a flow rate of approximately five milliliters per minute. Tenth milliliter aliquots from each fraction were counted in Bray's scintillation fluid. Gradients were changed immediately after the elution of MVA and 25-30 fraction thereafter. For convenience, the fraction volume was occasionally doubled to 16 milliliters per fraction, which allowed for complete elution of all the acid stable intermediates in approximately 30 fractions with no consequential loss in resolution.

Isolation of Labelled Prenols for Gas Chromatography

Phosphorylated isoprenoid intermediates were converted to their corresponding free alcohols by alkaline phosphatase digestion or acid hydrolysis. Alkaline phosphatase treatment causes no rearrangement during phosphate cleavage, so that the natural isoprenoid is released as a primary alcohol. On the other hand, acid hydrolysis produces a mixture of the primary alcohol and allylically rearranged tertiary alcohol. The latter product is the predominant isomer under acid hydrolysis (33). Prenols were released by either method according to the procedure described by Popják (124). Diethyl ether was used as the extracting solvent. All extractions were carried out in a cold room to minimize losses through volatilization of the free prenols.

Gas Chromatography of Labelled Prenols

Gas chromatography of free prenols, produced by either alkaline phosphatase or acid hydrolysis, was carried out on a Beckman GC-4 gas-liquid chromatograph equipped with a hydrogen flame ionization detector and splitter. The splitter diverted nine-tenths of the column effluent through a Nuclear Chicago ionization detector, Model 4998. Both radioactivity and mass peak responses eluted from the column were monitored simultaneously on a Sargent dual

recorder, Model DSRG, equipped with dual Disc integrators. A 6' x 1/4" glass column was packed with 80 mesh Chromosorb W coated with 20% butanediol succinate polyester, which was prepared according to the procedure of VandenHeuval (86). Best results were achieved by programming the GC-4 to run at an initial oven temperature of 80° for six minutes, then to rise linearly at 20° per minute to a maximum temperature of 180°. The carrier gas was Argon with a flow rate of 40-50 milliliters per minute. An aliquot (50-100 µl) was injected into the gas chromatograph at 80°, then analyzed as described above. The distribution of radioactive label among the prenols was calculated, where necessary, by internal normalization of the integrator sweeps (78).

DEAE Cellulose Chromatography of Isoprenoid Intermediates

Water soluble intermediates were separated from crude incubates by DEAE cellulose chromatography in ammonium carbonate gradients. The method is very useful for concentrating each of the intermediates under very mild conditions, being particularly useful for isolating acid labile intermediates. Dugan, Rasson and Porter have discussed the major advantages of DEAE cellulose chromatography over other methods presently available for resolving the common water soluble isoprenoid intermediates (43).

An excess of DEAE cellulose was allowed to swell in distilled

water, then washed with several volumes of 1 N NaOH, 1 N HCl and 1 N NaOH, in succession, and then with several volumes of glass distilled water. Prewashed cellulose was packed into a column (1.5 x 10 cm bed volume) by gravity flow and equilibrated to pH 8.3 by passing several bed volumes of 0.01 M K_2HPO_4 buffer through the column. Excess phosphate was washed away with glass distilled water until the eluted solution exhibited neutral pH. The procedures for separating individual intermediates have been previously described (43). A slight modification in the gradients (see page 112) allowed for the rapid separation of MVA and other phosphorylated intermediates. Sixteen milliliter fractions were automatically collected on a fraction collector and aliquots counted in Bray's scintillation fluid. Radioactive peaks were lyophilized and residual traces of ammonium carbonate sublimed off at room temperature under vacuum. Labelled intermediates were taken up in dilute ammonia and stored at -15° until needed.

Assay of Water Imbibition During Germination

A set of 100 seeds, partially serrated to enhance a uniform uptake of water, was preweighed and then reweighed intermittently over a 24 hour period following imbibition. Excess water was drained from the seeds by pressing an adsorbent cloth around the set of seeds prior to weighing. The difference between dry and wet weight was then

calculated and plotted as a function of germination.

Protein Determinations

All protein determinations were made according to the method of Gornall, Bardawill and David (67). Biuret reagent was mixed with protein solution in a ratio of 4:1, respectively, and read 45 minutes later on a Beckman DB spectrometer at 540 nm against a buffer blank. A standard protein curve was prepared from crystalline bovine serum albumin (BSA) over a range of approximately 3-30 milligrams in a total volume of 10 milliliters. Protein solutions were diluted appropriately with phosphate buffer to fall within the middle range of the curve, between 0.250 and 0.400 O. D. units, and the dilution factor noted for calculation of the initial protein concentrations.

Preparation of the Phosphate Buffered 40,000 g Supernatant

The crude homogenate was prepared essentially according to the method of Capstack et al. (17). One hundred seeds (approximately 23 g dry weight) previously germinated for a specified period were rinsed in distilled water and transferred to a Waring blender containing 75 ml of 100 mM fresh phosphate buffer, pH 7.4, which was 10 mM in $MgSO_4$, 10 mM reduced glutathione and 0.45 M sucrose. The seeds were homogenized for five seconds at low speed and 25 seconds at high speed. After passing the slurry through four layers of cheese

cloth, the crude homogenate was centrifuged at 40,000 g for ten minutes in a refrigerated Spinco Model L centrifuge to yield a clear, cell-free supernatant.

Preparation of the Tris-HCl Buffered 40,000 g Supernatant

All conditions and methods of preparation were identical to the phosphate buffered supernatant except 100 mM Tris-HCl buffer, pH 7.4, which was 10 mM in reduced glutathione was used as the homogenizing medium.

Preparation of the 30,000 g Yeast Supernatant

A crude yeast extract was prepared by autolyzing 50 g of commercial dry baker's yeast with 75 ml of glass distilled water and 7.5 ml of toluene for one hour at 37°. The yeast mixture was brought to room temperature, diluted with 100 ml of glass distilled water, and the pH maintained for an additional three hours at 7.4 by the addition of 5 N KOH at 30 minute intervals. The final autolysate was spun for ten minutes at 0°. The pellet and toluene layers were discarded and the middle layer retained. This layer has the capacity to synthesize all of the terpenoid intermediates through and including squalene from mevalonic acid.

Pork Liver F₃₀⁶⁰ Enzyme Solution

Pork liver homogenates were prepared by the method of Popják (124). Further purification and fractionation of these homogenates leads to a reportedly active squalene synthesizing system which may be used to biosynthesize two important intermediates of the isoprenoid pathway, IPP and FPP, to be used as substrates for enzymatic assays of IPP isomerase and squalene synthetase, respectively. Figure 5 briefly outlines the purification and fractionation scheme for making the F₃₀⁶⁰ solution, starting from fresh pork liver.

One hundred grams of fresh pork liver chilled in ice was finely minced with a pair of scissors or meat grinder and homogenized in a Waring blender for 30 seconds with 250 ml of 100 mM ice cold Tris-HCl buffer, pH 7.5, which was 5 mM in MgCl₂, 2 mM MnCl₂ and 30 mM nicotinamide. The homogenate was first centrifuged at 700-800 g for 20 minutes at 0° to remove heavy cell debris, filtered through eight layers of cheese cloth, and then centrifuged for 30 minutes at 10,000 g at 0°. The supernatant was decanted through another eight layers of cheese cloth, previously moistened with ice cold Tris buffer, followed by microsomal fractionation by one of two methods: protamine sulfate precipitation or high speed centrifugation. Where protein determinations were to be made on the F₃₀⁶⁰ solution, the former method was less desirable, since the protein contribution arising from added protamine sulfate was unknown and very difficult

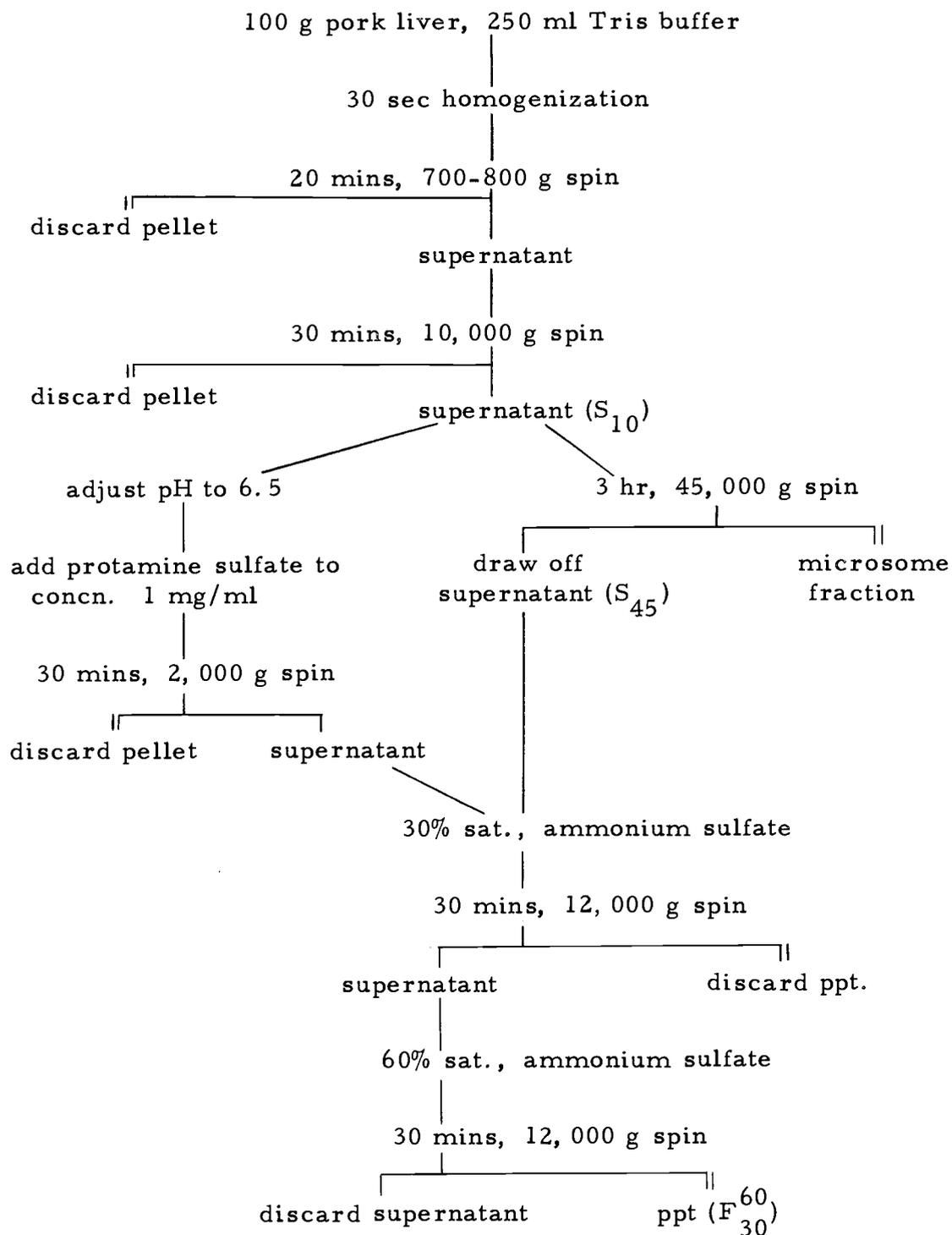


Figure 5. Preparation of Pork Liver F_{30}^{60} Fraction.

to estimate with any certainty.

Microsomal Fractionation

Protamine Sulfate. The pH of the 10,000 g supernatant was adjusted to 6.5 with 1 N acetic acid and then protamine sulfate added to a final concentration of 1 mg/ml. After allowing the mixture to stand for 20-30 minutes, the precipitated microsomal fraction was centrifuged at 2,000 g for 30 minutes at 0°. The supernatant was retained for subsequent ammonium sulfate fractionation.

High Speed Centrifugation. Microsomes were sedimented by spinning the 10,000 g supernatant for three hours at 45,000 g and 0°. The supernatant derived after sedimentation has been designated as the S₄₅ solution (124). The microsomal pellet is easily disturbed and contains considerable phosphatase and squalene synthetase activity. Contamination of the S₄₅ solution by either of these enzymes must be scrupulously avoided, if IPP and FPP are to be successfully prepared at a latter stage. Care was taken, consequently, to minimize microsomal contamination while gathering the S₄₅ solution. The top two-thirds of the 45,000 g supernatant from each tube was drawn off slowly with a Pasteur pipette, leaving the remaining third closest to the microsomal pellet untouched. The S₄₅ solutions were pooled together and then treated to ammonium sulfate fractionation as described in the next paragraph.

Ammonium Sulfate Fractionation

The volume of supernatant recovered free of microsomes by high speed centrifugation or protamine sulfate treatment was recorded and 17.6 g of ammonium sulfate per 100 ml of supernatant added slowly with stirring to bring the solution to 30% saturation. When the desired saturation was achieved, the mixture was allowed to stand an additional five to ten minutes. Protein precipitating out at 30% saturation was spun out at 12,000 g for 30 minutes at 0° and discarded. The remaining 12,000 g supernatant was measured for total volume and then brought to 60% saturation by adding 19.8 g of ammonium sulfate per 100 ml of supernatant. Centrifugation of the newly precipitated protein was carried out as before. Protein precipitating between 30 and 60% was rolled in Whatman #1 filter paper to take up excess buffer, then frozen at -15 to -20° as a crumbly crystalline precipitate. This fraction of the original pork liver homogenate has been designated the F_{30}^{60} preparation (124). Prior to enzymatic use, the F_{30}^{60} precipitate was dialyzed against 0.02 M KHCO_3 for four to five hours to remove excess ammonium sulfate. Dialyzed F_{30}^{60} solution can be stored for several weeks at -20° with no serious losses of isoprenoid synthesizing capacity from MVA (124).

Fructose-1, 6-diphosphate Aldolase Assay

Fructose-1, 6-diphosphate (FDP) aldolase was assayed essentially according to the method of Jagannathan, Singh and Damodaran (88). Slight modifications were made on the enzymatic control and in the order of reagents and enzyme addition. Cleavage of FDP was followed spectrophotometrically through the reaction of hydrazine sulfate with the product of the reaction, 3-phosphoglyceraldehyde, forming the hydrazone derivative which absorbs strongly at 240 nm.

Reagent solution (A) was made up as 3.5 mM hydrazine sulfate which was 0.1 mM in EDTA and adjusted to pH 7.5 with a few drops of 1 N NaOH. FDP, as substrate (B), was made up in water to 12 mM and adjusted to the same pH. The order of addition to a 1.0 cm silica cell was as follows: 2.0 ml of (A), 0.10 ml of 40,000 g supernatant and 1.0 ml of (B). A blank cell received the same solutions except 1.0 ml of water was substituted in place of (B). The cells were compared against one another on a Beckman DB spectrophotometer and recorder at 240 nm. A nonenzymatic assay was also run excluding the 40,000 g supernatant. The difference in initial velocity between the enzymatic and non-enzymatic assays was determined and taken as a measure of true aldolase activity. One enzyme unit was defined as $\Delta O.D._{240nm} \text{ min}^{-1} \text{ ml}^{-1}$ of reaction mixture.

Alkaline Phosphatase Assay

Alkaline phosphatase was assayed by the method of Garen and Levinthal (51) with a few modifications. p-Nitrophenyl phosphate (NPP) served as the substrate. Hydrolysis of NPP by alkaline phosphatase yields p-nitrophenol (NP) and inorganic phosphate. Since the former product absorbs strongly at 410 nm, phosphatase activity is most conveniently assayed spectrophotometrically.

To a 1.0 cm silica cell was added 0.20 ml of 40,000 g supernatant and 3.0 ml of 1.0 M Tris-HCl buffer, pH 8.4, which was 5 mM in MgSO_4 and 1 mM in NPP. A blank cell received the same solutions, except NPP was absent from the buffer. The absorbance at 410 nm as a function of time was recorded directly with a Beckman DB spectrophotometer and recorder. Phosphatase activity was determined from the initial slope achieved within seconds after adding NPP to the experimental cell. One unit of alkaline phosphatase activity was defined as $0.0618 \times \text{O.D.}_{410\text{nm}} \text{ min}^{-1} \text{ ml}^{-1}$ of reaction mixture.

In Vivo Assays of MVA-2-¹⁴C Incorporation and Distribution Among Products of the NSF

Five seeds, which were serrated, were germinated for a specified period in a small volume (3-4 ml) of distilled water with 20-25 μl of MVA-2-¹⁴C (2 μC , specific activity, 3-5 $\mu\text{C}/\mu\text{M}$). At the end of the incubation period the seeds were carefully washed free of residual

MVA-2-¹⁴C, ground in liquid nitrogen to a fine powder in a mortar and pestle and then transferred in a small volume of water to a glass stoppered extracting tube. Five to ten milliliters of 20% KOH was added to the aqueous slurry and the resulting solution saponified for four hours on a steam bath. The nonsaponifiable fraction (NSF) was extracted from the solution with four to five volumes (20-25 ml/ volume) of diethyl ether, concentrated to a small volume on a steam bath and then quantitatively transferred to a graduated test tube. An aliquot (1/100) was removed and counted in nonaqueous scintillation fluid to determine the total radioactivity incorporated into the NSF. An additional aliquot was chromatographed by TLC, from which the distribution of label among NSF products was calculated. The latter calculation was made from a radiochromatographic scan of the plate. Total MVA-2-¹⁴C taken up by the seeds was determined from the difference between the initial radioactivity added and the residual amount remaining in the washings (an aliquot of the washings was counted in Bray's scintillation fluid). The distribution of radioactivity among the NSF products was then expressed as a percentage of MVA-2-¹⁴C taken up by the seeds. By incubating seeds for varying periods, the capacity to incorporate label into NSF products was followed as a function of seed germination.

In Vitro Assays of MVA-2-¹⁴C
Incorporation into the NSF (Squalene)

Three milliliter aliquots of the 40,000 g supernatant were thermally equilibrated to 24° in a constant temperature water bath for 2-3 minutes. To each sample was then added 100 μl of ATP solution (181 μmoles), 100 μl of a mixture of glucose-6-phosphate (G-6-P) and NADPH (10 μmoles/181 μmoles), 10 μl of G-6-P dehydrogenase (2 units, P-L Biochemicals) and 20 μl of MVA-2-¹⁴C (2 μC, specific activity, 5.9 μC/μM). Following a one hour incubation, each assay was terminated with 1.5-2.0 ml of 20% KOH and saponified for two hours on a steam bath. The NSF was then extracted in four volumes of diethyl ether (20-25 ml/volume). The ether layer was evaporated off under a hood on a steam bath and the remaining NSF transferred in ether (5-10 ml) to a graduated test tube. Aliquots (1/100) of the final NSF were removed and counted in either Bray's or nonaqueous scintillation fluid. Quenching corrections were made with a toluene-¹⁴C standard by internal spiking. Aliquots of the NSF were also subjected to TLC to ascertain the distribution of label among NSF products. Although the ether extract has been designated the NSF, subsequent analysis showed that in all cases only labelled squalene was present. The 40,000 g supernatant lacked the capacity to make tetra- and pentacyclic products. The capacity to incorporate MVA-2-¹⁴C into squalene as a function of germination was expressed in units of

$\mu\text{moles min}^{-1} \text{mg}^{-1}$ of total protein.

Preface to Subsequent Methods and Enzyme Assays

Subsequent assays of the individual enzymes of the isoprenoid pathway are complex and, in most cases, require the preparation of substrates not commercially available. Hence, the following methods may not be treated routinely, if an intelligent evaluation of the final results is to be achieved. A more detailed description of each enzyme and the conditions by which it was assayed is given. In general, the preparation of each substrate (where necessary), its identification and final criteria of purity are discussed, followed by a brief description of the appropriate enzyme assay. In a later section (General Results and Discussion), these individual studies are integrated into a more dominant theme, by which this thesis may be more coherently discussed.

Corrections for isotopic dilution of added substrates by endogenous pools have been neglected. Quantitative estimates and variations in pool sizes, which may markedly affect the flow of intermediates through the isoprenoid pathway, are unknown.

Mevalonic Kinase Assay

As reported in the literature, mevalonic kinase has been assayed in three ways: (i) radiochromatographically using

Dowex-formate columns (159), for the measurement of the rate of disappearance of MVA-2-¹⁴C; (ii) by paper chromatography (41, 160) and thin layer chromatography (124) for the measurement of the appearance of phosphorylated products; (iii) spectrophotometrically (160) as a coupled reaction with pyruvate kinase and lactate dehydrogenase to measure NADH consumption.

There are certain disadvantages with all of the assay procedures. The spectrophotometric assay may only be used with relatively pure preparations of the kinase, as it is ultimately dependent upon the measurement of ADP formed during conversion of MVA to MVAP by ATP. However, reactions such as the further conversion of MVAP to MVAPP or of MVAPP to IPP utilize ATP and also produce ADP in the process. Furthermore, preparations of MVA kinase are frequently contaminated by enzymes catalyzing these two reactions and as a consequence activity measurements by this procedure may be high. Other difficulties encountered using this assay for crude preparations have also been discussed (124). The assay involving paper and thin layer chromatography of the extract for quantitation of the products of MVA metabolism must be carried out on small volumes to avoid overloading of the paper or plate. Although they have the advantage of requiring very small amounts of substrate, the paper chromatographic assay, at least, suffers from a relatively large inaccuracy (160). The dowex-formate column chromatography of the

assay mixture is the most rigorous method for the measurement of disappearance of substrate, but it is also the most laborious.

A new procedure for the assay of MVA kinase has been developed (70), based upon measurement of the rate of disappearance of MVA-2-¹⁴C under conditions of first order kinetics with respect to the substrate. The residual MVA in the extracts is removed by a modification of a procedure of Porter and Guchhait for the isolation of MVA lactone (132). That the residual radioactivity is associated only with MVA lactone is confirmed by paper and gas chromatography. In addition, all radioactive metabolites of MVA in the extract were identified by ion exchange and paper chromatography, confirming the fact that disappearance of MVA must have occurred only through the kinase. Phosphatase activity in the extract has no influence on the assay.

A series of tubes, each containing 3.0 ml of the crude 40,000 g supernatant and 0.18 micromoles of ATP, were thermally equilibrated in a water bath to 24° and 10 µl of R, S-MVA-2-¹⁴C solution added to each tube. After incubating the tubes for various intervals of time, 2.0 ml of hot 20% KOH was added to each tube to terminate the reaction and then 2 mg of cold MVA lactone was added to each sample as carrier. Each sample was transferred quantitatively to a continuous ether extractor, the solutions acidified to pH 1 with 12 N H₂SO₄, and allowed to stand at 37° for 15-30 minutes to lactonize

non-metabolized MVA. The incubations were terminated with 20% KOH rather than acid because it was found that protein precipitated in the presence of acid and made the quantitative transfer of the solution to the extractor difficult. The alkali treatment resulted in a clear solution. Following lactonization, the samples were continuously extracted with diethyl ether for 12-14 hours under reflux. The ether extracts were concentrated to a small volume on a steam bath under a hood, transferred to graduated test tubes in tetrahydrofuran (5-10 ml), and aliquots (1/100) removed for scintillation counting in Bray's scintillation fluid. Each sample aliquot was internally spiked with a toluene- ^{14}C standard in order to detect quenching. The decrease in radioactivity of the ether extract with time of reaction provided the basis of the mevalonic kinase assay. For routine measurements of kinase activity in pea seed homogenates, an incubation time of 2.0 minutes was used.

Results and Discussion

For the assay to be valid it was necessary to establish the following: (i) that under the conditions of the assay the total ^{14}C content of the ether extract was truly mevalonolactone; (ii) that the enzyme assayed was mevalonic kinase; (iii) that the assay was not influenced by phosphatase; and (iv) that the mevalonolactone was not bound to protein.

To substantiate that the ^{14}C content of the ether extract represented MVA-2- ^{14}C lactone, aliquots from incubations allowed to proceed for different lengths of time were subjected to gas and paper chromatography. Gas chromatography was carried out on the same column used for the analysis of prenols, as previously described, and was run isothermally at 195° . Not until more than 90% of the R-MVA had been metabolized, were labelled metabolites detected in the ether extract. From Figure 6 it can be seen that this represents an incubation time slightly greater than six minutes. Where other labelled compounds are found in the ether extract, internal normalization of the radiochromatographic tracings with respect to mevalonolactone can be used to determine the proportion of ^{14}C associated with mevalonolactone. Sensitivity of the counting equipment permitted the detection of less than 2% of the emerging radioactivity in a single peak. Ascending paper chromatography of the extract in solvent systems I and IV also demonstrated no other contaminating component to be present until 90% of the R-MVA had been metabolized. The measure of the ^{14}C content of the diethyl ether extract under the conditions of the assay was then concluded to represent the non-metabolized MVA.

It was necessary to correlate the disappearance of MVA with the appearance of products resulting from metabolism through the kinase. An enzyme aliquot in which 32% of the MVA had metabolized

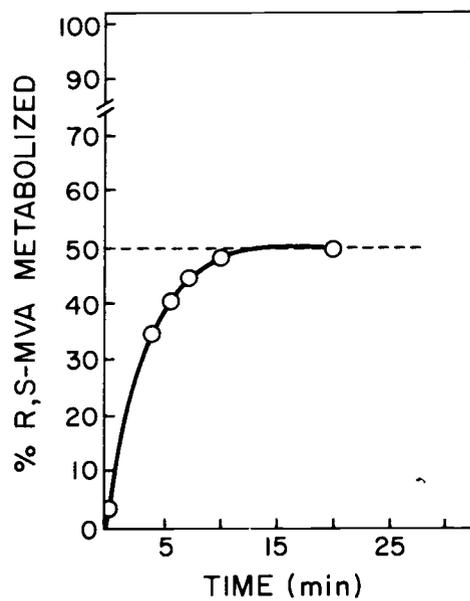


Figure 6. Stereospecificity of MVA kinase for one enantiomer of R,S,-MVA-2-¹⁴C.

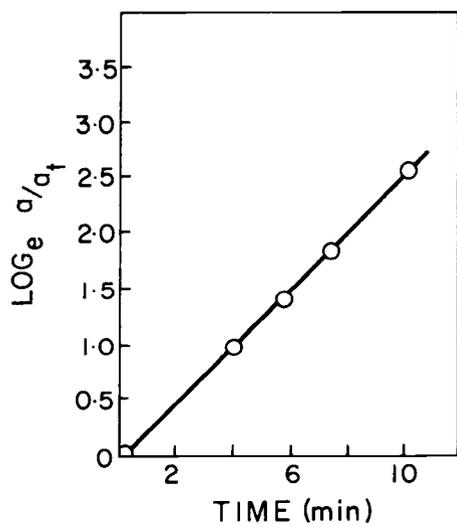


Figure 7. First order kinetic assay of MVA kinase.

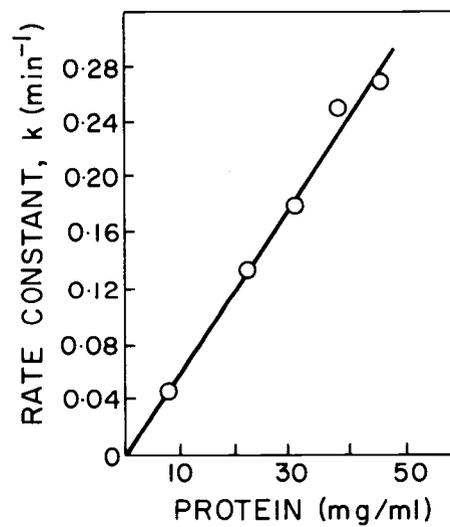


Figure 8. Demonstration of linear response of the MVA kinase assay to changes in enzyme concentration.

was subjected to the following procedures:

a) DEAE cellulose chromatography under conditions that separated MVA from the other radioactive components. These metabolites were subjected to paper chromatography in solvent systems I, II and IV for further identification.

b) Dowex-formate chromatography of the acid stable intermediates.

c) Acid hydrolysis and evaporation of volatile compounds for the determination of allyl derivatives (160). By these methods the distribution of radioactivity in the enzyme aliquot was found to be: MVA (68%), MVAP (6-7%), MVAPP (9-10%), IPP (4-5%), DMAPP (11%) and IP (1%). No higher allyl phosphates or pyrophosphates other than DMAPP were observed. By these procedures, all of the radioactivity in the incubate was accounted for as products resulting from metabolism of the MVA through MVA kinase.

For phosphatase to interfere with the assay, only the hydrolysis of MVAP back to MVA need be considered. In a separate study, the specific activity of MVAP kinase in the pea seed supernatant was measured¹ according to the method of Tchen (160). An excess of MVAP was added to several samples of the supernatant to test for phosphatase interference over different periods of time. Paper

¹See page 65, Phosphomevalonic Kinase Assay.

chromatographic analysis of the incubation products in solvent system IV revealed that no MVA accumulated during an incubation time three times the duration of the routine MVA kinase assay.

First order binding of MVA to protein would be indistinguishable from MVA kinase activity. The association of MVA with protein may take place during the enzymatic incubation period and/or during the isolation process following termination of the assay. In either case an anomalously high MVA kinase activity would be measured. Binding under the conditions of isolation could conceivably arise by acid-catalyzed esterification of MVA to a number of residues on protein (ser, thr, asp, glu, etc.). That this does not occur is evidenced by complete recovery of mevalonolactone at zero time incubation (Figure 7). The absence of any significant binding during incubation was shown by incubating a typical enzyme preparation for approximately ten minutes with a known amount of R, S-MVA and determining the proportion of ^{14}C recoverable by dialysis. According to the lactone assay, ten minutes is sufficient time to convert 92% of the R-MVA into products (Figure 6). Even under very mild conditions of termination with 0.10 M EDTA and subsequent dialysis, essentially all of the ^{14}C was recovered in the dialysate (Table 2). From these results, it appears that binding of MVA to protein does not interfere with the kinase assay.

Stereospecificity for the substrate was observed, although the

actual enantiomer metabolized was not determined. Metabolism of R, S-MVA ceased when 50% of the racemic substrate initially added had been consumed (Figure 6). A number of authors have isolated MVA kinase from various plant and animal sources and have shown it to be stereospecific for phosphorylation of racemic MVA (47, 107, 159). The absolute stereochemistry of the utilizable enantiomer has been established as R-mevalonate (34). Accepting the available substrate to be one-half the total added to the enzyme preparation, changes in the concentration of the utilizable enantiomer during the period of the assay can be calculated. The first order rate equation used for the initially racemic substrate is:

$$(1)^2 \quad \log_e 0.5A_o / (A_t - 0.5A_o) = kt$$

Figure 7 shows the linear relationship between reaction time and a logarithmic function of substrate disappearance relative to initial substrate, and Figure 8 the linear relationship between the first order rate constants and enzyme concentration.

$$^2 \log_e 0.5A_o / (A_t - 0.5A_o) = \log_e a_o / a_t$$

where A_o = total racemic substrate at $t = 0$
 a_o = total available substrate at $t = 0$
 A_t = total substrate remaining at time t
 a_t = available substrate remaining at time t

then $a = 0.5A_o$ and $a_t = A_t - 0.5A_o$ so that the typical ratio of initial to final substrate concentration, a_o/a_t , becomes $0.5A_o / (A_t - 0.5A_o)$.

Table 2. Influence of Protein Binding on Recovery of Radioactivity.¹

Initial MVA-2- ¹⁴ C x 10 ⁻⁶ dpm	Non-dialyzable ¹⁴ C ² x 10 ⁻⁶ dpm	% Dialyzed ¹⁴ C
0.7065	.016	98

¹ Refers to dialyzable ¹⁴C from a ten minute incubation in the presence of MVA-2-¹⁴C.

² Label remaining in the dialysis bag after 18 hour dialysis against several changes of water with intermittent changes against saturated NaCl solution. The incubated sample was terminated with 3.0 ml of 0.1M EDTA instead of KOH solution.

Having established the validity of the assay, the endogenous activity of MVA kinase was assayed in the 40,000 g supernatant as a function of germination period. The 40,000 g supernatant was prepared as described earlier on sets of seeds germinated for various periods. Enzyme activity was expressed in terms of the first order rate constant per milligram of total protein. Table 3 presents the experimental measurements in tabular form. Figure 9 is a plot of MVA kinase specific activity versus germination period. The specific activity of MVA kinase as a function of germination was relatively constant, exhibiting a slight decrease over the first 32 hours of germination.

Biosynthesis of MVAP-2-¹⁴C

MVAP-2-¹⁴C has been biosynthesized from MVA-2-¹⁴C with

Table 3. Assay of MVA Kinase by First Order Kinetics versus Germination Period.

Germination (hours)	Supn ^t (ml)	Time (min)	Protein (mg)	k ¹ (min ⁻¹)	Specific activity (k mg ⁻¹)
2.0	3.0	2.0	98.0	0.554	5.64 x 10 ⁻³
4.0	"	"	131.5	0.900	6.85
8.0	"	"	129.5	0.877	6.78
12.0	"	"	126.0	0.785	6.23
14.0	1.5	"	69.0	0.392	5.69
20.0	"	"	71.5	0.346	4.84
28.1	"	"	67.3	0.382	5.69
32.1	"	"	64.9	0.282	4.35

¹First order rate constant
 Initial R, S-MVA-2-¹⁴C = 159 μmoles/assay

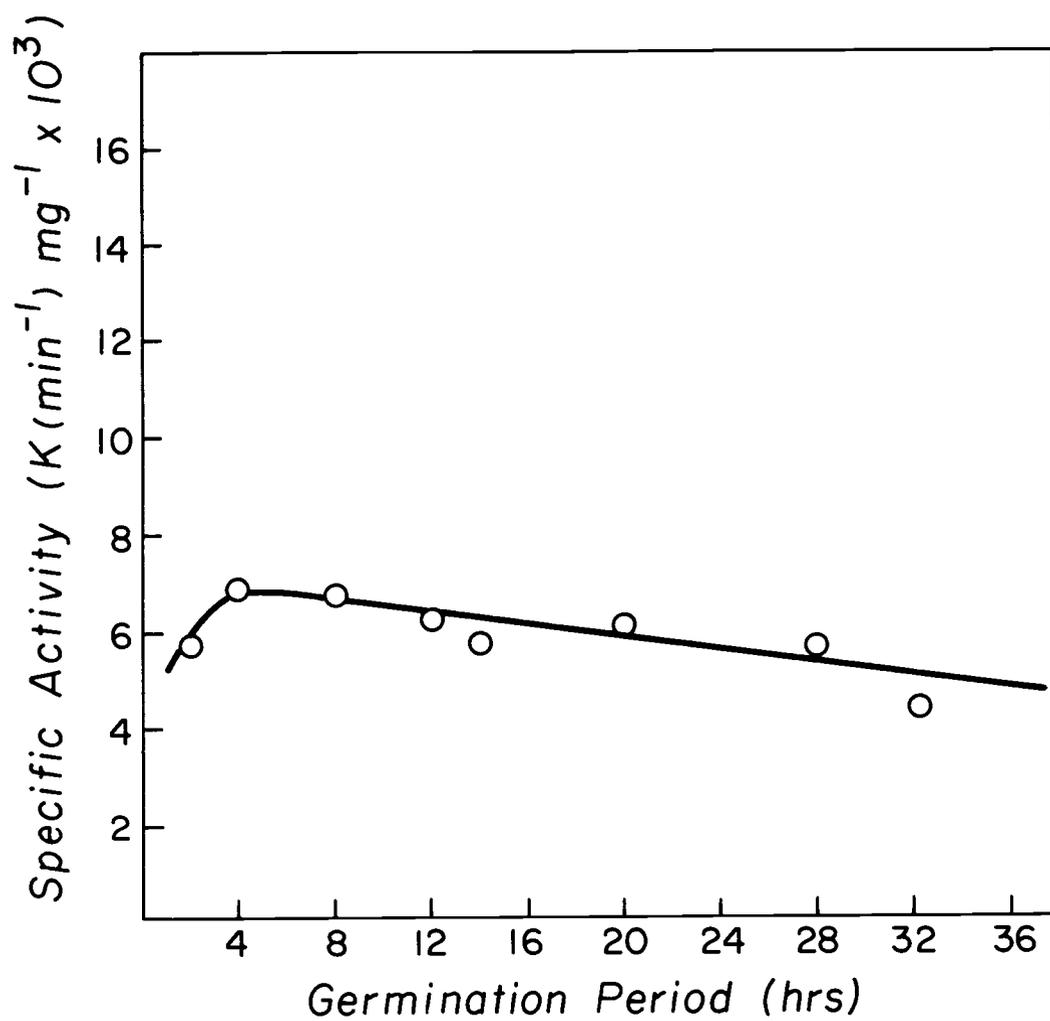


Figure 9. Specific activity of MVA kinase as a function of germination.

yeast (161) and pork liver preparations (124). By either method, the basic principle has been to isolate and purify MVA kinase from contaminating MVAP kinase, so that MVAP may be caused to accumulate as the end product. Two alternate methods are described below, one of which is considerably simpler than any method previously described (see method B).

Method A

MVAP-2-¹⁴C was biosynthesized from an F₃₀⁶⁰ enzyme preparation derived from pork liver. This fraction was first described by Popják as a convenient source for the biosynthesis of IPP and FPP (124). After preparing the F₃₀⁶⁰ fraction according to the method of Popják, it was found that MVA kinase activity consistently exceeded the activity of MVAP kinase by several fold. This difference was enhanced in the presence of five millimolar iodoacetamide. Under such conditions, a short term incubation with excess labelled MVA led to reasonably large accumulations of MVAP-2-¹⁴C.

To 0.10 ml of the F₃₀⁶⁰ fraction (10-20 mg total protein) was added 1.8 ml of 0.10 M phosphate buffer which was 10 mM in MgSO₄, 30 mM KF and 5 mM iodoacetamide. After thermally equilibrating the enzyme solution to 37^o, 0.10 ml of 70 mM ATP solution and 0.04 ml of R,S-MVA-2-¹⁴C (4 μC, specific activity 1.5-6.0 μC/μM) were added. Fifteen minutes later the protein was removed by the addition

of five drops of 10% HClO_4 . The precipitated protein was removed by centrifugation on a clinical centrifuge and the supernatant neutralized with 20% KOH. KClO_4 was similarly sedimented after the neutralized solution had been chilled to approximately 0° . The supernatant was retained for isolation and purification of MVAP-2- ^{14}C .

Method B

Fleischmann dry baker's yeast (50 g) was mixed with 75 ml of glass distilled water and 7.5 ml of toluene. The mixture was autolyzed for one hour at 37° ; a 30,000 g supernatant was prepared as previously described. A portion of the 30,000 g supernatant (25-50 ml) was then dialyzed overnight against three liters of 0.05 M phosphate buffer, pH 7.4, in a cold room set at approximately 6° . One-half milliliter of the dialyzed 30,000 g yeast supernatant was incubated at 37° for one hour with 0.05 ml of R,S-MVA-2- ^{14}C ($5 \mu\text{C}$, diluted with cold MVA to a specific activity of $0.099 \mu\text{C}/\mu\text{M}$) and 25 ml of 32 mM Tris-HCl buffer, pH 7.4, which was 4.8 mM in iodoacetamide, 6.4 mM sodium EDTA, 9.6 mM MgCl_2 , 4.8 mM ATP and 48 mM KF. The incubation was terminated by heat denaturing the yeast incubate for 5-10 minutes and the precipitated protein spun out at 10,000 g for ten minutes at 0° . The supernatant was retained for isolation of biosynthesized MVAP-2- ^{14}C .

It is tempting to ascribe the success of the latter method to a

tanning effect associated with the Fleischmann yeast. The 30,000 g yeast supernatant was observed to tan noticeably, turning progressively darker during dialysis. If the solution was used immediately, such aliquots of the yeast supernatant readily incorporated labelled MVA into higher products other than MVAP. Advantage of this fact is taken for the synthesis of IPP (page 80). A dialyzed aliquot, incubated under identical conditions, allowed only MVAP to accumulate (Figure 10). Recently, evidence has been presented which suggests that MVAP kinase is a sulfhydryl sensitive enzyme (124). Hence, the preferential inactivation of MVAP kinase, as opposed to MVA kinase, might be anticipated under mild tanning conditions. The presence of a potential tanning agent (TBA), t-butyl anisol, was noted to be an additive of Fleischmann's yeast, not found in other commercial brands. Other sources of yeast extract have been subjected to dialysis with no serious losses in MVAP kinase activity (147). Consequently, it would appear that MVAP-2-¹⁴C may be successfully prepared through exploitation of a tanning effect against MVAP kinase, as opposed to the more stable MVA kinase.

Isolation and Purification of MVAP-2-¹⁴C

MVAP-2-¹⁴C was most conveniently isolated as a single peak from a Dowex-formate column (1.5 x 10 cm) in a formic acid/ammonium formate gradient (Figure 10). The formic acid was taken off

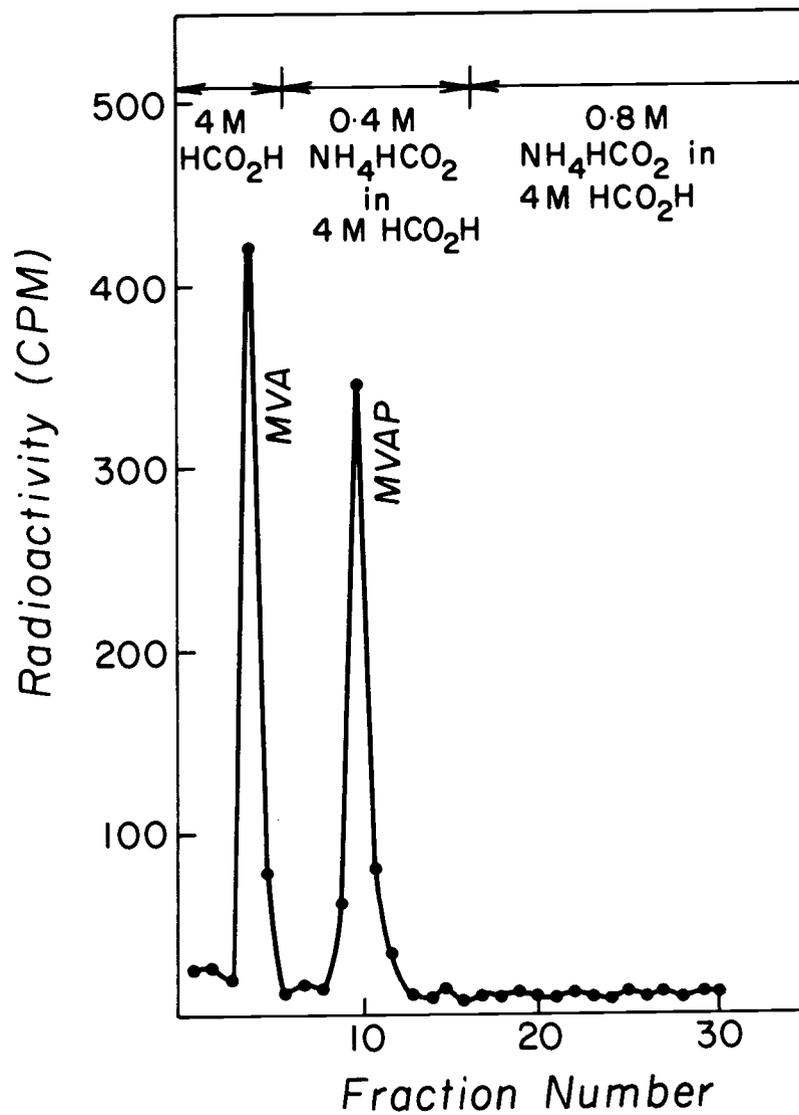


Figure 10. Isolation of newly biosynthesized MVAP-2-¹⁴C in the 30,000 g yeast supernatant by Dowex-1-formate column chromatography.

under vacuum at 45-50^o on a rotory evaporator, followed by sublimation of residual traces of ammonium formate. Radiochemically pure MVAP-2-¹⁴C (Figure 11) was stored at -15^o at neutral or slightly alkaline pH. Besides Dowex-formate chromatography, the identity and purity of MVAP-2-¹⁴C was ascertained by paper chromatography in solvent systems I and IV.

Phosphomevalonic Kinase Assay

The assay of MVAP kinase was carried out according to the method of Tchen (160). Ten microliters of MVAP-2-¹⁴C (11000-17,000 dpm, specific activity 0.099-1.54 $\mu\text{C}/\mu\text{M}$) was added to 0.10-0.30 ml of 40,000 g supernatant containing 6-20 μmoles of ATP. Following a short incubation period, the assay was terminated by adding an equal volume of 0.10 M EDTA solution to the incubation tube. The EDTA treated incubate was then transferred quantitatively to a Dowex-formate column and the remaining MVAP eluted off as a single peak with 0.4 M ammonium formate in 4 M formic acid. Fractions of the MVAP peak were combined and an aliquot removed and counted in Bray's scintillation fluid to determine the total radioactivity remaining. Quenching corrections were made using a toluene-¹⁴C internal spike. The difference between initial and final MVAP radioactivity was determined and expressed as MVAP kinase activity in units of μmoles of MVAP phosphorylated $\text{min}^{-1} \text{mg}^{-1}$ of

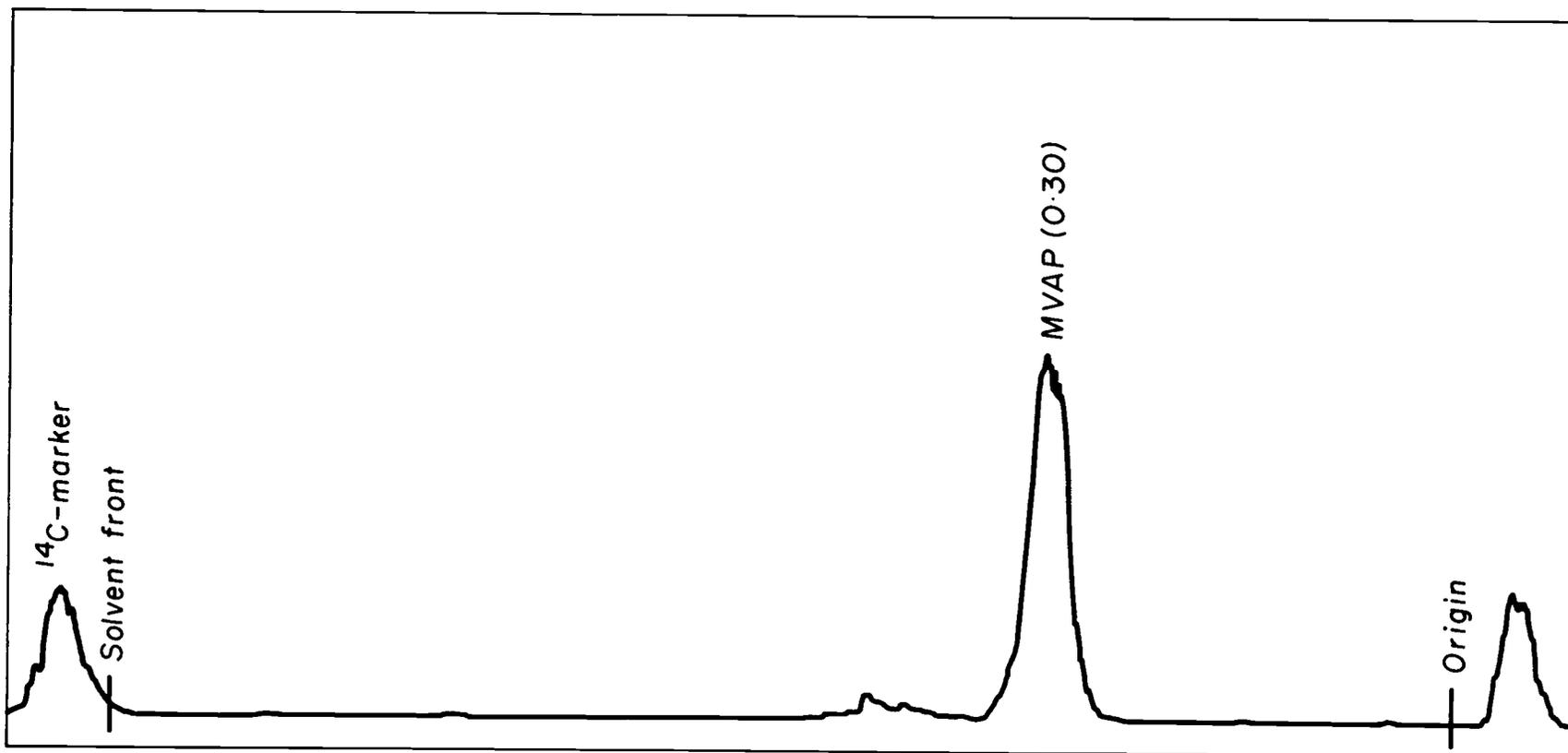


Figure 11. Paper radiochromatographic scan (solvent system I) of presumptive MVAP-2- ^{14}C isolated by Dowex-1-formate chromatography.

total protein.

Results and Discussion

The turnover of MVAP-2-¹⁴C was followed as a function of time on 0.30 ml aliquots of the 40,000 g supernatant prepared from a 16 hour set of germinated seeds (Figure 12). The rate of MVAP-2-¹⁴C was linear during the first four minutes of the assay. Subsequent assays were carried out on 0.10 ml enzyme aliquots incubated from 1.0-3.0 minutes. Apparently phosphatase activity did not compete for labelled MVAP, as seen by a nearly complete conversion of substrate into higher intermediates (Figure 12). Furthermore, at no time was MVA-2-¹⁴C detected in incubates treated to Dowex-formate chromatography, although MVAP-2-¹⁴C was readily recovered and quantitated.

MVAP kinase specific activity was measured over the first 20 hours of germination. Experimental measurements are summarized in Table 4 and a plot of specific activity versus germination period is given in Figure 13. During the first 12 hours of germination, the enzyme showed a 4-5 fold rise in activity.

Pyrophosphomevalonate Decarboxylase Assay

Although MVAPP is the natural substrate of MVAPP decarboxylase, it is not available commercially. A limited number of

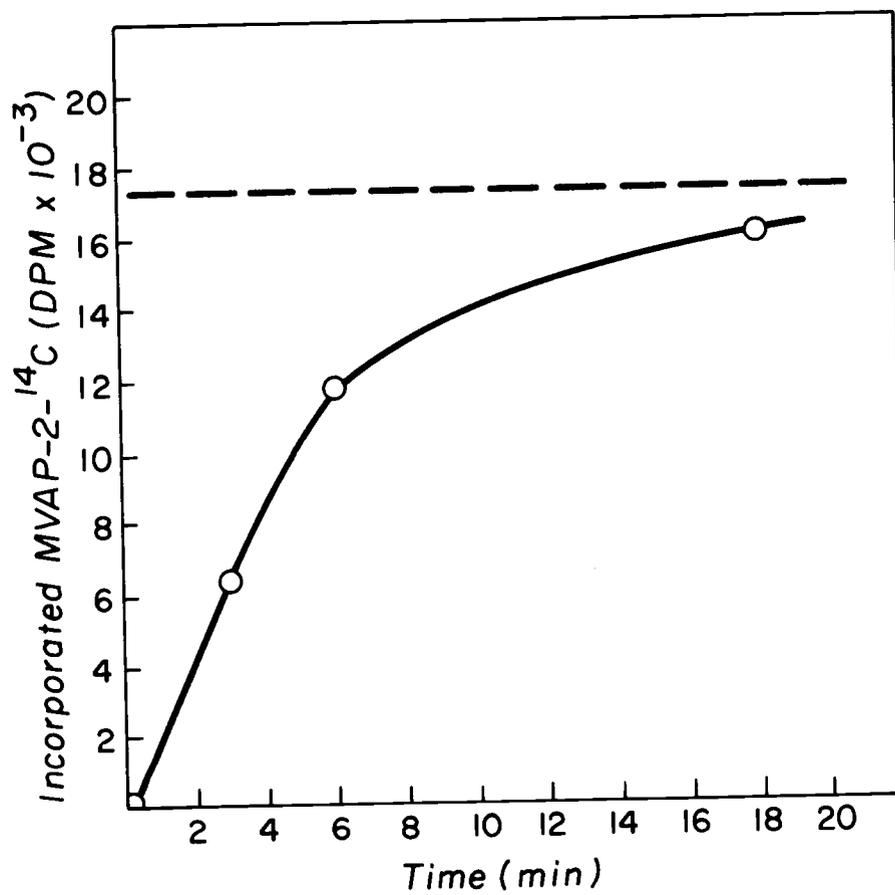


Figure 12. Turnover of MVAP-2-¹⁴C as a function of time. Dashed line indicates total MVAP-2-¹⁴C initially available.

Table 4. Assay of MVAP Kinase Specific Activity Versus Germination Period.

Germination (hrs)	Supn't (ml)	Time (min)	Protein (mg)	Incorporated MVAP (dpm)	Specific activity	
					(dpm min ⁻¹ mg ⁻¹)	(mμmoles min ⁻¹ mg ⁻¹)
2.0	0.10	2.0	2.98	680	114	3 x 10 ⁻²
4.0	0.10	3.0	4.40	2512	190	6
6.0	0.10	3.0	5.33	5718	358	11
8.0	0.10	3.0	4.46	6406	479	14
12.0	0.10	3.0	4.60	7406	536	16
15.3	0.10	1.0	4.30	2126	495	14
20.1	0.10	1.0	4.44	2359	531	16
2.0	boiled	-	-	0 ± 180	-	-

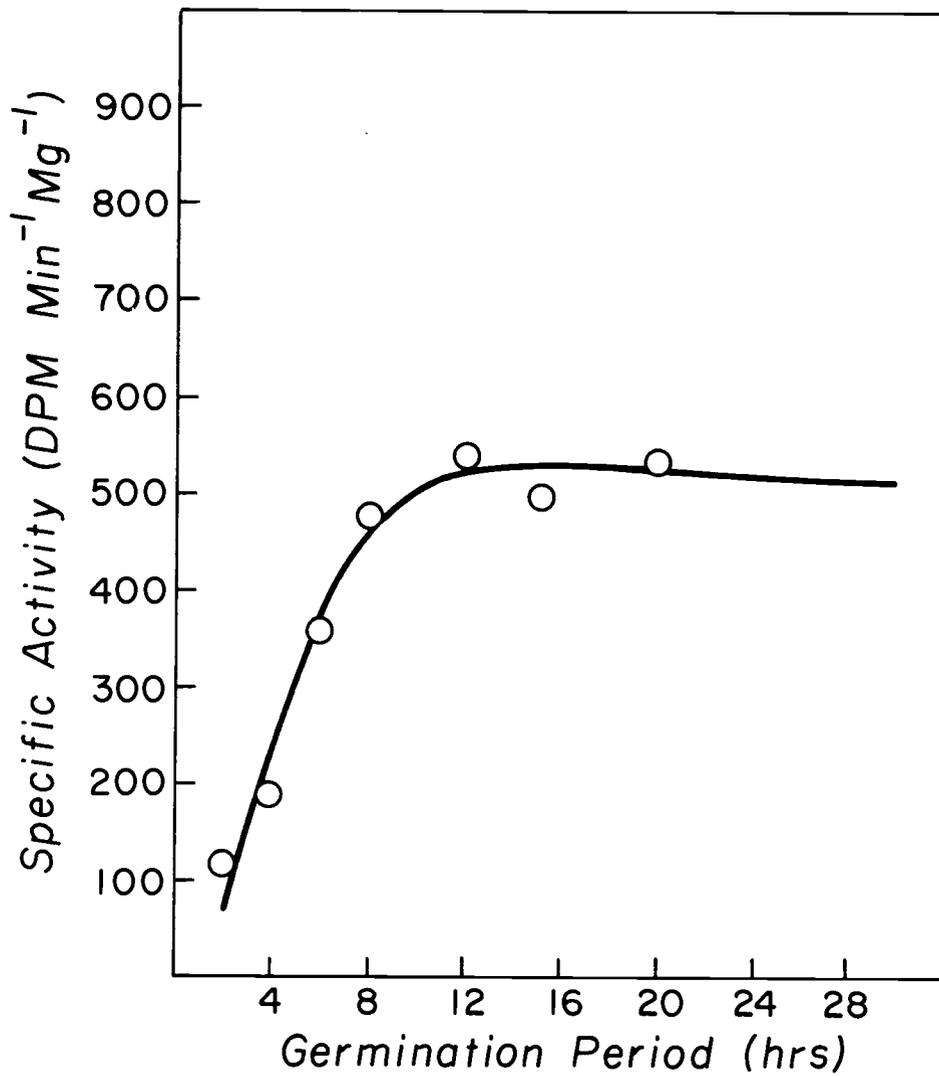


Figure 13. Specific activity of MVAP kinase as a function of germination.

biosynthetic methods have been developed for making MVAPP from MVA (124, 161). Such methods are quite tedious. Regardless of the enzyme source, the customary procedure has been to separately purify each of the two preceding enzymes, MVA kinase and MVAP kinase, from which MVAPP is made. MVA is first incubated with ATP and MVA kinase in a suitable buffer. Freshly synthesized MVAP, as the end product, is thus isolated from the incubation mixture and incubated under similar conditions with MVAP kinase to yield the final product. In assaying the decarboxylase, MVAPP-1- ^{14}C is frequently prepared, starting from MVA-1- ^{14}C . Hence, the rate of the reaction is conveniently followed by trapping and counting liberated $^{14}\text{CO}_2$ (124).

MVAPP decarboxylase may be assayed indirectly with commercially available MVA-1- ^{14}C , measuring the rate of $^{14}\text{CO}_2$ evolution under conditions in which the enzyme is the rate limiting step in converting MVA to IPP. By this latter method, the rate of $^{14}\text{CO}_2$ evolution is, in fact, a measure of the rate limiting step of the pathway lying between MVA kinase and MVAPP decarboxylase, responding directly in proportion to the turnover capacity of the slowest acting enzyme. If a short pulse of labelled MVA is run on an incubate under enzyme saturating conditions, then the rate limiting step, and hence the enzyme directly associated with $^{14}\text{CO}_2$ evolution, may be deduced by observing which precursors accumulate label. Concerning the three enzymes involved in transforming MVA into IPP, one of the

following conditions may be anticipated:

(i) Where MVA kinase is rate limiting, neither MVAP nor MVAPP will accumulate in pulsed incubates.

(ii) If MVAP kinase is the rate limiting step, MVAP will accumulate, but not MVAPP.

(iii) If the last enzyme in the series, MVAPP decarboxylase, is rate limiting, then label will be observed to accumulate in MVAPP.

(iv) Where the decarboxylase is rate limiting, the rate of $^{14}\text{CO}_2$ evolution must be less than the turnover rate of MVA and MVAP.

Conditions (iii) and (iv) must be satisfied, if the decarboxylase is to be assayed indirectly with MVA-1- ^{14}C .

The apparatus used for trapping and counting the $^{14}\text{CO}_2$ was patterned after an assembly originally described by Cuppy and Crevasse (37) and, more recently, by an improved version introduced by Habibulla and Newburgh (74). Ten milliliter glass flasks, serum caps and polyethylene center wells were purchased from Kontes Glass.³ To each flask was added one milliliter of 40,000 g supernatant and 0.10 ml of neutralized ATP solution (0.18 μmoles). Next, 0.10 ml of 20% KOH was pipetted into each center well and the entire serum cap assembly inserted over the flask opening to form an

³Catalog Item Nos. K-882300, K-882310 and K-882320.

air tight seal. Each flask was thermally equilibrated for approximately three minutes to reach 24° ; then 10 μ l of MVA-1- ^{14}C ($\approx 1\mu\text{C}$, specific activity 3.8 $\mu\text{C}/\mu\text{M}$) was injected through the serum cap into the enzyme solution. After a 20 minute incubation, each assay was terminated with 0.30 ml of 6 N HCl and incubated for an additional hour on a shaker bath at 37° to drive dissolved $^{14}\text{CO}_2$ into the alkaline center well. The center wells were removed from the incubating flasks and dropped directly into Bray's scintillation fluid for counting of the trapped $^{14}\text{CO}_2$. Each scintillation vial was carefully cooled and kept in the dark for several hours until fluorescence ceased. The samples were then counted and corrected for quenching by automatic external standardization (170)

Results and Discussion

Conditions (iii) and (iv) were found to exist in 40,000 g supernatants in terms of label distribution studies (Figure 14) and by assays of MVA and MVAP kinase⁴, thus allowing the use of MVA-1- ^{14}C as an indirect, but responsive substrate for assaying MVAPP decarboxylase. Figure 15 shows the kinetics of $^{14}\text{CO}_2$ evolution for two periods of germination: (a) at two hours, where the rate is minimal, and (b) at nine hours, where the rate reaches a maximum over

⁴See Tables 3, 4 and 5.

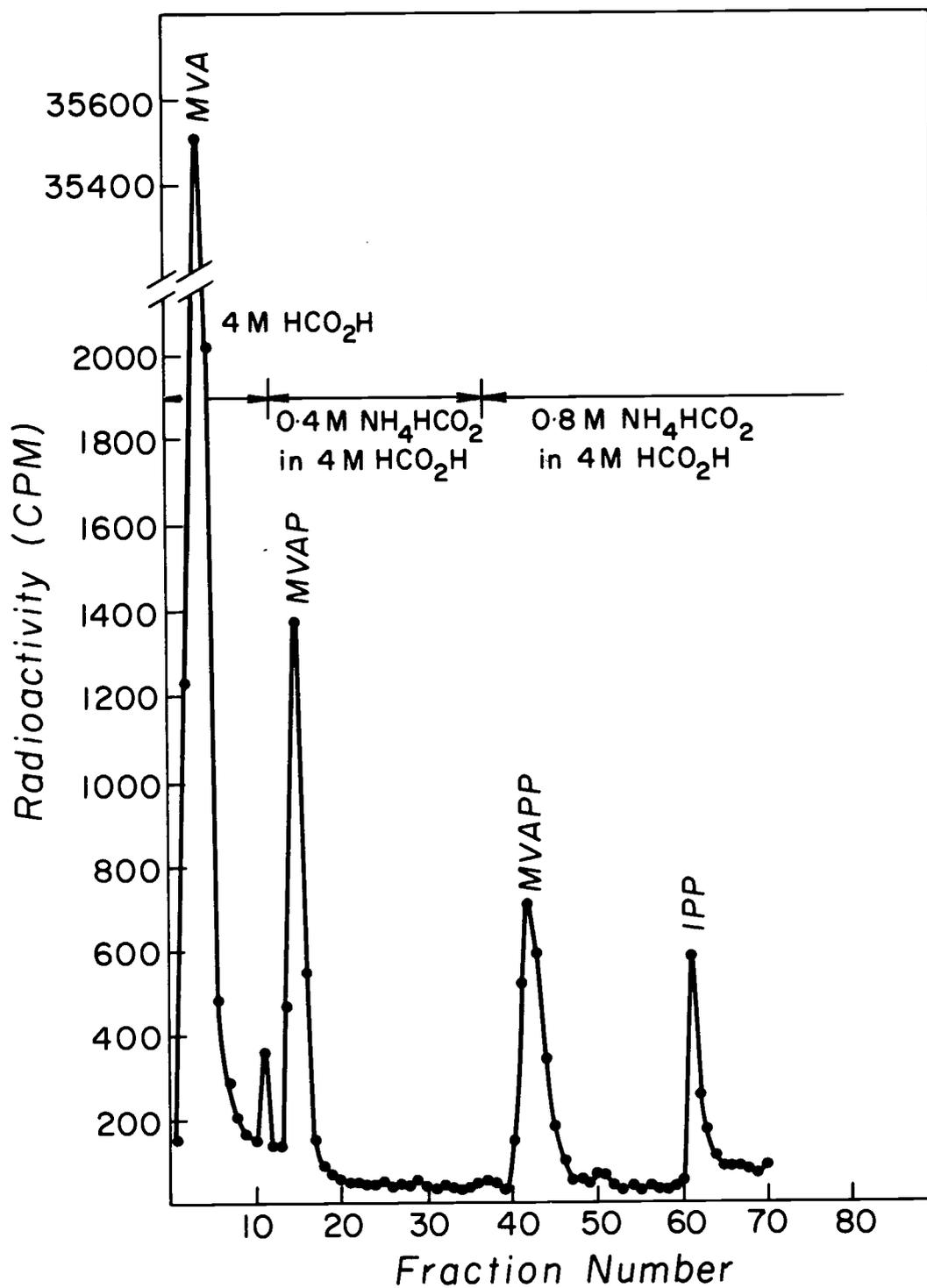


Figure 14. Dowex-1-formate chromatographic separation and distribution of acid stable isoprenoid products in the 40,000 g supernatant from a 30 min incubation with excess R, S-MVA-2-¹⁴C.

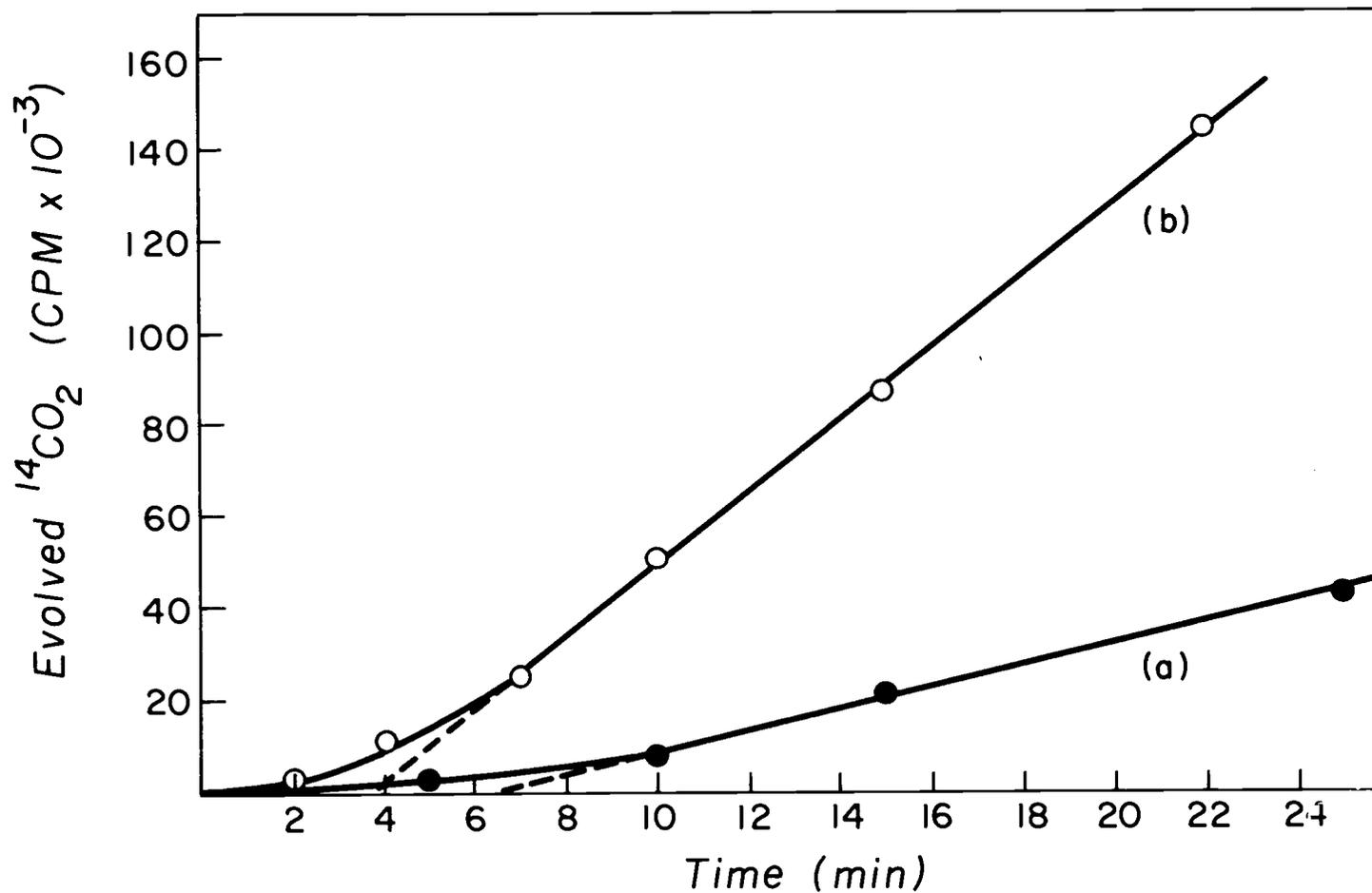


Figure 15. MVAPP decarboxylase. Kinetic assay of $^{14}\text{CO}_2$ evolution versus incubation period in the 40,000 g supernatant prepared at (a) 2 hrs and (b) 9 hrs germination. The lag period prior to linearity in $^{14}\text{CO}_2$ evolution was determined by extrapolating through the abscissa (dashed lines).

the germination interval assayed. The initial lag period prior to linearity is the time required to accumulate a saturating level of MVAPP-1-¹⁴C, starting from MVA-1-¹⁴C as the initial substrate. Thereafter, the decarboxylase remains saturated with an excess of MVAPP-1-¹⁴C. The decrease in this lag period arises through a preferential increase in the activity of MVAP kinase over the decarboxylase during the first nine hours of germination. Hence, the time required to saturate the decarboxylase at nine hours is less than at two hours because of the increased capacity of MVAP kinase to saturate the decarboxylase. The proportional increase in MVAP kinase activity over decarboxylase activity may be estimated to be slightly greater than 180%, judging from the decrease in lag period from 6.5 to 3.7 minutes over this same interval (Figure 15). Supernatants derived from each germination interval were assayed in triplicate and the average value taken. The average deviation from the mean for triplicate samples was less than $\pm 1\%$.

For comparative purposes, the effect of MVAP kinase on the decarboxylase assay must be taken into account. Since the lag time is known at two germination intervals, estimates of the lag time at each germination stage may be made by graphically correlating changes in MVAP kinase activity with proportional changes in the lag time as intercepts of the ordinate (Figure 16). Corrected values for decarboxylase activity were calculated by subtracting out the lag time

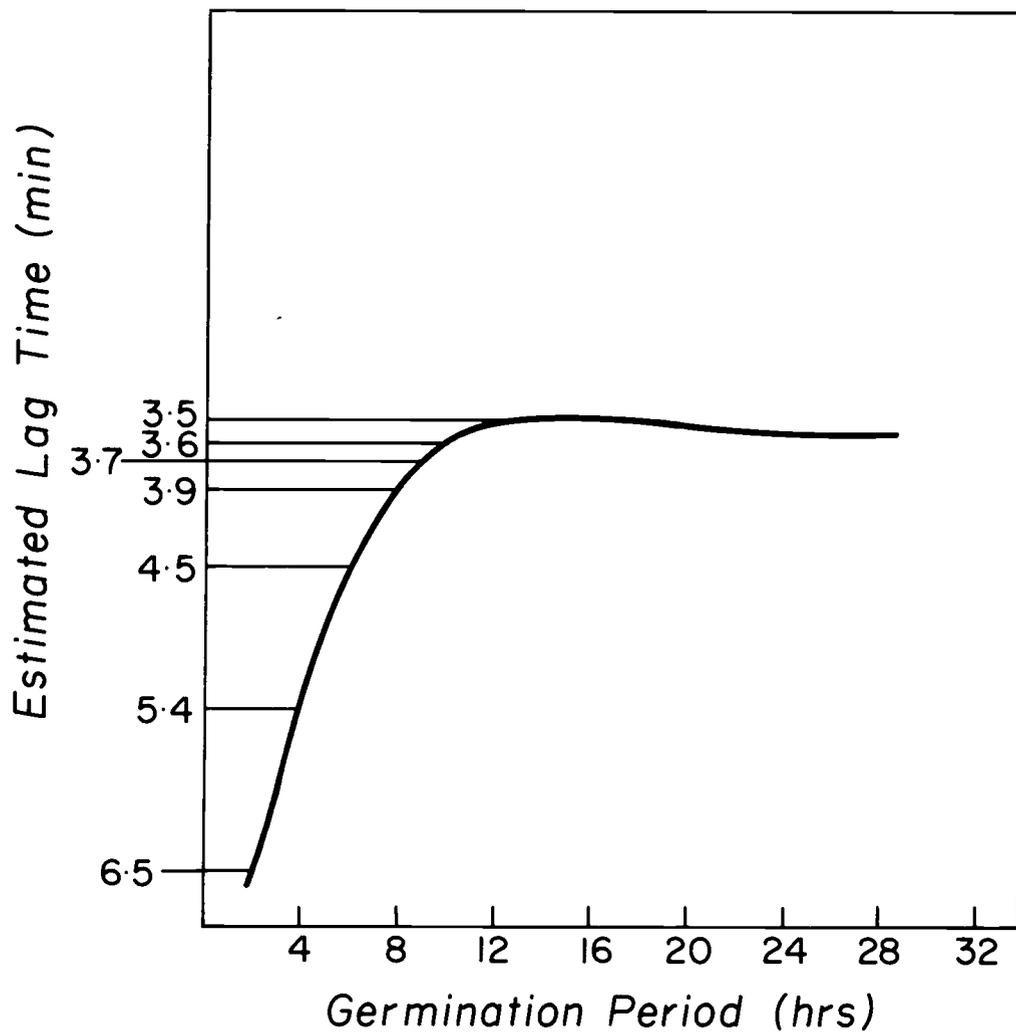


Figure 16. Graphical estimation of the lag period prior to linearity in $^{14}\text{CO}_2$ evolution as a function of germination.

Table 5. Assay of MVAPP Decarboxylase Specific Activity Versus Germination Period.

Germination (hrs)	Supn't (ml)	Time (min)	Protein (mg)	¹⁴ CO ₂ (dpm)	Specific Activity		
					(dpm min ⁻¹ mg ⁻¹)		(mμmoles min ⁻¹ mg ⁻¹)
2.0	1.0	20.0	31.0	41400	67	99 ¹	1.2 x 10 ⁻²
4.0	1.0	20.0	37.6	96563	128	175	2.1
6.0	1.0	20.0	42.8	169984	198	256	3.0
8.0	1.0	20.0	42.0	184065	220	273	3.2
10.0	1.0	20.0	42.0	188939	225	273	3.2
12.0	1.0	20.0	42.6	177453	208	252	3.0
14.5	1.0	20.0	45.2	198971	220	266	3.2
16.0	1.0	20.0	44.8	185385	207	251	3.0
18.0	1.0	20.0	45.6	184342	202	246	2.9
20.0	1.0	20.0	43.8	179317	205	248	2.9
22.0	1.0	20.0	47.2	194735	206	249	2.9
24.0	1.0	20.0	45.1	187149	208	251	3.0
8.0	boiled	-	-	450	-	-	-

¹ Corrected for MVAP kinase effect on apparent decarboxylase activity. Corrections against the boiled blank were negligible.

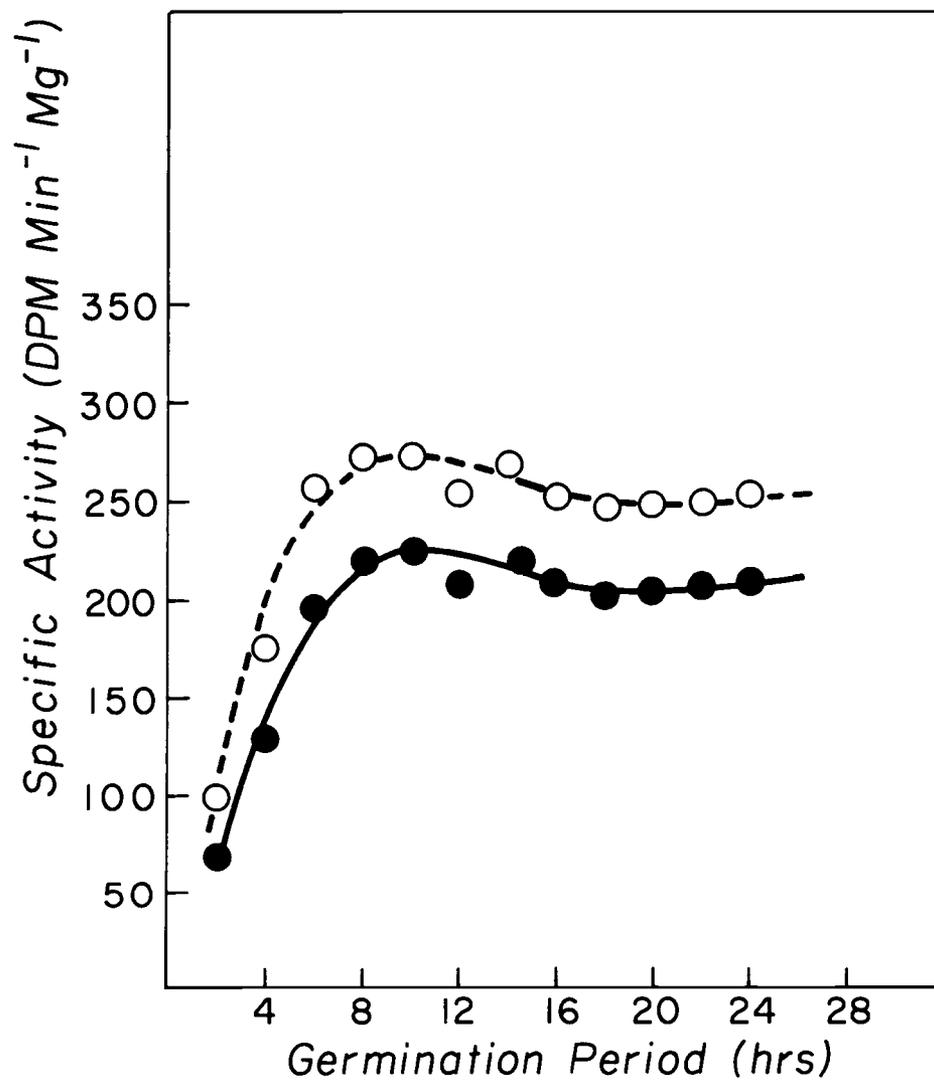


Figure 17. Corrected (○) and uncorrected (●) specific activity measurements of MVAPP decarboxylase as a function of germination.

incurred prior to linearity in $^{14}\text{CO}_2$ evolution. The specific activity for MVAPP decarboxylase was expressed in units of $\mu\text{moles of CO}_2$ formed $\text{min}^{-1} \text{mg}^{-1}$ of total protein (Table 5). Figure 17 is a plot of variations in decarboxylase specific activity for corrected and uncorrected values over a 24 hour germination period. Based on the corrected values, the decarboxylase experiences approximately a three fold rise in specific activity during the first nine hours of germination.

Biosynthesis of IPP-4- ^{14}C

Two methods, A and B, for the biosynthesis of IPP-4- ^{14}C , starting from MVA-2- ^{14}C , are presented, although only one of the methods (B) was successful.

Method A

Pork liver homogenates were prepared and fractionated according to the method of Popják (124). The final enzyme preparation, designated as the F_{30}^{60} fraction, was reported to accumulate substantial quantities of labelled IPP, when incubated with MVA-2- ^{14}C in the presence of iodoacetamide (124). Approximately 15 mg of dialyzed F_{30}^{60} solution was preincubated for 10-15 minutes at 37° with 0.90 ml of 100 mM phosphate buffer, pH 7.4, which was 5 mM in iodoacetamide, 5 mM MgCl_2 , 2 mM MnCl_2 , 7 mM ATP and 30 mM KF. Forty microliters of MVA-2- ^{14}C ($\sim 4 \mu\text{C}$, specific activity $1.54 \mu\text{C}/\mu\text{M}$)

was then added to the buffered solution and incubated for one hour at 37° . Incubation was terminated with a few drops of ice cold 10% HClO_4 . After sedimenting the protein on a clinical centrifuge, the supernatant was neutralized with 20% KOH, chilled to near 0° , and the precipitated KClO_4 centrifuged down at 10,000 g for five minutes in a refrigerated Sorval centrifuge set at 0° . An aliquot from the final supernatant was paper chromatographed in solvent system IV (Figure 18a) to determine the distribution of labelled products.

Figure 18b is the distribution of acid stable intermediates accumulated in the absence of iodoacetamide. Radiochromatographic peaks were identified by paper chromatography in three additional solvent systems (I-III) as MVA, IP and MVAP, respectively. Although the pork liver isomerase, as a sulfhydryl sensitive enzyme, was adequately blocked with iodoacetamide, a degradative process evidently consumed newly synthesized IPP, preventing accumulation of the pyrophosphate as an end product. Since IPP is stable against chemical degradation at neutral pH (13), formation of IP was attributed to enzymatic action on IPP. A most likely enzymatic competitor for IPP might very well occur through alkaline phosphatase contamination of the F_{30}^{60} fraction free of microsomal phosphatase, the presence of a rather active alkaline phosphatase was observed.

Table 6 is a summary of phosphatase activity measured essentially according to the method of Garen and Levinthal (51), except the pH of

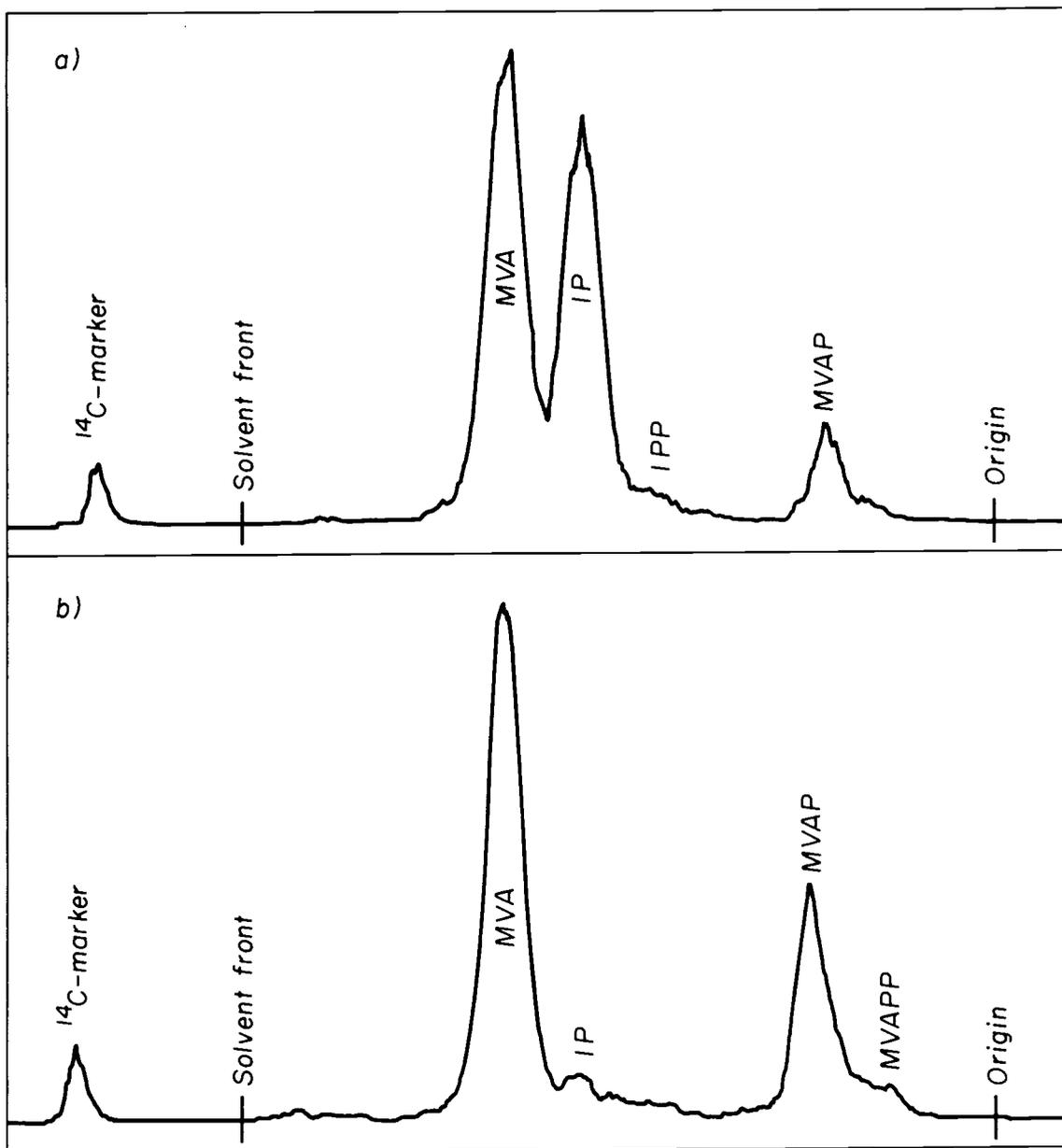


Figure 18. Incorporation and distribution of ^{14}C label from MVA-2- ^{14}C among acid stable isoprenoid products in the F_{30}^{60} pork liver fraction: paper radiochromatographic scans (solvent system IV) for products accumulated in (a) the presence and (b) the absence of iodoacetamide.

Table 6. Assay of Alkaline Phosphatase - Inhibitory Studies on the F_{30}^{60} Fraction Derived from Pork Liver.

F_{30}^{60} Enzyme (ml)	Inhibitor	Phosphatase ¹ initial velocity ($\Delta O.D._{410} \text{ min}^{-1}$)	Inhibition (%)
0.10	-	0.133	0
0.10	10 mM tartrate	0.115	14
0.10	0.5 mM $ZnSO_4$	0.108	19
0.10	30 mM KF	0.074	46
0.10	2% BSA	0.045	66
0.10	100 mM phosphate	0.008	99+

¹ All assays were carried out on the same F_{30}^{60} fraction in 100 mM Tris-HCl buffer, pH 7.5, which was 5 mM in $MgCl_2$ and 2 mM $MnCl_2$. p-Nitrophenyl phosphate at 1 mM in the same buffer served as substrate. Assays were carried out in a total volume of 3.1 ml on a Beckman DB spectrophotometer.

the buffer solution was identical with that of the biosynthetic experiments. Common inhibitors of alkaline phosphatase were also tested for their effectiveness against endogenous phosphatase found in the F_{30}^{60} fraction (124, 156, 172). Several attempts to accumulate IPP- $4-^{14}C$ in the presence of KF, BSA and inorganic phosphate did not improve the yield. An incubation carried out in the presence of all three phosphatase inhibitors, followed by Dowex-formate chromatography of the acid stable intermediates, demonstrated considerable incorporation of MVA- $2-^{14}C$ into IP- ^{14}C with the remainder in MVAP- ^{14}C , MVAPP- ^{14}C and an almost negligible trace in IPP- ^{14}C (Figure 19). Treatment of the F_{30}^{60} fraction to calcium phosphate gel (166) or to Hydroxylapatite (102), likewise, failed to improve IPP- ^{14}C accumulation. Following Hydroxylapatite treatment, phosphatase could not be detected, even in prolonged incubations, as assayed by the method of Garen and Levinthal.

An enzyme or enzymes apparently exist in the F_{30}^{60} fraction that are capable of degrading IPP at a rate nearly equal to or exceeding the capacity of MVAPP decarboxylase to turn over its precursor substrate, MVAPP. Eight individual trials with pork livers, each obtained fresh and prepared according to the method of Popjak, failed to yield an enzyme fraction capable of accumulating IPP. Several assays were run on each F_{30}^{60} fraction; a number of modifications to achieve better yields of IPP- ^{14}C were attempted. All efforts proved in vain.

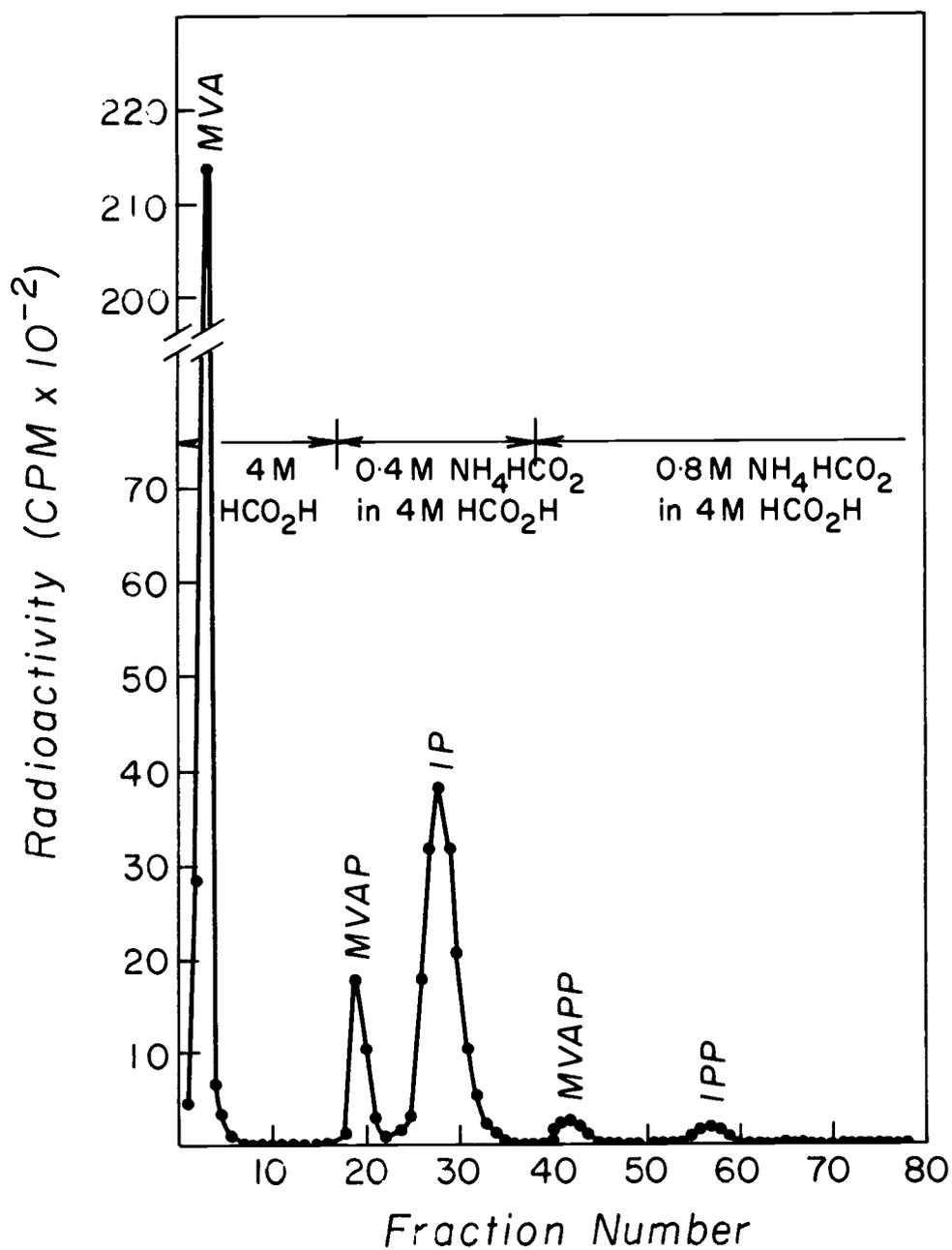


Figure 19. Dowex-1-formate column chromatography of acid stable isoprenoid products accumulated in the F₃₀⁶⁰ pork liver fraction from MVA-2-¹⁴C in the presence of inorganic phosphate, KF, BSA and iodoacetamide.

An alternate method for the biosynthesis of IPP-4-¹⁴C was sought.

Method B

Yeast extracts have a high capacity to biosynthesize IPP-4-¹⁴C from MVA-2-¹⁴C (1, 13, 111). IPP-4-¹⁴C was prepared, with some modifications of the original yeast extraction procedure, according to the method of Salokangas, Rilling and Samuels (147). One-half milliliter of 30,000 g yeast supernatant was incubated at 37° for one hour with 50 μ l of MVA-2-¹⁴C (5 μ C, specific activity 4.7 μ C/ μ M) and 25 ml of 32 mM Tris-HCl buffer, pH 7.4, which was 4.8 mM in iodoacetamide, 6.4 mM sodium EDTA, 9.6 mM MgCl₂, 4.8 mM ATP and 48 mM KF. The incubation was terminated by heat denaturing the yeast incubate in a boiling water bath for 5-10 minutes. Precipitated protein was centrifuged down at 10,000 g for ten minutes at 0° and discarded. Dowex-formate column chromatography of the acid stable intermediates revealed a nearly quantitative turnover of MVA-2-¹⁴C into IPP-¹⁴C (Figure 20). The identity of IPP-¹⁴C, as the major product other than MVA, was also confirmed by paper chromatography of the protein free solution in solvent systems I and IV. Finally, purified presumptive IPP-4-¹⁴C was treated to alkaline phosphatase digestion, and the subsequent ether extract gas chromatographed on a 20% butanediol polyester succinate column on Chromosorb W. Radioactivity, measured on a Nuclear Chicago ionization detector,

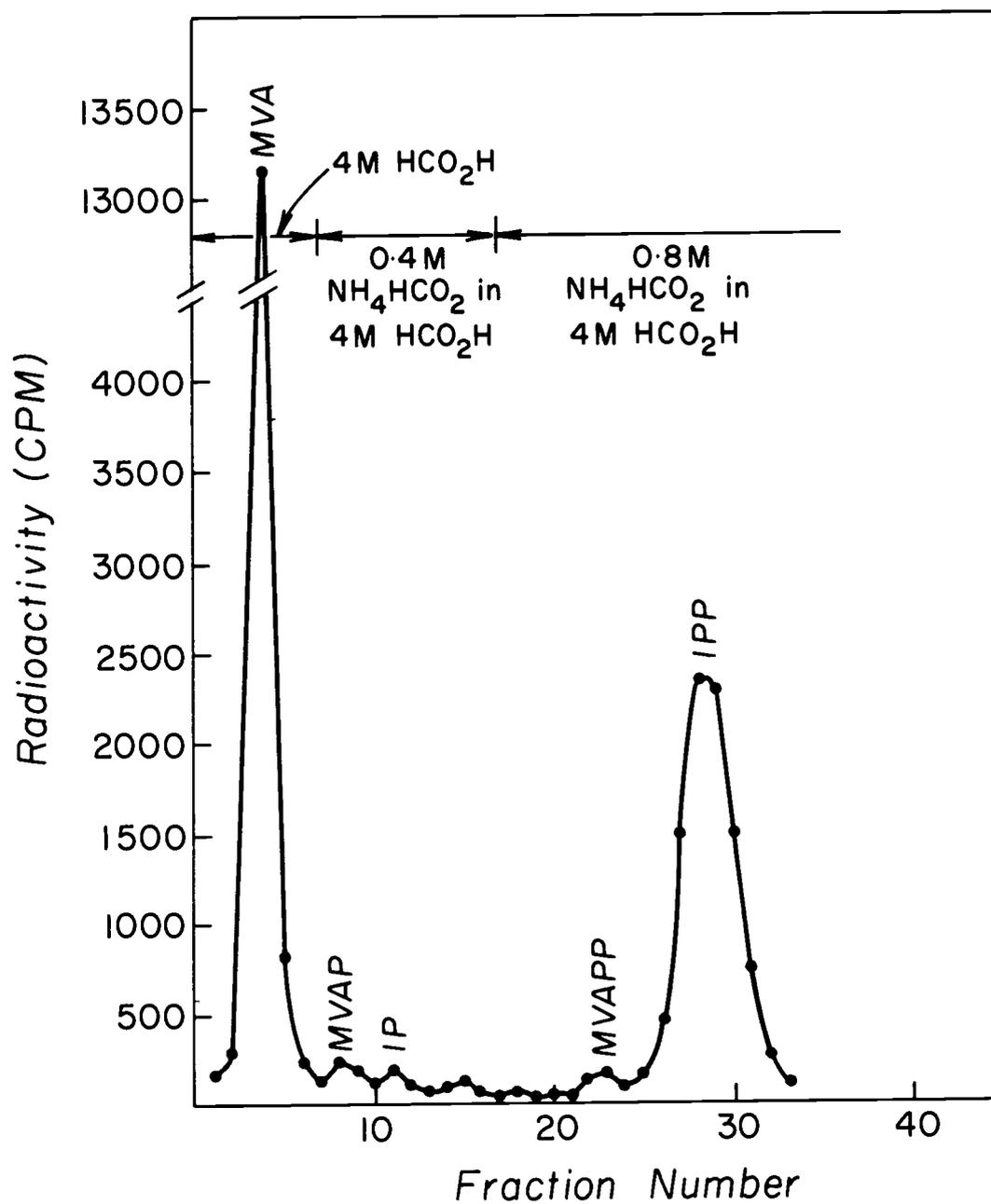


Figure 20. Dowex-1-formate column chromatography of acid stable isoprenoid products accumulated in the 30,000 g yeast supernatant from MVA-2-¹⁴C in the presence of KF and iodoacetamide.

emerged from the column with a retention time identical with that of isopentenol (Figure 21). The capacity to biosynthesize IPP-4-¹⁴C from MVA-2-¹⁴C was estimated at approximately 3-5 $\mu\text{moles hr}^{-1}$ ml^{-1} of yeast extract, based on dpm's of product formed per unit time and the specific activity of labelled MVA.

Isolation and Purification of IPP-4-¹⁴C

IPP-4-¹⁴C may be isolated from crude incubated solutions by Dowex-formate chromatography, paper chromatography, or TLC. The choice of isolation procedure depends rather heavily on the losses of IPP which can be tolerated during subsequent purification steps. On a macro-scale, small losses can be tolerated with preparative methods of isolation such as Dowex-formate chromatography. Such a method generally requires a second step purification, to remove break-down products incurred by the isolation technique and during the initial steps of purification. Silicic acid columns have frequently been used as a final purification step, where minor contaminants such as IP or its diol analogue (13) are present (33). The silicic acid column is useful mainly as a tool to resolve prenyl monophosphates from their corresponding pyrophosphates, but fails to resolve the prenyl pyrophosphates from one another (33). Such columns are, consequently, applicable only in the final stages of purification.

Attempts to purify micromolar quantities of IPP-¹⁴C by

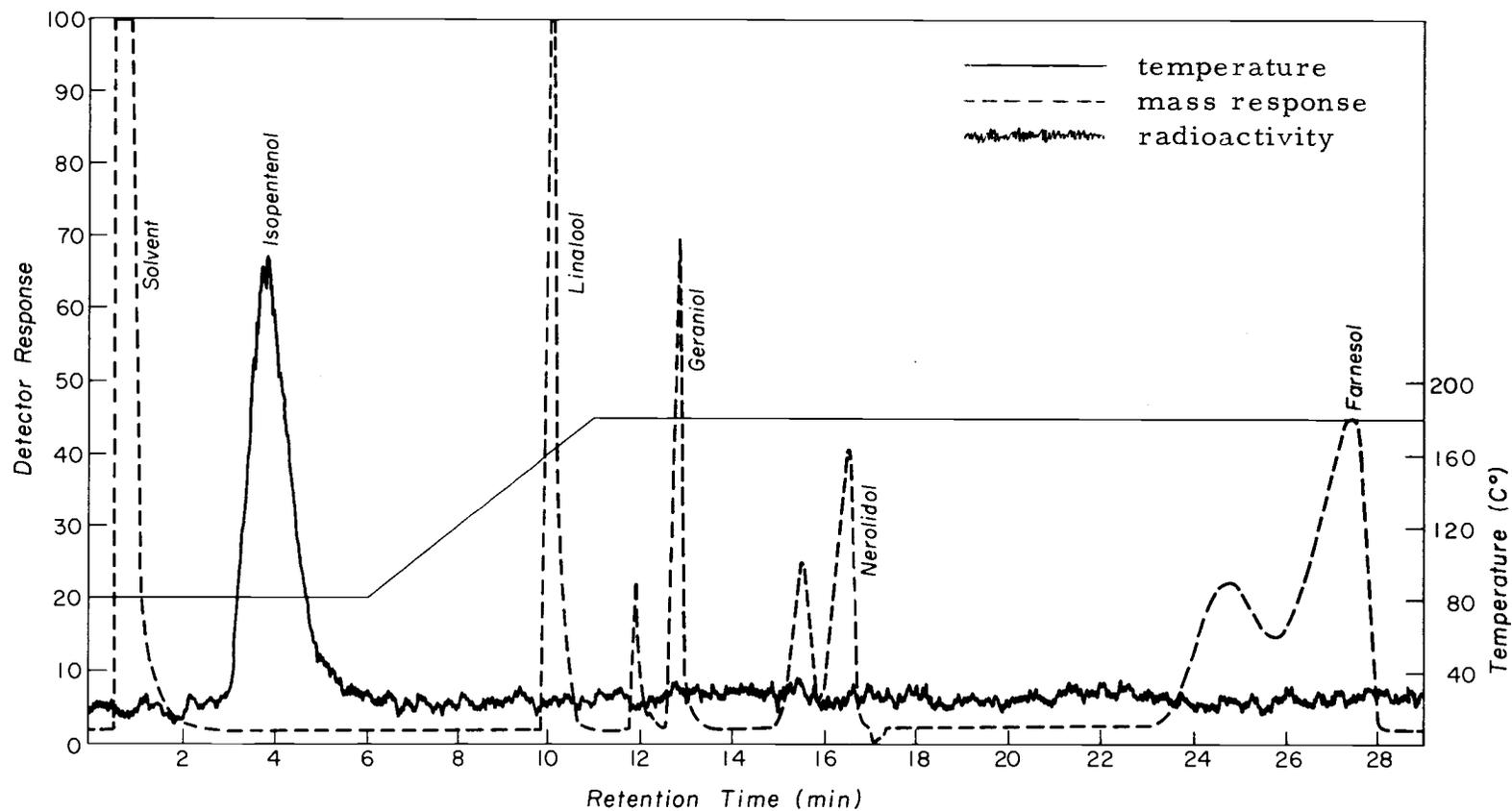


Figure 21. Detection of the isopentenyl- ^{14}C moiety. Gas chromatography of an alkaline phosphatase treated aliquot of the yeast incubated solution.

Dowex-formate chromatography were largely unsuccessful, even though IPP was easily resolved from all other radioactive compounds as a single radioactive peak (Figure 20). Major difficulties were encountered in removing the formic acid/ammonium formate solvent, which is eluted off the Dowex-formate column with IPP. When formic acid was first distilled off on a rotatory evaporatory at 45-50° under vacuum, and then the ammonium formate sublimed off at 45° under vacuum (13, 161), the radioactivity remaining was predominantly IPP-¹⁴C. Treatment of the IPP elutant to Dowex-50 cation exchange chromatography to remove ammonium formate (153), followed by distillation of the formic acid, gave similar results. An improvement in preserving IPP was achieved, however, by distilling off the formic acid at 15-20° as an azeotrope with toluene (171). Nevertheless, side products were unavoidable (Figure 22).

IPP-¹⁴C was separated from other compounds on a series of paper strips approximately 5 x 45 cm in solvent system IV, which stabilized the pyrophosphate moiety against hydrolysis to orthophosphate. Portions of the paper strips associated with IPP-¹⁴C were cut out and eluted into a round bottom flask with 0.01 M ammonia. After pooling the elutions from several chromatograms, the ammonia solution was concentrated on a rotory evaporator to a small volume under vacuum at 10-15°, then lyophilized to dryness. IPP-¹⁴C remaining in the flask was taken up in a small volume of 0.01 M ammonia and

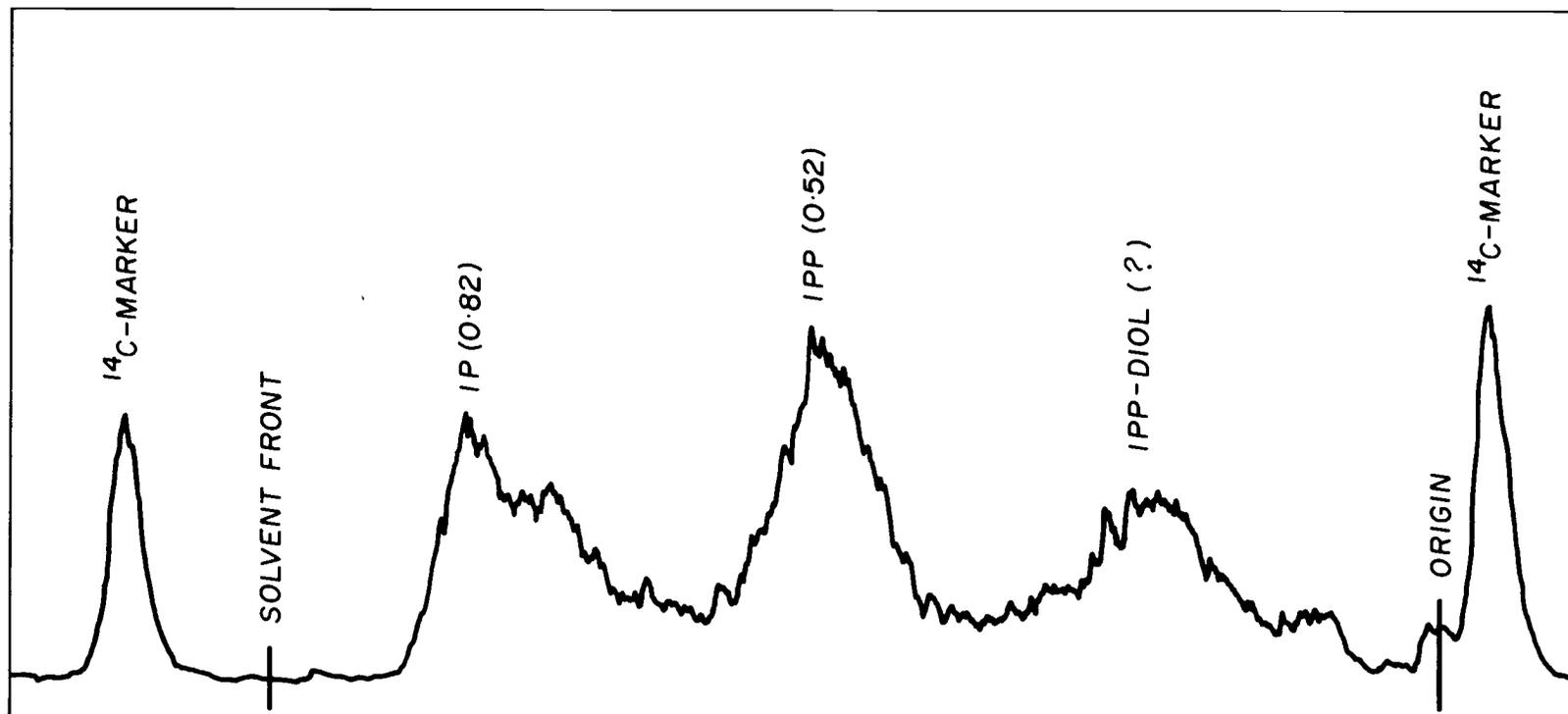


Figure 22. Paper radiochromatographic scan (solvent system IV) of IPP-4- ^{14}C following Dowex-1-formate chromatography and subsequent removal of the formic acid/ammonium formate solution.

stored in a freezer at -15° for future use. Yields of pure IPP- ^{14}C recovered by paper chromatography were low, however, due to inefficient elution from the paper and the limited supply of crude solution which could be resolved per paper strip.

The most successful method in terms of pure product and preparative capacity for isolation of IPP- ^{14}C was by TLC. Aliquots of the crude solution were applied in strips to silica gel H plates (20 x 20 cm, 0.25 mm thickness) and developed in n-propanol:ammonia:water (6:3:1). IPP- ^{14}C ran with an R_f of 0.17-0.28. Silica gel associated with IPP- ^{14}C was scraped from the TLC plates, transferred to conical centrifuge tubes, and the gel washed three to four times with 0.01 M ammonia. Each washing was preceded by a short centrifugation on a clinical centrifuge to pellet the gel, so that the supernatant could be drawn off essentially free of silica scrapings. The combined washings were concentrated to a small volume on a rotary evaporator at $10-15^{\circ}$ under vacuum, and residual traces of silica gel completely removed by centrifuging the remaining solution at 10,000 g for ten minutes at 0° . Aliquots from the final supernatant were chromatographed in solvent systems I and IV and also treated to alkaline phosphatase digestion, ether extraction, and subsequent gas chromatography. Based on the paper and gas chromatographic analysis, the final supernatant was judged to be radiochemically pure IPP-4- ^{14}C . The solution was stored at -15° in 0.01 M

ammonia (pH \sim 8) until needed. Table 7 is a summary, or guide, for the various methods used to isolate pure IPP-4- 14 C. Where micromolar or millimicromolar quantities of IPP must be isolated, TLC on silica gel H is by far the superior method of choice.

Table 7. Summary of methods for IPP-4- 14 C isolation.

Method	Time Required	Capacity
Dowex-formate and silicic acid chromatography	32 hrs	millimolar
paper chromatography	16 hrs	micro-, millimicromolar
TLC - silica gel H	4 hrs	micro-, millimicromolar

Isopentenyl Pyrophosphate Isomerase Assay

IPP isomerase stereospecifically converts IPP into its isomeric product, DMAPP (1, 111). In crude enzyme solutions contaminated with several of the higher isoprenoid synthesizing enzymes, DMAPP is readily incorporated into higher isoprenoid products through a series of condensations (20). Quantitation of IPP isomerase activity amounts to estimating the total units of DMAPP produced per unit time, whether such units are accumulated as free product or incorporated into the backbone of higher isoprenoids. As DMAPP is the "starter" C₅ unit of each higher isoprenoid, then it represents

one-half of a C₁₀, one-third of a C₁₅ isoprenoid, etc. Products of the isomerase are chemically isolated into two distinct groups, the NSF and prenol fraction. Each fraction is analyzed in terms of product composition, from which the units of DMAPP may be deduced. Using IPP-4-¹⁴C, the total rate may be calculated by analyzing the radioactive distribution among the products and summing the radioactivity which has arisen through DMAPP. IPP isomerase activity is conveniently expressed per unit time by the following equations:

$$(2) \quad \frac{-d(\text{IPP})}{dt} = \frac{d(\text{DMAPP})}{dt} \text{ total}$$

$$(3) \quad \frac{-d(\text{DMAPP})}{dt} \text{ total} = \frac{d(\text{DMAPP})}{dt} + \frac{1}{2} (\text{GPP}) + \frac{1}{3} (\text{FPP}) \\ + \frac{1}{3} (\text{squalene})$$

To a five milliliter conical centrifuge tube was added 0.10 ml of 40,000 g supernatant. The enzyme solution was thermally equilibrated in a constant temperature water bath to 24° for two to three minutes and then mixed with 20 μl of IPP-4-¹⁴C (18,000 dpm, specific activity 4.7 μC/μM). After a two minute incubation, the reaction was stopped with two drops of 20% KOH and extracted four to five times with one milliliter volumes of diethyl ether. The combined ether extracts were evaporated to dryness on a steam bath, then counted in

Bray's scintillation fluid to determine the total counts of IPP-4-¹⁴C incorporated into the NSF fraction. Only squalene appears in the NSF fraction in the 40,000 g supernatant. Acid labile prenyl phosphates were subjected to acid hydrolysis by adding a few drops of 12 N H₂SO₄ and maintaining the solution at 37° for 15 minutes. During acid hydrolysis, the conical centrifuge tube was kept tightly sealed with a cork to prevent losses of the more volatile prenols. The acidic solution was then chilled in an ice tray, brought to alkaline pH with 5 N KOH, and extracted with five to six volumes of diethyl ether. Sufficient salt was generated in neutralizing the acidified incubate to aid extraction of the prenol fraction. An aliquot of the prenol fraction was subjected to gas chromatography to determine the distribution of labelled prenols. Simultaneously, an aliquot was counted in Bray's scintillation fluid to determine the total radioactivity. Corrections for quenching were made by internal spiking with a toluene-¹⁴C standard. From the distribution analysis by gas chromatography and an estimate of total radioactivity incorporated into the prenol fraction, the radioactivity incorporated into each prenol was calculated. Units of DMAPP were determined by summing together all of the values derived from the prenol and NSF fractions as outlined in equation (3). Enzymatic activity was expressed in units of μmoles of DMAPP produced min⁻¹ mg⁻¹ of total protein.

Results and Discussion

Three factors that may be modified to obtain zero order kinetics are: (i) substrate concentration, (ii) total enzyme units, or (iii) the incubation period. The latter two factors were adjusted appropriately to achieve a linear rate of IPP-4-¹⁴C isomerization, thus conserving substrate. The turnover of IPP-4-¹⁴C as a function of time was measured on 0.20 ml aliquots of 40,000 g supernatant prepared from a 16 hour set of germinated seeds (Figure 23a). Under these conditions, the rate of isomerase activity was linear over the first three minutes. For subsequent assays, the enzyme aliquot was halved to 0.10 ml and a two minute incubation period chosen to insure zero order kinetics.

It is interesting to note the short lag period prior to linearity of squalene formation (Figure 23b). This may be compared with the instantaneous and linear turnover of IPP-4-¹⁴C. There are two enzymes acting sequentially on IPP, one directly (IPP isomerase) and the other indirectly on the product(s) of the isomerase. The relationship of squalene formation to isomerase activity is interpreted as evidence for a rate limiting step between IPP isomerase and squalene synthetase. Furthermore, gas chromatographic analysis of the prenols released by acid hydrolysis revealed the presence of DMAPP, but no other allyl intermediates. Hence, the prenyltransferase is most likely the rate limiting enzyme.

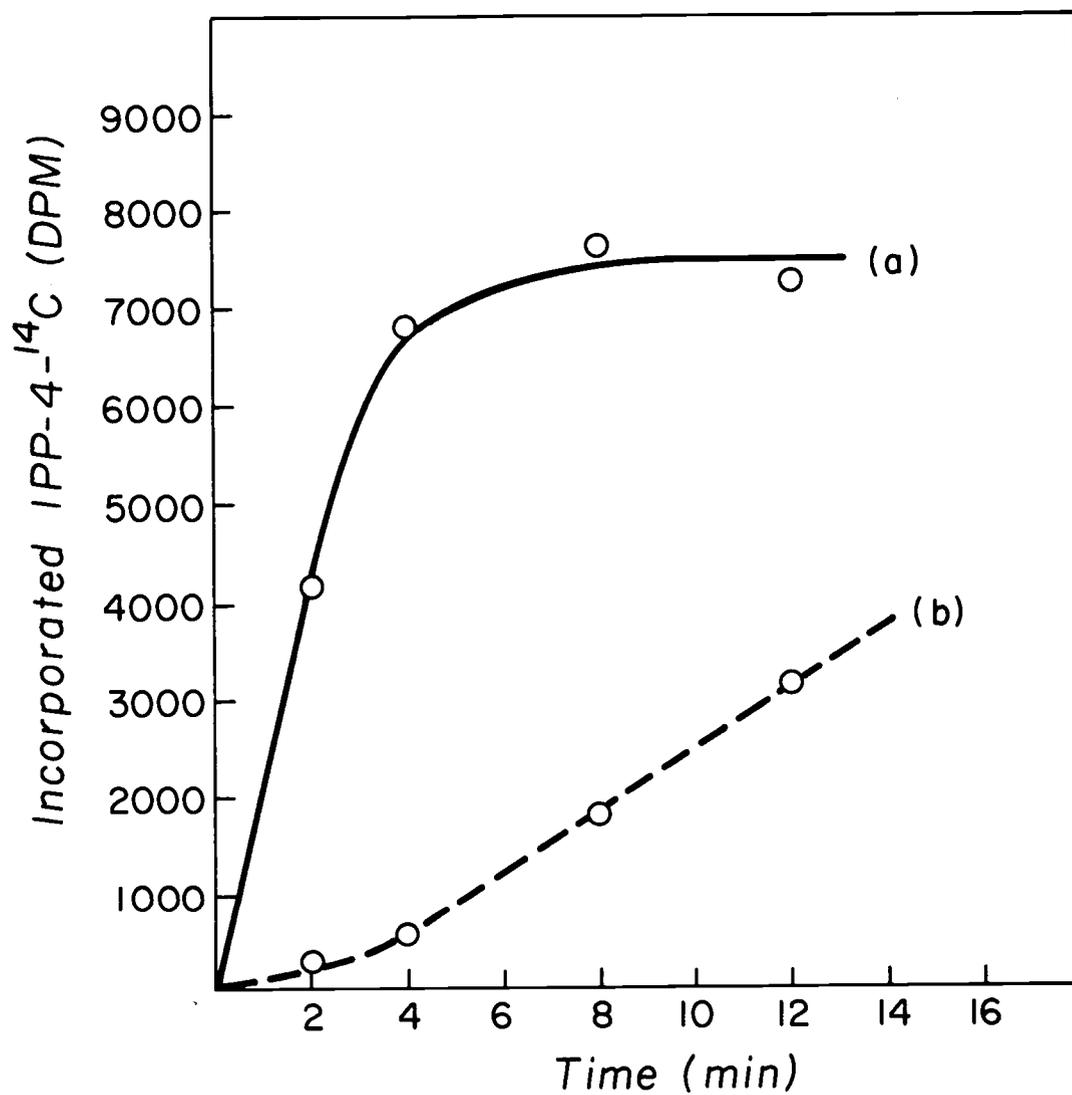


Figure 23. Kinetic assay of IPP isomerase. Incorporation of IPP-4-¹⁴C into (a) DMAPP and (b) squalene as a function of incubation period.

Table 8. Assay of IPP Isomerase Specific Activity Versus Germination Period.

Germination (hrs)	Supn't (ml)	Time (min)	Protein (mg)	NSF (dpm)	DMA ¹ (dpm)	Specific activity ²	
						(dpm min ⁻¹ mg ⁻¹)	(mμmoles min ⁻¹ mg ⁻¹)
2.0	0.10	2.0	3.24	52	1541	240	2.3 x 10 ⁻²
4.0	0.10	2.0	3.92	56	2350	303	2.9
6.0	0.10	2.0	4.34	65	3362	390	3.7
8.0	0.10	2.0	4.38	85	3510	402	3.9
12.0	0.10	2.0	4.58	124	3679	406	3.9
16.0	0.10	2.0	4.60	127	4061	446	4.3
24.0	0.10	2.0	4.39	106	3890	447	4.3
34.0	0.10	2.0	4.06	139	3450	430	4.1
8.0	boiled	-	-		96 (total)	-	-

¹Dimethylallyl alcohol

²See equation (3), page 94. Corrections against the boiled blank were negligible.

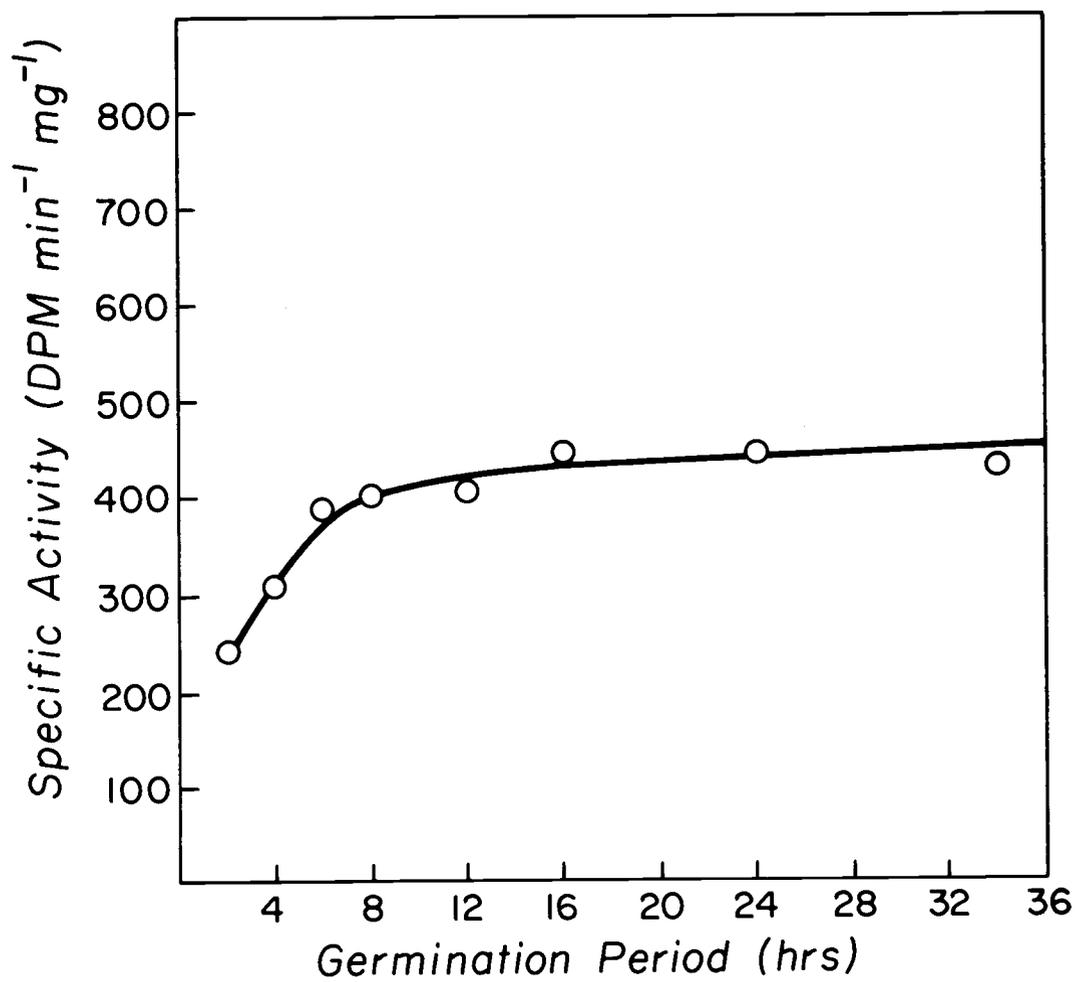


Figure 24. Specific activity of IPP isomerase as a function of germination.

Table 8 tabulates the experimental findings in assaying IPP isomerase over the first 34 hours of germination. A plot of IPP isomerase specific activity as a function of germination (Figure 24) shows a total change of approximately two fold during the first 16 hours of germination. Thereafter, the activity remains essentially constant.

Trans-Prenyltransferase Assay

A precise definition of what is collectively referred to as the prenyltransferases is somewhat nebulous in light of recent work on this enzyme system. The prenyltransferases characteristically catalyze the condensation of a prenyl pyrophosphate, such as DMAPP or GPP, with IPP to yield inorganic pyrophosphate and either GPP or FPP. The two condensations leading to FPP formation were previously defined as distinct enzyme reactions, designated as dimethylallyltransferase and geranyltransferase, respectively (84). The stereochemistry of these condensations and order of substrate binding has been thoroughly discussed by a number of authors (9, 32, 84). Recently, Holloway and Popják reported a 100 fold purification of prenyltransferase from pork liver, in which preference for either DMAPP or GPP as the condensing substrate with IPP was indistinguishable (84). In terms of specific activity, the ratio of DMAPP transferase activity to GPP transferase activity remained constant

throughout each purification step. The same authors reported that addition of DMAPP and IPP to the partially purified transferase resulted in FPP formation, but no GPP. Based on this data, Holloway and Popják concluded that dimethylallyltransferase and geranyltransferase are one and the same enzyme (i. e., only one enzyme catalyzes the condensation of DMAPP and IPP into FPP). Whether two distinct transferases, or one collective transferase, catalyzes the condensation of DMAPP with IPP, culminating in the formation of FPP, remains to be resolved. Aspects of this problem in relation to regulatory changes, which apparently alter the flow of prenyl pyrophosphates through the isoprenoid pathway, will be discussed in a later section.

Since the 40,000 g supernatant is heavily contaminated with isomerase, prenyltransferase may not be assayed by conventional methods (84, 124). However, observations cited in the isomerase assay and subsequent assays of squalene synthetase suggested that the prenyltransferase was the rate limiting enzyme lying between DMAPP and squalene formation. For the purposes of this study, an excess of IPP-4-¹⁴C was allowed to isomerize to DMAPP-4-¹⁴C, which then condensed with excess IPP-4-¹⁴C via the prenyltransferase. The rate of squalene formation rapidly approached linearity, following a short preincubation period with labelled IPP. Since the relative ratio of enzyme activities was observed to be the following,

IPP isomerase >> IPP/DMAPP prenyltransferase << squalene synthetase

the steady state rate of squalene formation is dependent upon the rate of IPP/DMAPP condensation (where IPP serves as the initial substrate). This rate was measured and calculated in terms of IPP-4-¹⁴C consumed and defined as prenyltransferase activity in units of $\mu\text{moles of IPP condensed min}^{-1} \text{ mg}^{-1}$ of total protein. At no time did the specific activity of prenyltransferase approach that of IPP isomerase or squalene synthetase.

To each of four conical centrifuge tubes was added 0.20 ml of the 40,000 g supernatant and 10 μl of NADPH (100 μmoles). Each enzyme solution was thermally equilibrated for 2-3 minutes to 24^o in a water bath. Samples were then incubated with 20 μl of IPP-4-¹⁴C (18,000 dpm, specific activity 4.7 $\mu\text{C}/\mu\text{M}$) and terminated with 5-6 drops of 5 N KOH at various intervals over a 20 minute period. The NSF (squalene) fraction from each sample was extracted with four volumes of diethyl ether, transferred to a scintillation vial, evaporated to dryness under nitrogen, and then counted in Bray's scintillation fluid. Quenching corrections were made by internal spiking with a toluene-¹⁴C standard. By plotting the dpm's of squalene-¹⁴C produced versus incubation time, the slope, or rate of squalene formation, was determined. Prenyltransferase activity was then calculated as described earlier.

Results and Discussion

Figure 25 is a kinetic plot of squalene formation from homogenates at various germination intervals as a function of time. Following a 2-3 minute lag period, the rate of squalene formation was linear with time. Slopes were determined from the linear portions of the graph. A summary of prenyltransferase specific activity during the first 29 hours of germination is given in Table 9. As defined in this study, the specific activity of the prenyltransferase was observed to rise approximately two fold in the first 12 hours of germination, then to fall off sharply over the next few hours, leveling off at approximately one-half of its initial activity (Figure 26).

Biosynthesis of FPP-4, 8, 12-¹⁴C

FPP-¹⁴C has principally been biosynthesized from MVA-2-¹⁴C in pork liver preparations derived through successive fractionation schemes by high speed centrifugation and ammonium sulfate fractionation (124). There are certain disadvantages inherent in such a method. Among these are: (i) a requirement for obtaining fresh pork liver; (ii) a disproportionate amount of time and effort is devoted to isolating active enzyme; (iii) frequent contamination of the preparation with phosphatase, which diminishes the yields of final product through competition for phosphorylated intermediates; and (iv) a wide

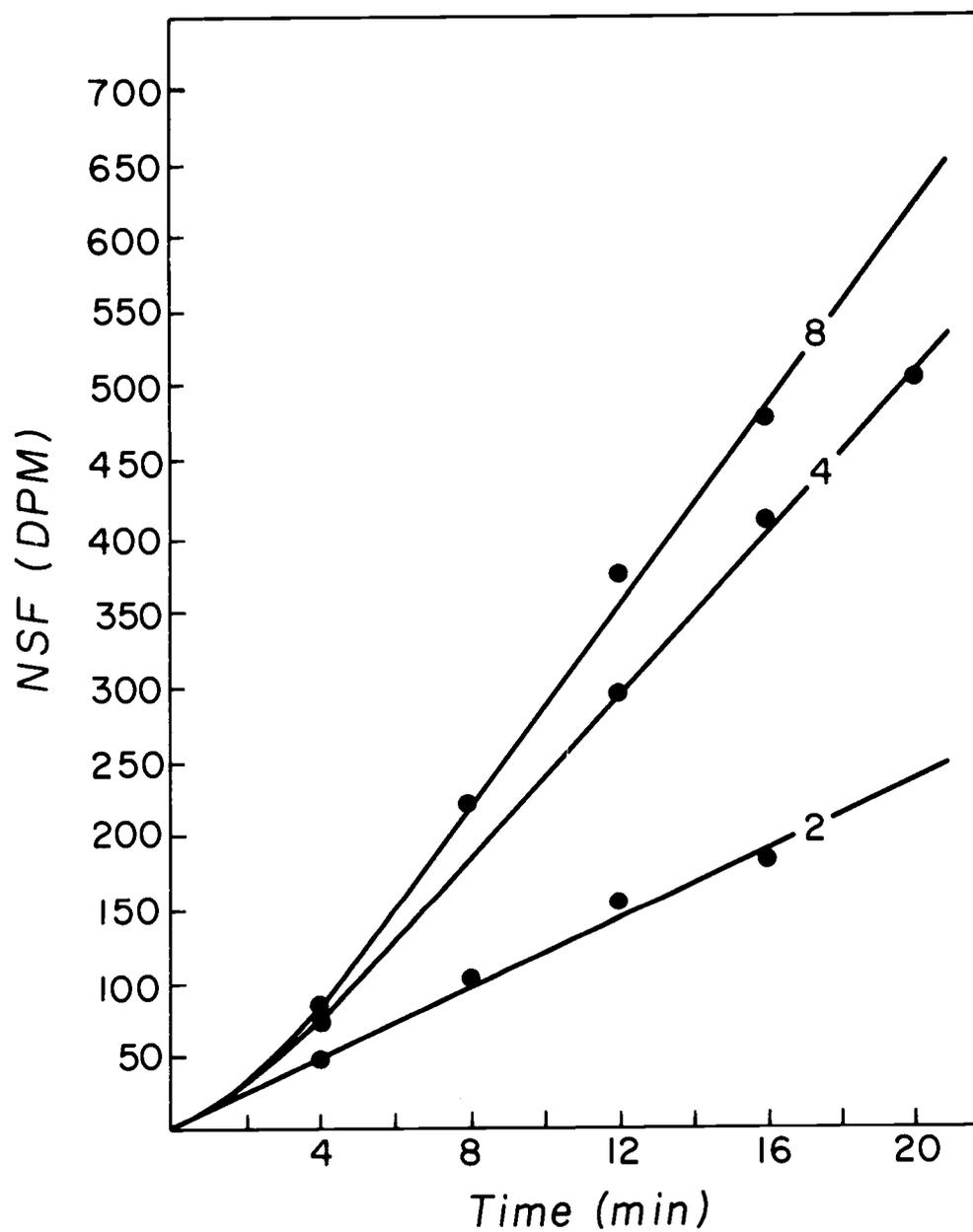


Figure 25. Kinetic assays of IPP/DMAPP prenyltransferase activity at specific periods of germination as noted.

Table 9. Assay of Prenyltransferase Specific Activity Versus Germination Period.

Germination (hrs)	Supn't (ml)	Protein (mg)	NSF slope (dpm min ⁻¹)	Specific activity ¹	
				(dpm min ⁻¹ mg ⁻¹)	(μmoles min ⁻¹ mg ⁻¹)
2.0	0.20	6.28	12	127 x 10 ⁻²	1.22 x 10 ⁻⁴
4.0	0.20	8.28	27	217	2.06
6.0	0.20	9.20	31	224	2.14
8.0	0.20	8.24	34	272	2.60
12.0	0.20	8.20	36	280	2.77
14.0	0.20	8.48	35	277	2.65
18.0	0.20	7.76	9	79	0.75
24.0	0.20	8.56	12	90	0.86
29.0	0.20	8.48	9	66	0.64

¹ Calculated in terms of IPP-4-¹⁴C incorporation.

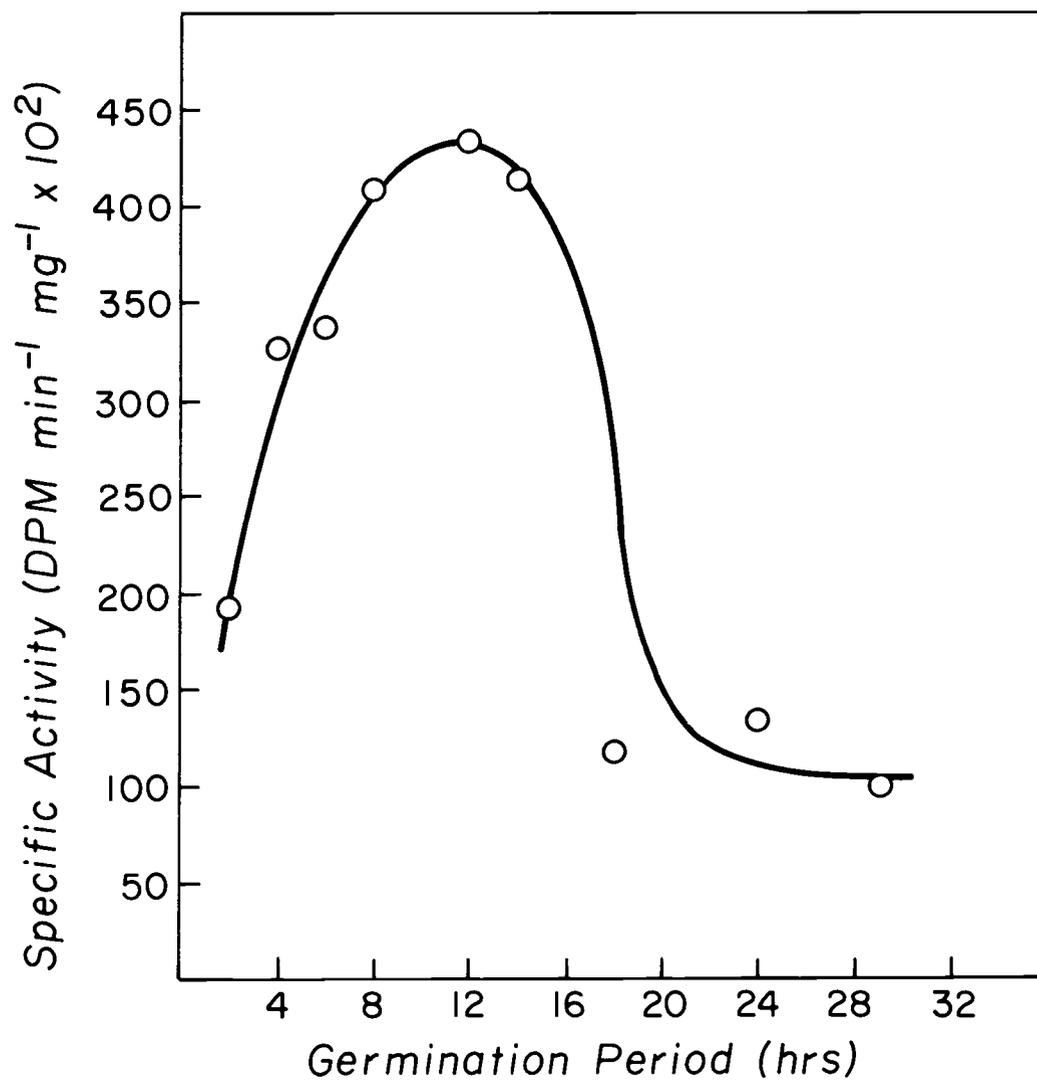


Figure 26. Specific activity of IPP/DMAPP prenyltransferase as a function of germination.

distribution of labelled products are accumulated during the biosynthesis of FPP-¹⁴C, thus requiring very selective isolation procedures which markedly reduce the recovery of pure product.⁵ Several attempts to biosynthesize FPP-¹⁴C from such preparations were unsuccessful. For these reasons, an alternate method of preparing FPP-¹⁴C was sought.

Yeast autolysates were prepared and subjected to high speed centrifugation to sediment microsomally associated squalene synthetase and phosphatases (56, 96, 110, 124), which normally consume FPP, preventing its accumulation in crude incubates. A clear, soluble yeast fraction was prepared, requiring little time and effort, which converted MVA-2-¹⁴C almost quantitatively into FPP-¹⁴C with few side product contaminants. Commercial dry baker's yeast may be purchased immediately before use, or stored in the laboratory until needed, then autolyzed, centrifuged, and incubated with labelled MVA-2-¹⁴C to yield an abundant supply of FPP-¹⁴C in half the time required to prepare the pork liver fraction previously used for biosynthesizing FPP-¹⁴C.

An aliquot of a 30,000 g supernatant, prepared from commercial dry baker's yeast (Red Star) as previously described, was transferred to a high speed centrifuge tube and centrifuged for three hours

⁵ Recovery of pure product is reportedly between 30-40% at best (124).

at 45,000 g and 0° to sediment the microsomal fraction. One-half milliliter of soluble supernatant, free of microsomes, was drawn from the upper portion of the centrifuge tube and mixed with 25 ml of 32 mM Tris-HCl buffer, pH 7.4, which was 6.4 mM in EDTA, 9.6 mM MgCl₂, 4.8 mM ATP and 48 mM KF. The solution was incubated for one hour at 37° with 50 μl of MVA-2-¹⁴C (5 μC, specific activity 4.7 μC/μM) and terminated by heat denaturing the solution in a boiling water bath. Precipitated protein was centrifuged down at 10,000 g for ten minutes and discarded. The supernatant was then adjusted to pH 7.4 and stored at -15° for future purification of the newly synthesized FPP-¹⁴C.

Gas chromatography of an acid hydrolyzed aliquot of the protein-free supernatant revealed the presence of the farnesyl moiety (converted to its isomerically more stable analogue, nerolidol-¹⁴C, via acid catalysis) as the major allyl intermediate (Figure 27). Paper chromatography of an additional aliquot in solvent system IV showed only one peak running coincident with MVA-2-¹⁴C (Figure 28). Although this system fails to resolve FPP from MVA, it clearly demonstrates a nearly clean conversion of label into the former product with only negligible traces of phosphorylated precursors.

Isolation of FPP-¹⁴C

FPP-¹⁴C was isolated by DEAE cellulose chromatography of the

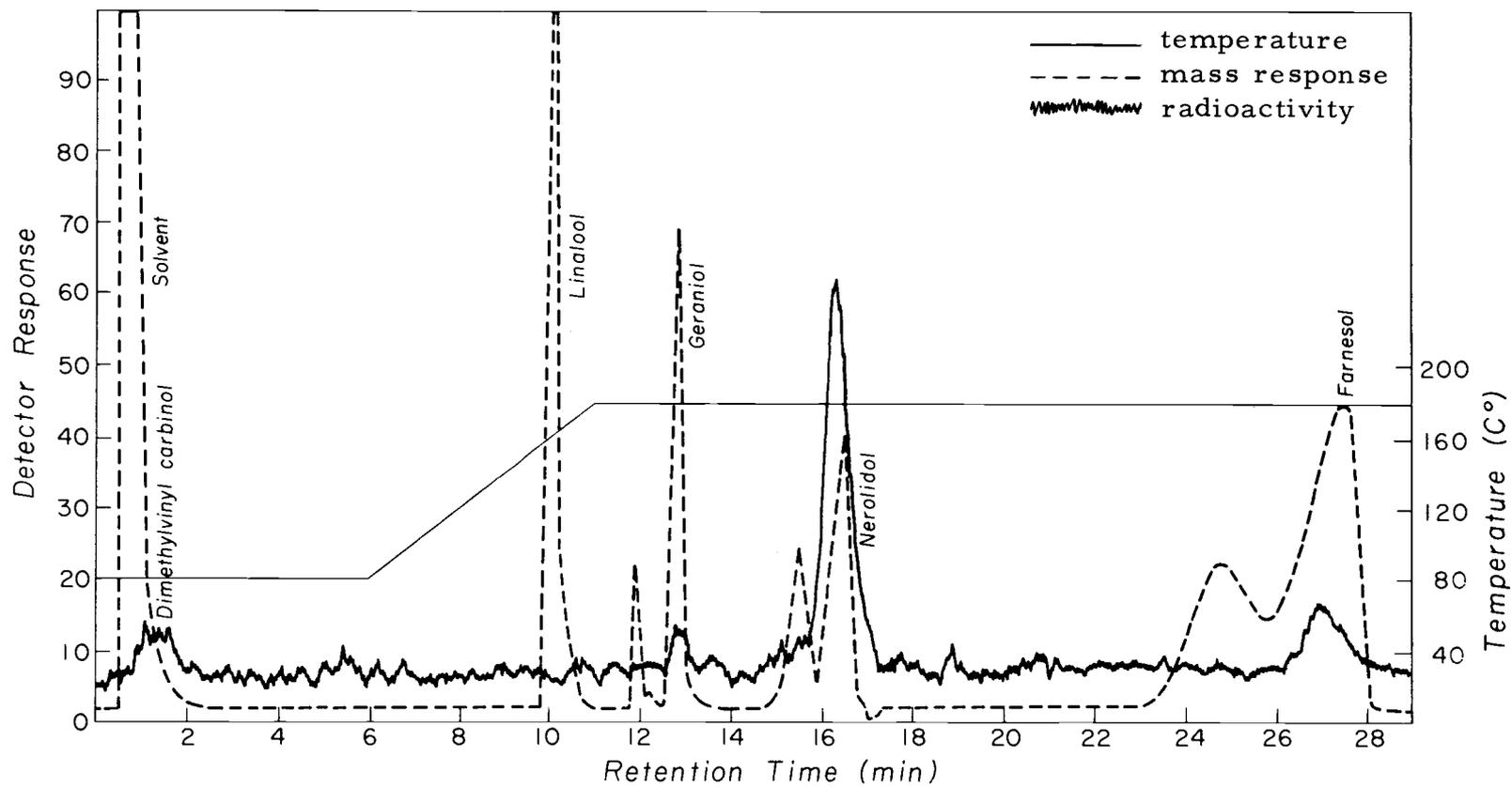


Figure 27. Detection of the farnesyl-¹⁴C moiety. Gas chromatography of an acid-hydrolyzed aliquot of the yeast incubated solution.

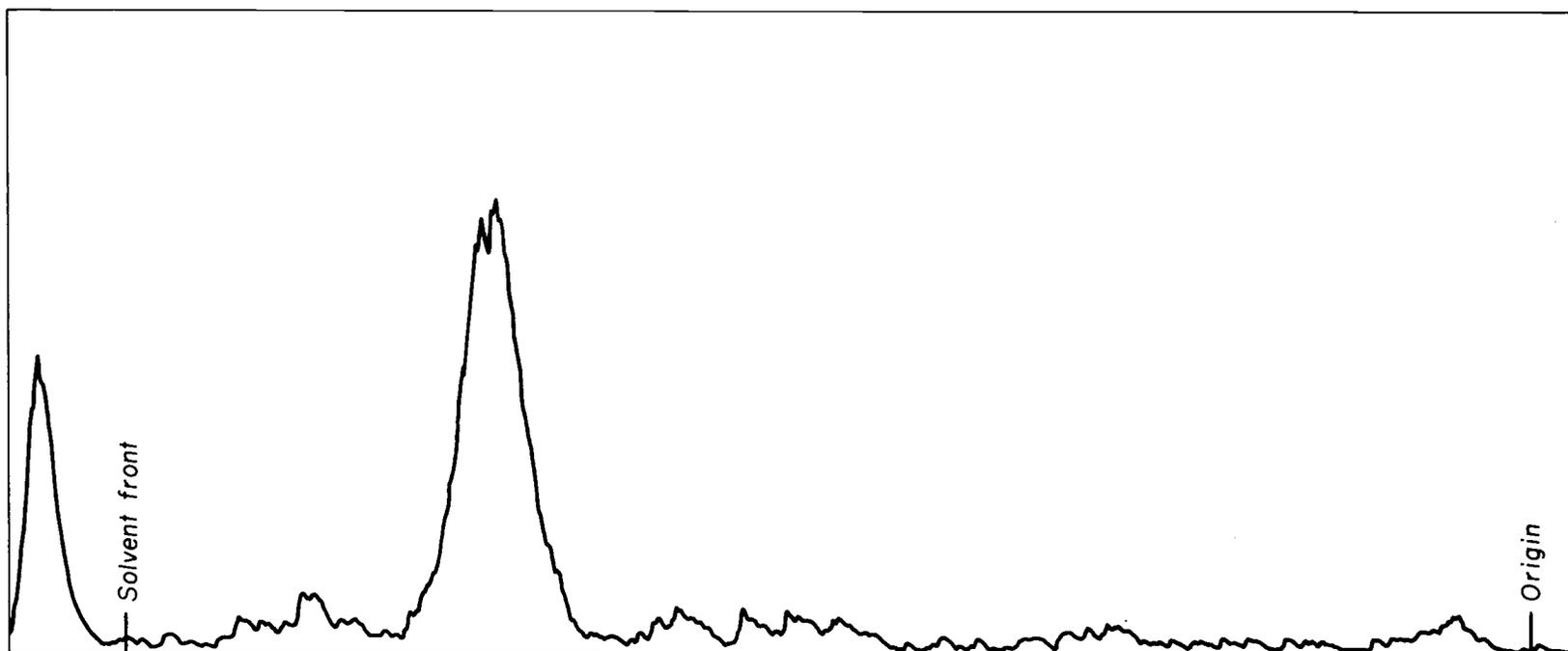


Figure 28. The biosynthesis of FPP-4, 8, 12- ^{14}C from MVA-2- ^{14}C . Paper radiochromatographic scan (solvent system IV) of a protein-free aliquot from an incubated solution of the 45,000 g yeast supernatant.

yeast incubated solution. An aliquot of the yeast solution (5.0 ml) was diluted with 0.01 M ammonia solution to a total volume of 15 ml and passed through a 1.5 x 10 cm DEAE cellulose column. Gradients of 0.01 and 0.10 M $(\text{NH}_4)_2\text{CO}_3$ were used, as shown in Figure 29, to fractionate prenyl pyrophosphates free of contaminating MVA- ^{14}C . Sixteen milliliter fractions were automatically collected and aliquots from each fraction counted in Bray's scintillation fluid. Fractions 10 and 11 were pooled in a round bottom flask and concentrated to a small volume on a rotatory evaporator at 15-20 $^\circ$ under vacuum. The solution was then lyophilized to dryness and residual traces of $(\text{NH}_4)_2\text{CO}_2$ sublimed off at room temperature. Radiochemically pure FPP- ^{14}C (> 90%) which remained in the flask was taken up in a small volume of 0.01 M ammonia and stored at -15 $^\circ$ until needed. An aliquot of the presumptive FPP- ^{14}C was chromatographed on paper in solvent system V and an R_f of 0.80-0.93 found (literature value = 0.85-0.90). The solution was slightly contaminated with traces of GPP- ^{14}C and DMAPP- ^{14}C . Gas chromatography of the free prenol revealed essentially the same distribution as previously shown.

Squalene Synthetase Assay

In biochemical terms, a satisfactory definition of squalene synthetase activity has not been developed. The capacity to make squalene has generally been associated with the microsomal fraction (56, 96,

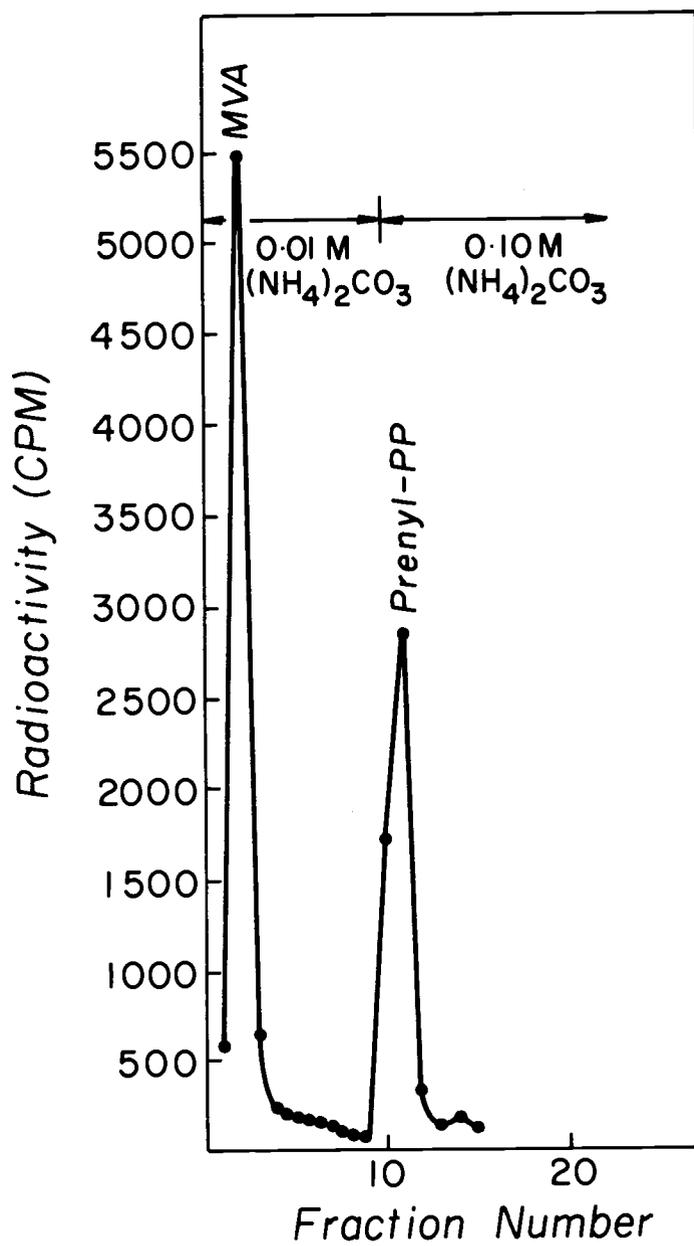


Figure 29. Isolation of FPP-¹⁴C from the crude yeast incubation mixture by DEAE cellulose chromatography.

110, 124). Squalene formation arises from a condensation of two molecules of FPP, in which the C-1 hydrogen from one of the farnesyl residues is stereochemically displaced by a hydride ion from reduced pyridine nucleotide (3, 124, 128). The condensation takes place with an inversion of configuration at C-1 of the second farnesyl residue (30, 124, 128, 129). Nerolidyl-PP has also been proposed as a precursor of squalene synthesis (28, 56, 110, 123, 128). However, Rilling has shown the latter intermediate is not incorporated into squalene (155). The possibility still exists, though, in which a farnesyl residue may rearrange to the nerolidol structure as an "enzyme-bound" intermediate. Recently, a C₃₀ precursor of squalene formation has been identified in yeast and pork liver fractions, which accumulates in the absence of reduced pyridine nucleotide (95, 138). This latter precursor was reported to be an "enzyme-bound" intermediate of squalene synthetase by Krishna et al. (95). There are still a number of conflicting reports on the precise nature of squalene synthetase (95, 124, 126, 140). Whether squalene synthetase is a distinct enzyme, or a multi-enzyme complex, remains to be shown. For the purposes of this study, squalene synthetase has been defined as the catalytic capacity to convert FPP, in the presence of NADPH, into squalene. The specific activity of this enzyme was expressed in units of $\mu\text{moles of squalene produced min}^{-1} \text{ mg}^{-1}$ of total protein.

To 0.10 ml of the 40,000 g supernatant was added 10 μl of

NADPH (100 μ moles). Following thermal equilibration to 24^o in a constant temperature water bath, 20 μ l of FPP-¹⁴C (22,000 dpm, specific activity 4.7 μ C/ μ M) was added and the sample incubated for a short period of time (8.0-16.0 mins). The reaction was stopped by the addition of 5-6 drops of 5 N KOH. Squalene-¹⁴C was then extracted in 4-5 volumes of diethyl ether, transferred to a scintillation vial and the ether evaporated off under a stream of nitrogen. The sample was then counted in Bray's scintillation fluid and corrected for quenching by internal spiking with a toluene-¹⁴C standard.

Results and Discussion

Figure 30 is a kinetic assay of squalene-¹⁴C formation versus incubation time which was carried out on the 40,000 g supernatant of a 28 hour germinated set of seeds. The turnover of FPP-¹⁴C into squalene-¹⁴C is instantaneous and linear over the first 24 minutes assayed. All subsequent assays were run for a period of 16 minutes or less to insure enzyme saturation. A summary of specific activity changes over the first 28 hours of germination (Table 10, Figure 31) shows the squalene synthetase to rise approximately two fold in the first 10-12 hours. Thereafter, the specific activity remains relatively constant.

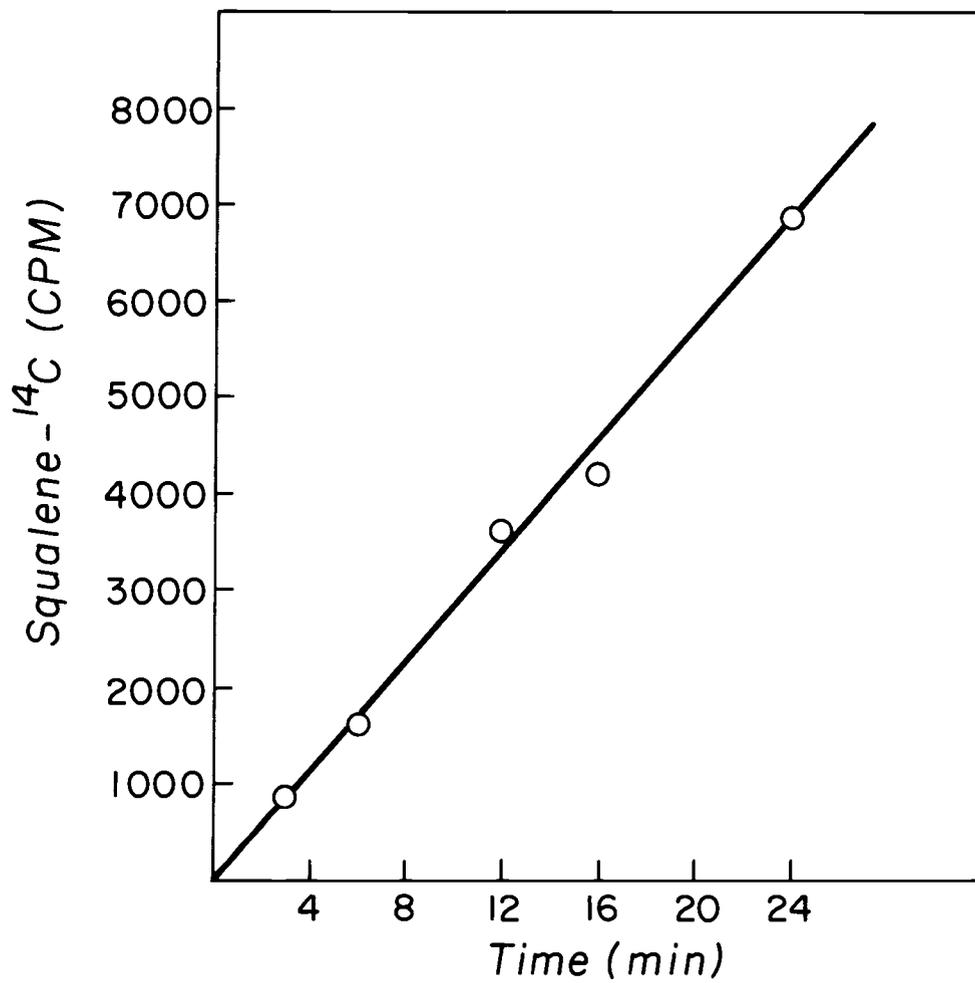


Figure 30. Kinetic assay of squalene-¹⁴C formation from FPP-¹⁴C.

Table 10. Assay of Squalene Synthetase Specific Activity Versus Germination Period.

Germination (hrs)	Supn't (ml)	Time (min)	Protein (mg)	Squalene (dpm)	Specific activity ¹	
					(dpm min ⁻¹ mg ⁻¹)	(μmoles min ⁻¹ mg ⁻¹)
2.0	0.10	8.0	3.20	1460	53	5.1 x 10 ⁻³
4.0	0.10	8.0	3.84	2485	81	7.8
5.0	0.10	16.0	3.54	5310	92	8.8
6.0	0.10	8.0	4.08	2981	88	8.4
8.0	0.10	8.0	4.28	2765	78	7.5
10.0	0.10	16.0	4.12	6390	95	9.1
12.0	0.10	16.0	4.22	5740	84	8.0
16.0	0.10	16.0	4.23	6055	88	8.4
22.3	0.10	16.0	4.32	6385	91	8.7
28.3	0.10	8.0	4.52	3100	83	7.9
8.0	boiled	-	-	111	-	-

¹ Corrected against boiled blank.

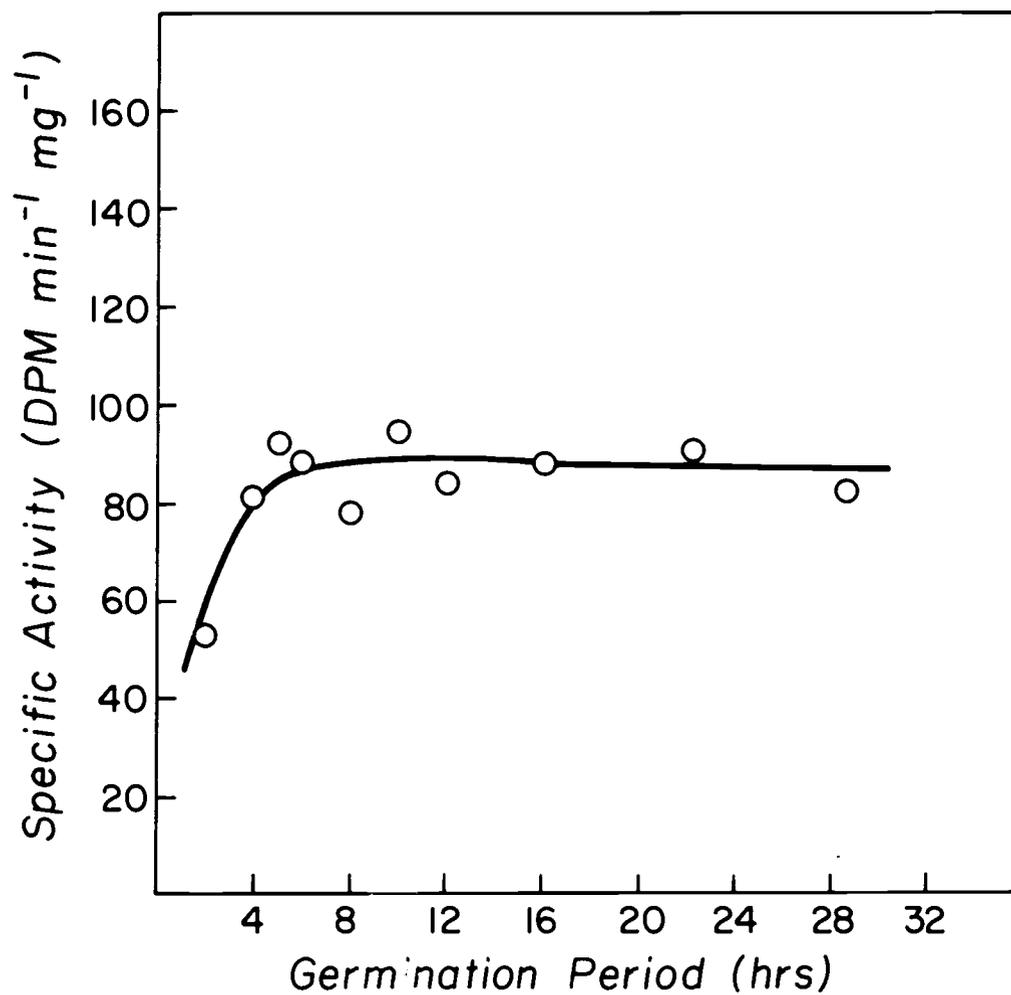


Figure 31. Specific activity of squalene synthetase as a function of germination.

RESULTS AND DISCUSSION

In Vivo Assay of MVA-2-¹⁴C Incorporation into NSF Products

Nes et al. (120) reported in vivo changes in the metabolic capacity of germinating pea seeds to incorporate labelled MVA into NSF products. The results suggested sequential incorporation of label into squalene, β -amyrin, and sterols. Of notable interest was an observation that the total capacity to make NSF products decreased with development. In terms of regulation, it is most significant that the seed selectively discriminated between tetra- and pentacyclic products, channelling the flow of carbon into pentacyclic products (β -amyrin) during the early stages of germination and, at later stages, reversing the flow into tetracyclic products (sterols). Since the latter two products must arise from the same common precursor, 2,3-oxidosqualene, the mechanism by which the seed establishes a priority for one product over the other is of considerable interest.

However, it was not clear from the experiments of Nes et al. whether the reported incorporations of MVA into NSF products were truly a measure of the capacity to make such products, or whether these changes represent steady state levels attained at different germination intervals. An apparent loss of capacity to incorporate MVA into the NSF may arise through an increase in the turnover rate of NSF products. What appears to be selective synthesis of one

product over the other, might equally be interpreted as a proportional change in the steady state. Consequently, the turnover rate of NSF products must be clearly defined before interpretations on the actual metabolic capacity of germinating seeds to make such products may be proposed. In the following paragraphs are described experiments which were carried out to (i) evaluate the capacity of seeds to make NSF products at very early stages of germination and (ii) to determine to what degree these latter products are turned over.

Several sets of seeds were germinated for various intervals in the presence of a short pulse of MVA-2-¹⁴C, which was added at the initiation of imbibition. An opportunity was thus allowed for the uptake and subsequent metabolism of MVA from time zero. Hence, the fate of NSF products could be ascertained by observing the levels of radioactivity passing into the NSF. If there were a capacity to transform any of the NSF products into new compounds not normally isolated in the NSF, and if that capacity depended upon germination, then the accumulation of radioactivity should rise through a maximum and then decline. If, on the other hand, NSF products were stable against further metabolism, the radioactivity associated with the NSF would be anticipated to rise to a maximum and remain constant thereafter. The actual experimental results may be seen in Table 11 and Figure 32.

It may be seen from Figure 32 that the capacity of seeds to

Table 11. In Vivo Incorporations of MVA-2-¹⁴C into NSF Products.

Germination (hrs)	Incorporation of MVA-2- ¹⁴ C ¹			
	Total NSF (%)	Squalene (%)	Triterpene (%)	Sterols (%)
4.0	7	7	-	-
8.0	7	7	-	-
16.0	16	14	2	-
25.1	29	20	9	-
48.2	29	5	23	1
72.2	30	2	25	3

¹In terms of R,S-MVA-2-¹⁴C taken up during imbibition. Methods for determining the distribution of label among NSF products are described in the text.

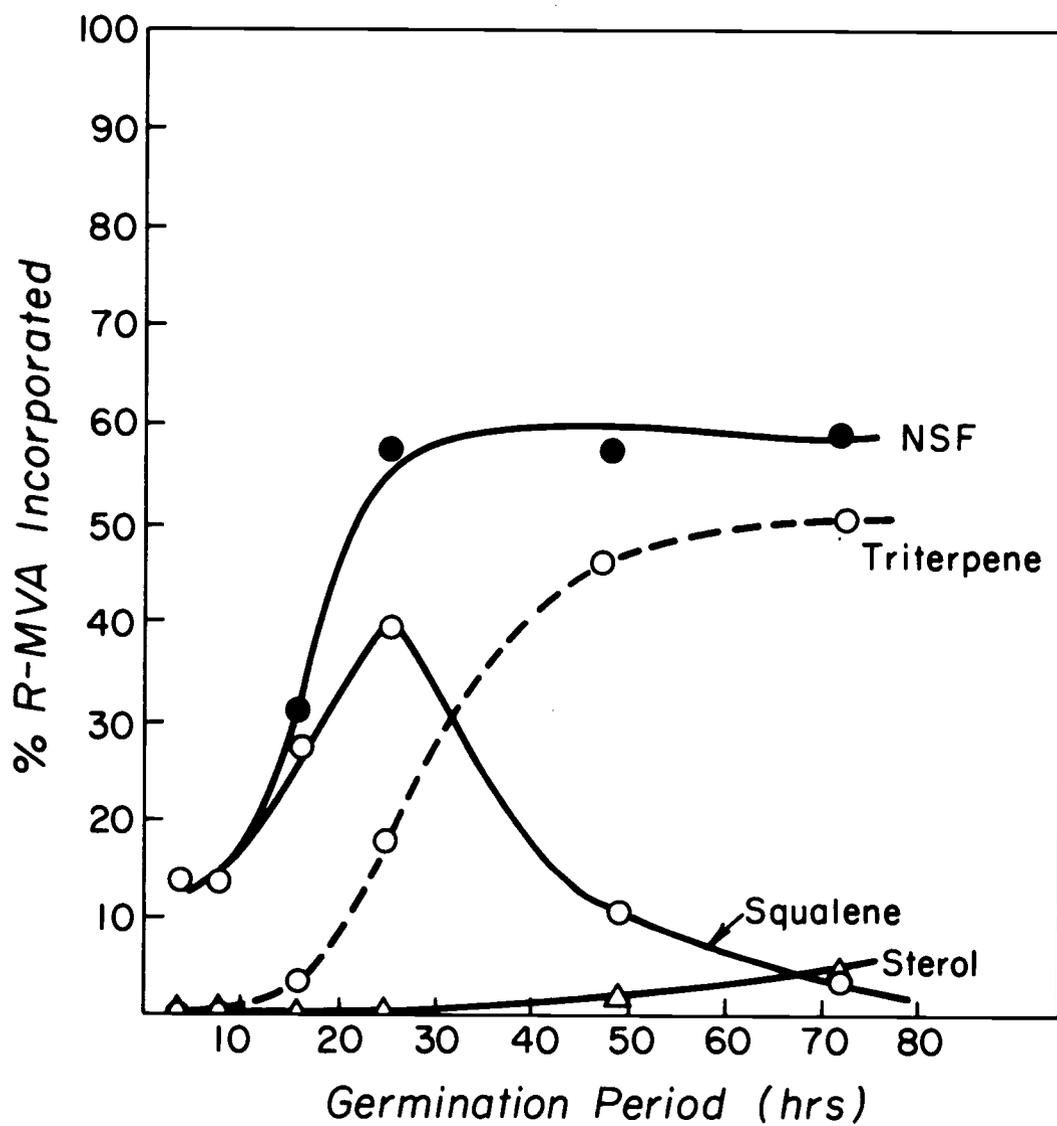


Figure 32. In vivo incorporation of MVA-2-¹⁴C into NSF products over the first three days of germination.

incorporate endogenously administered MVA into squalene rises sharply over the first several hours of germination. Over a similar period, the capacity to make tetra- and pentacyclic products is absent, with the exception of β -amyrin, which may be estimated to arise sometime shortly after eight hours. By 16 hours germination, the capacity to make β -amyrin is still quite low, however, but rises rather rapidly thereafter. Between the second and third days of germination, small traces of labelled sterol were first detected.

As evident from Figure 32, radioactivity incorporated into the NSF rises to a maximum level around 24 hours germination and remains constant. Since this level does not fall off, variations in the distribution of label among the NSF products as a function of germination must truly represent changes in the metabolic capacity of the squalene epoxidase⁶/oxidosqualene cyclase system. Similarly, the overall decrease in MVA incorporated into NSF products during germination, as reported by Nes et al., must be accounted for in terms of a decreased capacity to make such products. The factor (or factors) which suppresses these incorporations must act at some point in the isoprenoid pathway prior to squalene epoxidase.

⁶The existence of such an enzyme may only be inferred. Corey (23) was able to show that 2,3-oxidosqualene, the presumptive product of the epoxidase, is an intermediate in the pathway. When oxidosqualene was added to pea seed homogenates, it was shown to cyclize to β -amyrin.

Furthermore, this factor is temporal, since the capacity to make NSF products from MVA shows an initial rise during the first 24 hours of germination.

If the flow of carbon among the NSF products is examined in light of the above experiments, it is apparent that metabolically rate limiting steps exist, which change in an orderly and well defined pattern during germination. Initially, large levels of labelled squalene accumulated in the NSF. During such an interval, it is logical to conclude that squalene epoxidase is metabolically inactive (i. e., the rate limiting step). This interpretation does not necessarily imply the absence of such an enzyme, nor its presence in an inactive state, since it is equally conceivable that the enzyme and substrate are spatially segregated from one another, perhaps through compartmentation. The reasons for such metabolic inactivity are not available for interpretation under the conditions in which these experiments were carried out. It is important to note, however, that the subsequent transition between accumulation of squalene to production of pentacyclic product (β -amyrin) at no time reveals an accumulation of oxidosqualene. Hence, squalene epoxidase remains rate limiting during this transition. As this is the case, it is quite surprising to observe an increase in the activity of oxidosqualene cyclases at an even later stage of germination, as apparent from the increased capacities to make sterols. This clearly implies that such enzymes are

successfully competing for a portion of oxidosqualene. Since oxidosqualene was not observed to accumulate in the NSF, by what mechanism do the cyclases successfully compete for a common and obligatory precursor of such divergent pathways?

Enzyme Aggregates - A Regulatory Model

Figure 33 is a schematic model illustrating a potentially active complex consistent with the above experimental observations. By this model, cyclases for the synthesis of tetra- and pentacyclic products form enzyme aggregates with squalene epoxidases. Oxidosqualene remains as an enzyme-bound intermediate that is subsequently transformed into its ultimate cyclized product by the aggregate cyclase of the enzyme complex. As enzyme aggregates, two or even more divergent pathways would not compete for oxidosqualene, but for squalene. Experimentally, the latter product was observed to accumulate in excess and, hence, is capable of meeting the demands of either of the presumptive aggregates (squalene epoxidase/ β -amyrin oxidosqualene cyclase and squalene epoxidase/sterol oxidosqualene cyclase). With such a model, the flow of carbon would predictably accumulate in squalene, as the aggregate becomes rate limiting, with the next detectable product appearing as a cyclized intermediate (i. e., β -amyrin and/or sterol).

Bernard and Reid (10) have suggested that phyto- and methyl

sterols arise from divergent pathways. In the former case, cycloartenol is believed to be the likely precursor, while the methyl sterols are believed to possibly arise through lanosterol. Both cycloartenol and lanosterol have been implicated as the products of distinctly different cyclases (20). In either case, however, oxidosqualene serves as substrate. In light of recent work by Reid (136) on the accumulation of 2,3-oxidosqualene in leaf slices of N. tabacum, this aggregate concept is consistent with his experimental results. Reid incubated leaf slices of N. tabacum with MVA-2-¹⁴C during inhibition of phyto-sterol synthesis with tris(2-diethylaminoethyl)phosphate hydrochloride (SK&F 7997). The incorporation of label into oxidosqualene, phyto-sterols and methyl sterols was then compared to a control experiment that lacked inhibitor. In the presence of SK&F 7997, the accumulation of label into oxidosqualene increased some 9-17 fold, while phyto-sterols experienced a 0.1-0.15 fold decrease and methyl sterols a 0.5-0.7 fold decrease. Reid attributed the difference in inhibition of phytosterols, as opposed to methyl sterols, to selective suppression of biosynthetic components in the latter pathway. If reference is made to conventional flow diagrams of the common intermediates, an alternate reationalization of this data may be offered. Figure 34a represents the flow of MVA through a distinct squalene epoxidase step. The product, squalene oxide, then branches off to either phyto- or dimethyl sterol products. If an inhibitor then blocks one or both of

these divergent routes, then oxidosqualene must increase by a proportional amount. On the other hand, an aggregate model (Figure 34b) does not share the same relationship. As an enzyme aggregate, epoxidation of squalene normally can not exceed the rate of cyclization, since the enzyme-bound intermediate (oxidosqualene) and native conformation of the aggregate prevent additional molecules from attaching to the active site(s) of the complex. SK&F 7997 may cause a conformational change in the aggregate, or possibly complete dissociation, such that oxidosqualene is no longer efficiently transferred to the cyclase. Under such conditions, squalene epoxidation may exceed subsequent cyclization by prematurely releasing oxidosqualene that is normally bound to the enzyme aggregate until cyclized. Complete dissociation of the aggregate is less likely in this case, however, since the results of Reid's experiments are not compatible with the precursor/product relationship of Figure 34a (note that the dissociated aggregate is analogous to the flow diagram of Figure 34a). In essence, the inhibitor, which is a detergent, might drastically alter the efficiency of oxidosqualene transfer without severely affecting the catalytic capacity of either enzyme. (This could be accomplished through a conformational change.) Providing the epoxidation rate exceeds that of the cyclase, the overall yield of sterols will be only slightly depressed, while a disproportionately large accumulation of oxidosqualene occurs. This latter model more adequately rationalizes

the inhibitor studies of Reid, in which an extraordinarily large accumulation of oxidosqualene was accompanied with only slight decreases in sterol formation.

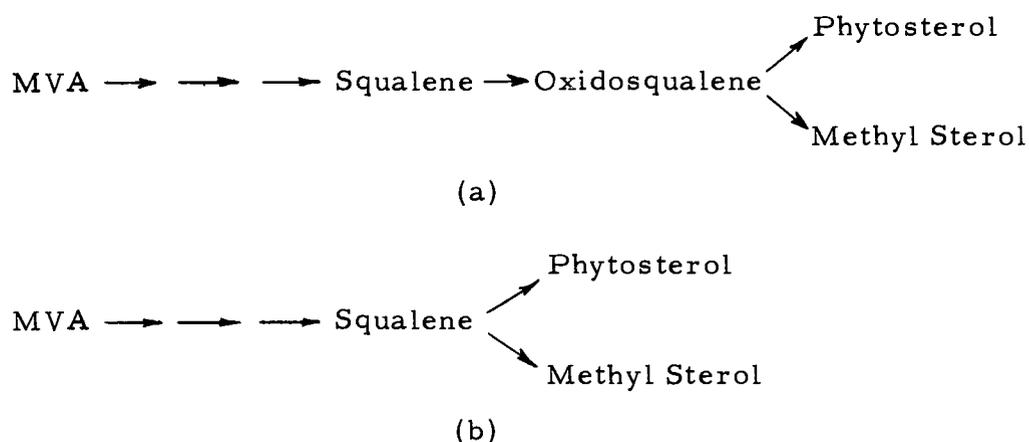


Figure 34. Schematic flow diagrams for squalene transformations into sterol products by (a) linear transformations between precursor and product and (b) direct transformations through enzyme aggregates.

Limitations of *in Vivo* Assays

The priority for making one product or the other may be regulated by variations in the enzyme levels (i. e., de novo synthesis and subsequent degradation) or, alternately, through any of a number of regulatory mechanisms such as zymogen formation and activation, enzyme inhibition and activation, substrate segregation (i. e., compartmentation), etc. An additional control for selectively channeling carbon into one product or the other might be through association and dissociation of the enzyme aggregate, in which only the associated

aggregate competes for substrate. A thorough characterization of apparent changes in enzymic capacities to make NSF products could not be suitably applied to in vivo assays. In short, rate limiting steps which may be observed to change during germination may be due to simple transport of substrate across membrane barriers. There is no satisfactory way of ascertaining to what extent a substrate penetrates to a metabolically active site. An in vitro enzyme preparation was therefore sought, which would allow a more direct approach in characterizing the factors responsible for the metabolic changes previously observed

In Vitro Incorporations of MVA-2-¹⁴C into NSF Products

It is perhaps not surprising that in vitro preparations of germinating seeds were considerably less active in biosynthesizing tetra- and pentacyclic products from MVA-2-¹⁴C. On the other hand, the incorporation of MVA-2-¹⁴C into the NSF in vivo and in vitro was quite comparable. TLC of appropriate aliquots from each NSF showed that in vitro incubations lacked the capacity to make cyclic products normally found in vivo. In very crude homogenates, heavily contaminated with cellular debris, β -amyrin could be made from MVA, but with much less efficiency than normally observed in vivo; the reproducibility of identically prepared homogenates to make β -amyrin from MVA was also quite poor. These difficulties were

most likely due to the particulate nature of the latter enzymes. Since cyclase-rich homogenates could not be prepared with activities comparable to those previously observed in vivo, it was necessary to abandon further studies on triterpene and sterol synthesis. Instead, an effort was made to study those isoprenoid enzymes most readily solubilized in vitro, which constituted all of the enzymes between MVA kinase and squalene synthetase, respectively.

The technique of preparing and assaying crude homogenates was examined for reproducibility by the following experiment: Five individual sets of seeds, each germinated for the same period, were homogenized and assayed by methods previously described. Subsequent incubations of aliquots from each homogenate with MVA-2-¹⁴C under identical conditions demonstrated that variations in NSF incorporations would not exceed, on the average, $\pm 5\%$ (Table 12). A lower limit was thus established regarding detectable metabolic changes. A clear, cell-free 40,000 g supernatant was finally chosen for all future studies, which converted MVA-2-¹⁴C into the NSF at a rate equal to that of crude homogenates (Table 13). Aliquots of this supernatant were homogenous, thus providing suitable samples to be correlated against one another during germination. This same supernatant contained considerable squalene synthesizing capacity from MVA-2-¹⁴C, but lacked the capacity to make tetra- and pentacyclic products. This conclusion was based on several assays at different stages of

Table 12. Reproduceability of Homogenate Preparation and Assays of NSF Incorporations from MVA-2-¹⁴C.

Trial (75 seeds)	Homogenate (ml)	Incorporation into NSF (dpm hr ⁻¹)	Deviation ¹ (dpm hr ⁻¹)
1st	5.0	435500	24540
2nd	5.0	489800	29760
3rd	5.0	468900	8860
4th	5.0	429700	30340
5th	5.0	<u>476300</u>	<u>16260</u>
	Average	460040	<u>±21952</u>
		460040 ± 4.7%	

¹To avoid averaging of errors, all deviations were considered to be positive. This then represents a maximal error in which averaging errors have been neglected.

Table 13. NSF Incorporating Capacity from MVA-2-¹⁴C. Distribution in Homogenate.

	Enzyme solution (ml)	NSF incorporation	
		%	R,S-MVA hr ⁻¹
Crude Filtrate	10.0	18.8	1.501x10 ⁶ dpm
40,000 g supernatant	10.0	18.8	1.505x10 ⁶ dpm
Pellet (resuspended) ¹	10.0	1.1	0.092x10 ⁶ dpm

¹The pellet of the 40,000 g supernatant was resuspended in 25 ml of phosphate buffer, from which an aliquot was then drawn as shown.

germination, followed by TLC of aliquots from the NSF on silica gel G and plates impregnated with 3% AgNO_3 . In all cases, radioactivity in the NSF ran coincident with authentic carrier squalene. No radioactivity was observed to migrate with authentic samples of β -amyrin, nor β -sitosterol.

Kinetics of Squalene- ^{14}C Formation from MVA-2- ^{14}C
in the 40,000 g Supernatant

Incorporation of MVA-2- ^{14}C into squalene was studied as a function of incubation time on 3.0 ml aliquots of a 40,000 g supernatant, prepared from a nine hour germinated set of seeds (Figure 35). An initial lag period of approximately 13 minutes was observed prior to linearity in squalene formation. It should be noted that the rate of squalene formation is dependent upon the slowest acting enzyme in the isoprenoid pathway leading to squalene. The initial lag in squalene formation then presumably results from the time required to accumulate sufficient precursors with which to saturate this rate limiting enzyme. Evidence to be cited in the following section suggests that the rate limiting step in squalene formation is dependent upon IPP/DMAPP prenyltransferase activity. Approximately 30% of the R-MVA-2- ^{14}C initially added to the incubates was converted into squalene within one hour. In a three hour incubation, incorporations averaged between 80-90% of the available enantiomer. On the basis of these experiments, an incubation period of 60 minutes was chosen for subsequent routine

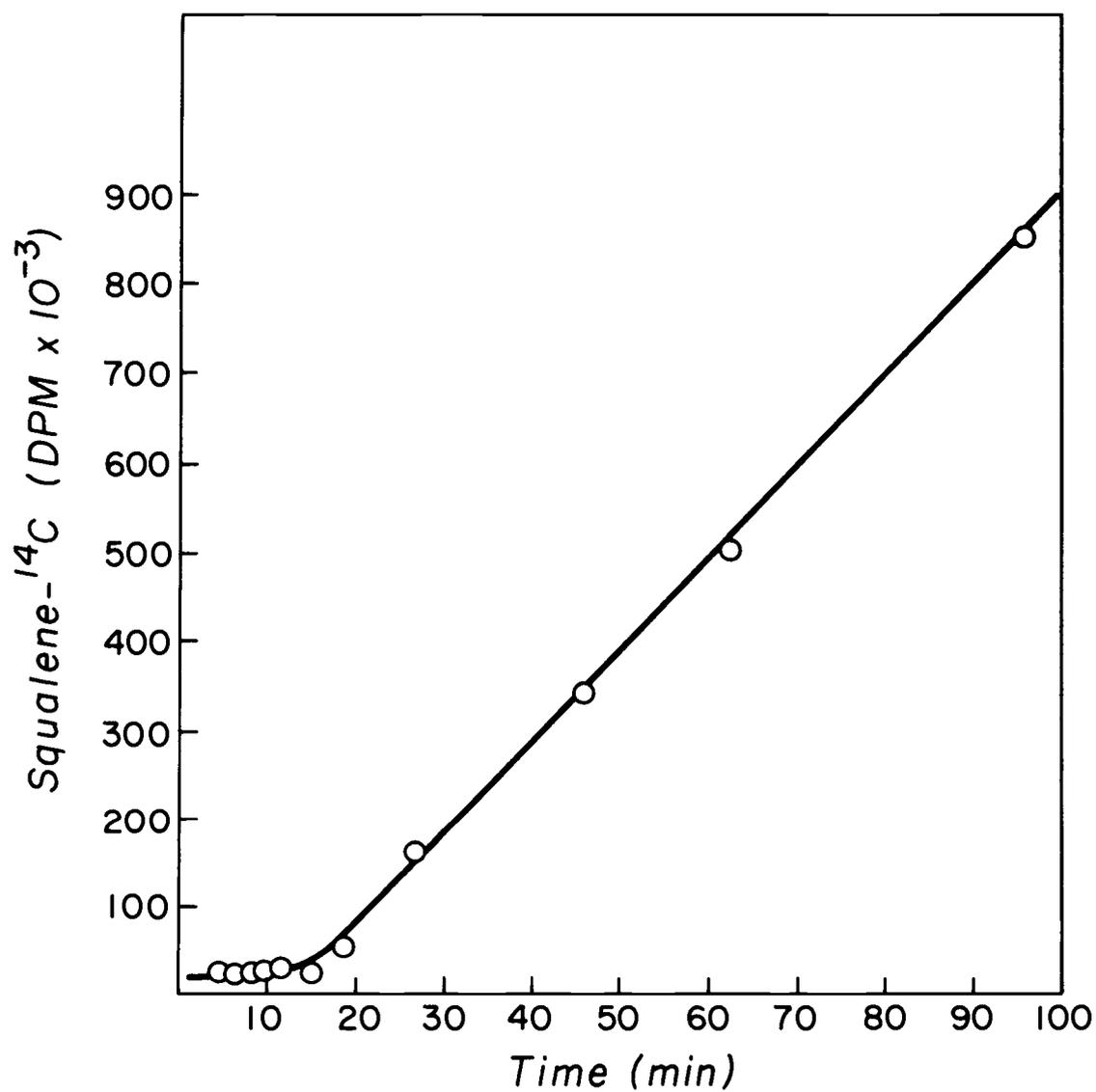


Figure 35. Kinetic assay of squalene formation from MVA-2-¹⁴C.

assays.

Squalene Synthesizing Capacity from MVA-2-¹⁴C
versus Germination Period

The capacity to biosynthesize squalene from MVA-2-¹⁴C was studied in two distinctly different media. Assays were carried out on 40,000 g supernatants, prepared as previously described, except the effect of sucrose on squalene synthesizing capacity was investigated. In one series of experiments, 0.45 M sucrose was retained in the homogenizing medium, while in the other, sucrose was omitted. All other conditions were kept the same. Table 14 summarizes the experimental results of each study. Included in Table 14 is the acid labile prenyl pyrophosphate(s) simultaneously accumulated in each incubate. For convenience, those metabolic changes observed with sucrose in the homogenizing medium will be referred to as (+) sucrose patterns; metabolic changes observed with the omission of sucrose from the homogenizing medium will be referred to as (-) sucrose patterns. The capacity to make squalene from MVA-2-¹⁴C and related levels of prenyl pyrophosphate(s) for (+) and (-) sucrose patterns have been superimposed upon one another in Figure 36.

An examination of the precursor/product relationships and the effects of sucrose on this balance provides an insight into the origin of the patterns.

Table 14. Squalene Synthesizing Capacity from MVA-2-¹⁴C versus Germination Period.

Germination (hr)	(-) Sucrose ¹		(+) Sucrose ¹	
	Squalene (dpm hr ⁻¹ ml ⁻¹)	Prenyl-PP (dpm hr ⁻¹ ml ⁻¹)	Squalene (dpm hr ⁻¹ ml ⁻¹)	Prenyl-PP (dpm hr ⁻¹ ml ⁻¹)
0.0	4500	15800	1733	5420
2.0	16640	87950	12810	6980
4.0	42600	221500	129100	42900
6.0	23550	181000	218250	43300
8.0	22190	210500	209000	41500
10.0	15900	202700	211900	43650
12.0	19000	109800	217000	39420
14.0	45800	-	246000	21500
16.0	47200	-	424000	29700
18.0	36800	-	398000	35700
20.0	20590	-	389000	19600
22.0	-	-	321000	8600
24.0	11200	-	347000	-
26.0	-	-	325000	-
28.0	-	-	333000	-
30.0	-	-	273000	-
32.0	-	-	296000	-

¹(-) and (+) sucrose is defined in the text. The specific activity of the MVA-2-¹⁴C used in all experiments was 5.9 $\mu\text{C}/\mu\text{M}$. Dashes indicate no assay was made.

Analysis of the (+) Sucrose Pattern

From Figure 36 it may be seen that the (+) sucrose pattern, in terms of squalene synthesizing capacity from MVA, passes through three transitions, designated as phases I, II and III. Phase III will be discussed at a later time (see page 145, Suppression of MVA-2-¹⁴C Incorporation into Squalene).

Reference to Figures 37a, b, and c is useful in analyzing the observed transitions between phases I and II. Conceptually, a hypothetical pathway may serve as a useful model in place of the more complex isoprenoid pathway. In this scheme, substrate A is transformed by enzyme (1) into B, which is subsequently transformed into product C by enzyme (2). Although specific activities of enzymes (1) and (2) are not known, conditions are such that addition of substrate A allows a linear rate of C formation. At any period of development, the flow of A into C is dependent upon the least active enzyme in the pathway (i. e., the rate limiting step of the pathway). Experimentally a biphasic curve (phases I and II) was observed during development, in terms of A incorporated into C. The transition between phase I and II may arise by either a sequential change in the rate limiting step, induced by development, or maintenance of the same rate limiting step throughout phases I and II. In the latter case, the rate limiting enzyme must rise in activity, level out and then rise once again. A particularly attractive example might be sequential activation of isozymes,

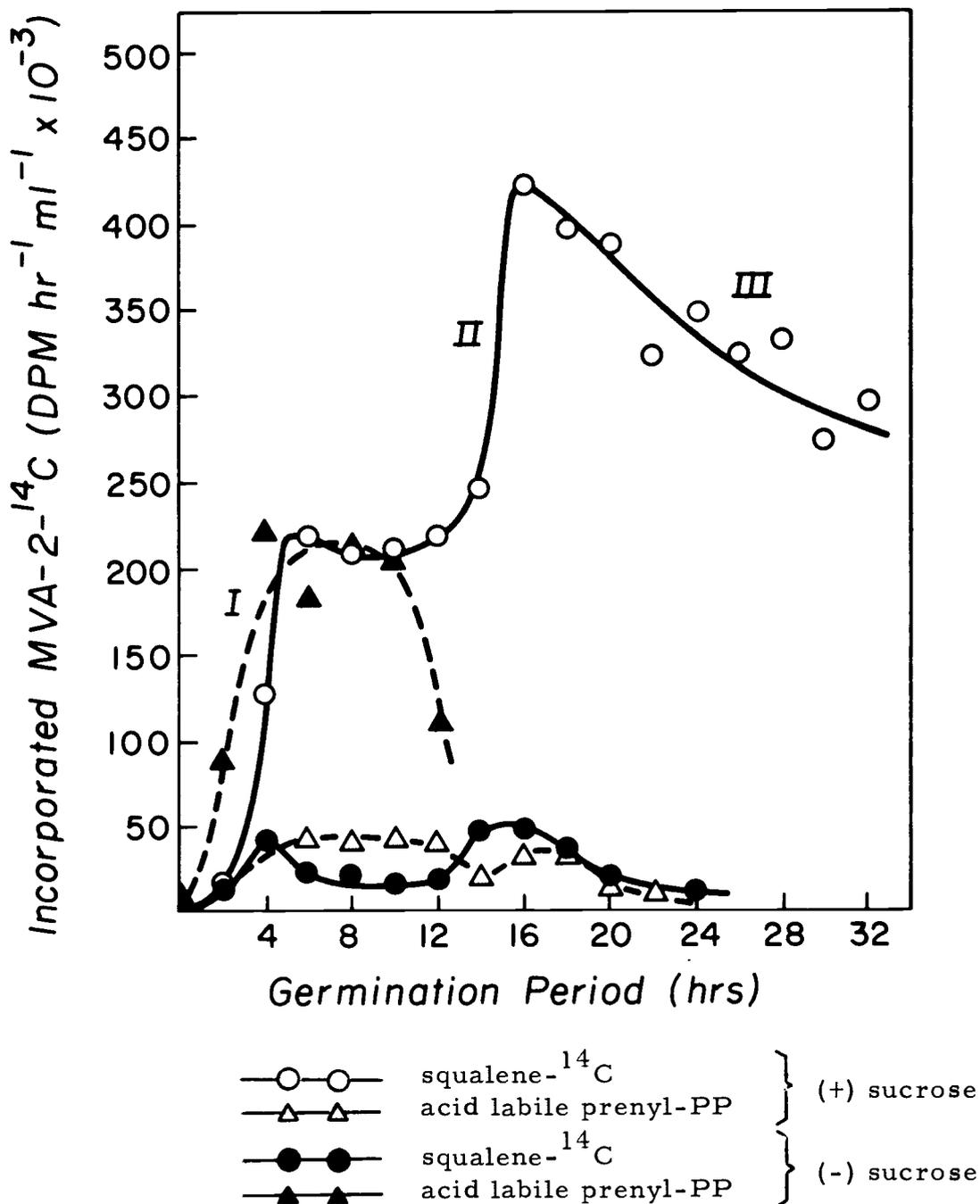


Figure 36. Effect of sucrose in the homogenizing buffer on the incorporation of MVA-2-¹⁴C into squalene and acid-labile prenyl pyrophosphates as a function of germination.

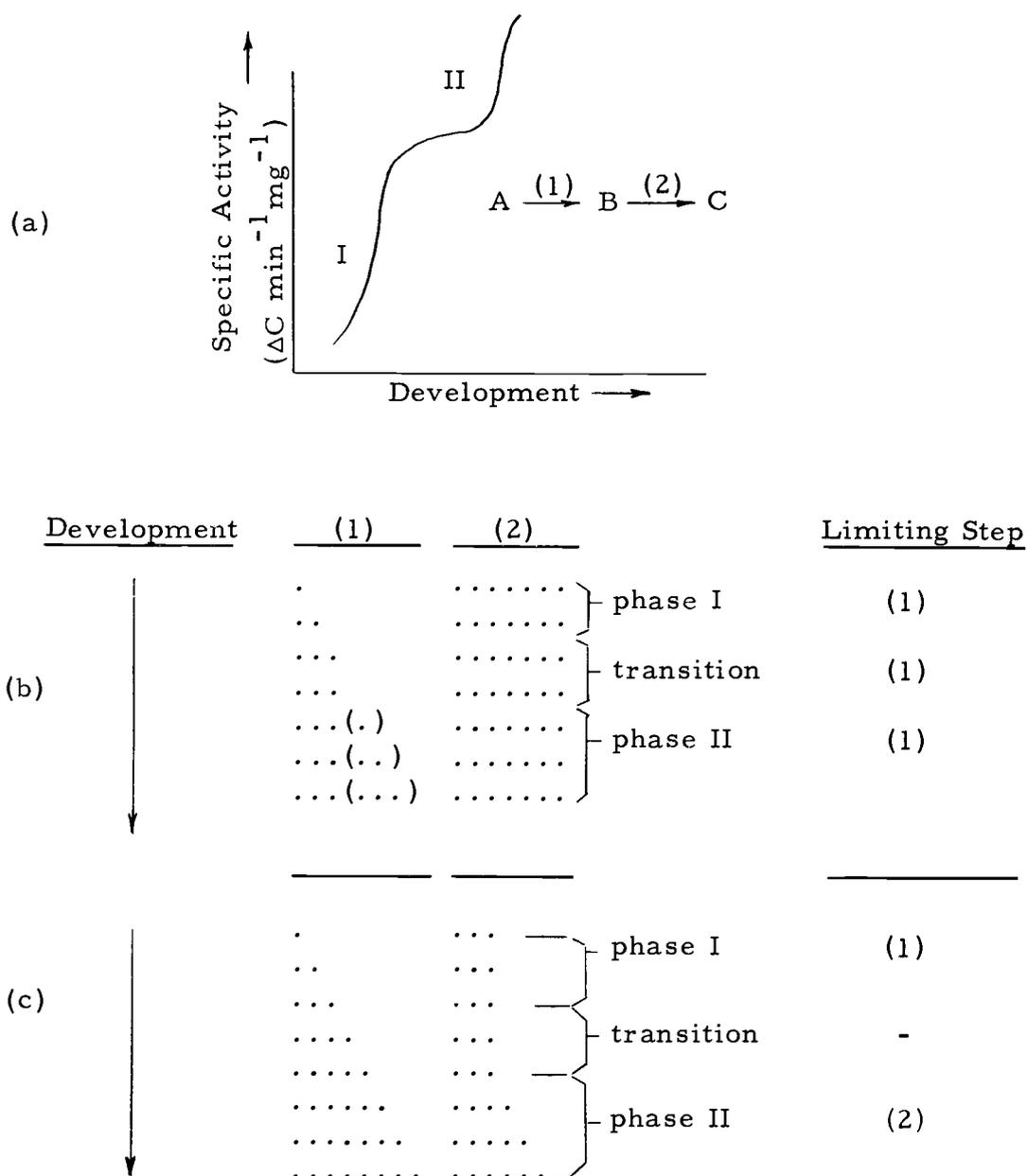


Figure 37. Schematic representation of enzymic transitions in a hypothetical pathway as a function of development: (a) hypothetical pathway and observed metabolic pattern during different stages of development; (b) and (c), ways by which the metabolic pattern of (a) may arise through changes in enzyme activity. Each dot represents one unit of activity.

or, possibly, sequential activation and intermixing (during homogenization) of cytoplasmic and organelle associated enzymes. A transition involving two rate limiting steps, on the one hand, or a transition with a common rate limiting step, on the other hand, may be schematically represented in terms of enzymes (1) and (2), as shown in Figures 37b and 37c. Each dot represents an arbitrary unit of activity. No statement regarding the origin of enzyme activity is intended (i.e., de novo synthesis, allosteric activation, etc.). In Figure 37c, parentheses are enclosed around new units of activity arising in phase II. The rate limiting step is arbitrarily assigned to enzyme (1), but could equally be assigned to enzyme (2). Alternately, this same metabolic pattern might arise through the transient appearance of a competitive pathway for product B, or, possibly, the transient appearance of an inhibitor during development of enzyme (1) or (2).

Analysis of the (-) Sucrose Pattern

It may be seen from Figure 36, that the total metabolic capacity to make prenyl(s) from MVA is quite similar to phase I of the (+) sucrose pattern, except labelled prenyl(s) accumulate as the major product of the pathway instead of squalene. Since squalene arises from prenyl pyrophosphate, any squalene which is biosynthesized from MVA must also indirectly represent the capacity to make prenyl

pyrophosphate. Table 15 summarizes the interrelationship between prenyl pyrophosphate and squalene formation and the effect of sucrose on this balance. It is evident that the metabolic capacity of supernatants in making prenyl pyrophosphates is unchanged, whether sucrose is present or absent from the homogenizing buffer. On the other hand, the capacity to make squalene from prenyl pyrophosphate(s) is lost with the omission of sucrose from the homogenizing buffer. It is therefore apparent that phase I is independent of squalene synthetase. By careful analysis, it may be deduced that the decline in prenyl pyrophosphate formation is either due to suppression of prenyltransferase or IPP isomerase activity. Direct assays of IPP isomerase over this same period demonstrated no suppression, while similar assays of prenyltransferase did. The latter two assays will be discussed in more detail in a later section.

Analysis of the Rate Limiting Step(s) Between Phases I and II

The detection of acid labile prenyl pyrophosphate(s) throughout phases I and II (Table 14) indicated that the rate limiting step(s) was beyond IPP isomerase, the first enzyme of the pathway leading into the prenyl pyrophosphates. Subsequent labelling experiments with MVA-2-¹⁴C led to the conclusion that the prenyltransferase (IPP/DMAPP) was rate limiting throughout phases I and II (phase II is defined for the (+) sucrose pattern only). The line of reasoning which

Table 15. Action of Sucrose on the Isoprenoid Pathway.

Incorporation of MVA-2- ¹⁴ C into -	Phosphate Buffer	
	(-) Sucrose	(+) Sucrose
	($\mu\text{moles ml}^{-1} \text{ hr}^{-1}$) ¹	($\mu\text{moles ml}^{-1} \text{ hr}^{-1}$) ¹
Squalene	3.3 (22000 dpm)	31.8 (209000 dpm)
Acid Labile Prenyl Pyrophosphate	<u>32.1</u> (211000 dpm)	<u>3.4</u> (21500 dpm)
Total	35.4	35.2

¹ Assays were made on 40,000 g supernatants from 8.0 hr sets of germinated seeds prepared as previously described.

allowed for such a conclusion may be more clearly illustrated by referring once again to the model pathway depicted in Figure 36a.

If an excess pulse of labelled A is allowed to pass through the pathway into C, and then intermediate B is examined, the rate-limiting step at any stage of development may be deduced. If B accumulates label, then enzyme (2) must be rate limiting. Conversely, the absence of labelled B indicates that enzyme (1) is rate limiting. Hence, the rate limiting enzyme(s) responsible for the transition between phases I and II may be deduced by determining whether label accumulates in B over the same developmental period. For the conditions illustrated by Figure 36b, labelled A and C would be observed over phase I and A, B and C over phase II. In Figure 36c, only labelled A and C would be detected throughout phases I and II.

Such labelling experiments were carried out on the isoprenoid pathway. Sets of seeds at 9 and 16 hours germination were homogenized and the 40,000 g supernatant prepared as previously described. Aliquots of each were incubated with MVA-2-¹⁴C for a sufficient time to allow for squalene-¹⁴C formation. Each reaction was then terminated and analyzed for labelled prenyl pyrophosphate accumulation. In each case, the last detectable intermediate of the pathway, other than squalene-¹⁴C, was DMAPP-¹⁴C. Similarly, assays of IPP isomerase, which were carried out over phases I and II with IPP-4-¹⁴C, showed the formation of DMAPP-¹⁴C and squalene-¹⁴C as the only labelled

products. The distribution of label throughout phases I and II among identifiable products of the isoprenoid pathway is summarized schematically in Figure 38. Metabolically, IPP/DMAPP prenyltransferase must therefore be the common rate limiting enzyme of both phases.

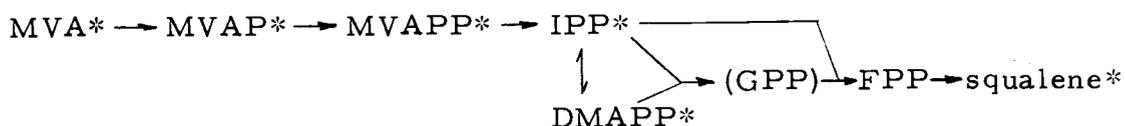


Figure 38. Deduction of the rate limiting step of the isoprenoid pathway: schematic labelling pattern from MVA-¹⁴C through squalene-¹⁴C.

Thus the following facts are known: (a) a metabolically rate limiting step is common to both phases I and II; (b) phase I is independent of sucrose effects; and (c) phase II is highly dependent upon sucrose effects. It is evident from (b) and (c), that the enzymic properties associated with phases I and II are quite divergent, although, metabolically, the rate limiting enzyme of either phase is the same. These facts suggest the presence of two prenyltransferases, one of which is independent of sucrose, and the other, highly dependent upon sucrose for in vitro activity. Nonsynchronous activation of two distinctly segregated prenyltransferases and subsequent intermixing of the enzyme pools during homogenization may clearly lead to the biphasic (+) sucrose pattern.

Suppression of MVA-2-¹⁴C Incorporation
into Squalene

Squalene synthesis from MVA-2-¹⁴C was sharply suppressed in supernatants derived from sets of seeds germinated longer than 16 hours (phase III, (+) sucrose pattern). As a working hypothesis, the apparent loss of metabolic capacity may occur from any one, or a combination, of the following conditions: (i) instability of the 40,000 g supernatant during the incubation period, which successively increases with each stage of development; (ii) an increased competition for cofactors through activation of alternate pathways and competing enzymes; (iii) increased competition for one or more precursors leading to squalene formation; (iv) an increased level of proteolytic activity; (v) temporal appearance of an inhibitor in the latter supernatants, capable of blocking one or more steps of the isoprenoid pathway.

Conditions (i)-(iv) do not appear to be major contributors in suppressing squalene formation, based on the results of the following experiments. Two sets of seeds, germinated for 16 and 39 hours, were homogenized simultaneously in the usual manner and the corresponding 40,000 g supernatants prepared by previously described methods. Aliquots from each supernatant, and from a 1:1 (v/v) mixture of the two supernatants, hereafter designated as a 16/39 hr mixture, were mixed with the appropriate levels of cofactors (ATP, NADPH) and allowed to stand for various intervals prior to the addition of labelled

MVA. Squalene synthesizing capacity as a function of standing time was then measured for each enzyme solution. One, or possibly a combination of the following results were anticipated: If conditions (i), (ii) and/or (iv) prevailed over squalene synthesizing capacity in the 39 hour supernatant, then the capacity of the latter supernatant to make squalene must decrease with standing time at a rate comparably greater than that of the 16 hour supernatant. Where either conditions (iii) and/or (v) dominated the capacity of the 39 hour supernatant to make squalene, the relative loss of synthesizing capacity of both the 39 and 16 hour supernatants must be similar. Under condition (iii) only, the 16/39 hr mixture would yield a similar stability pattern to both the 16 and 39 hour supernatants. On the other hand, condition (v) must cause a strong, time-dependent suppression of squalene synthesizing capacity in the 16/39 hr mixture. The results of the appropriate assays are summarized in Table 16. Both the 16 and 39 hour supernatants were comparably stable under the conditions of the assays, while the 16/39 hr mixture exhibited a very strong and time-dependent suppression against squalene synthesizing capacity from MVA. A normalized plot of enzyme activity versus standing time for each set of assays is presented in Figure 39.

Further studies relating to the same factor demonstrated that it is heat-labile and apparently of large molecular weight (nondialyzable). In one experiment, a 39 hour supernatant was heat denatured for 2-3

Table 16. Deactivating Factor in Squalene Synthesizing Capacity.

Enzyme solution	Standing time ¹ (min)	Activity ² ($\mu\text{moles hr}^{-1} \text{ml}^{-1}$)
16 hr supernatant	5.0	57
	10.0	61
	20.0	57
	35.0	61
	50.0	49
39 hr supernatant	5.0	17
	19.0	17
	47.0	15
16/39 hr mixture (1:1)	5.0	41
	16.0	11
	26.0	9
	39.0	6
	46.0	5
	56.0	3

¹ 3.0 ml aliquots were allowed to stand for the indicated time with co-factors added prior to initiation of the assays with MVA-2-¹⁴C.

² Expressed as squalene-¹⁴C synthesized from MVA-2-¹⁴C.

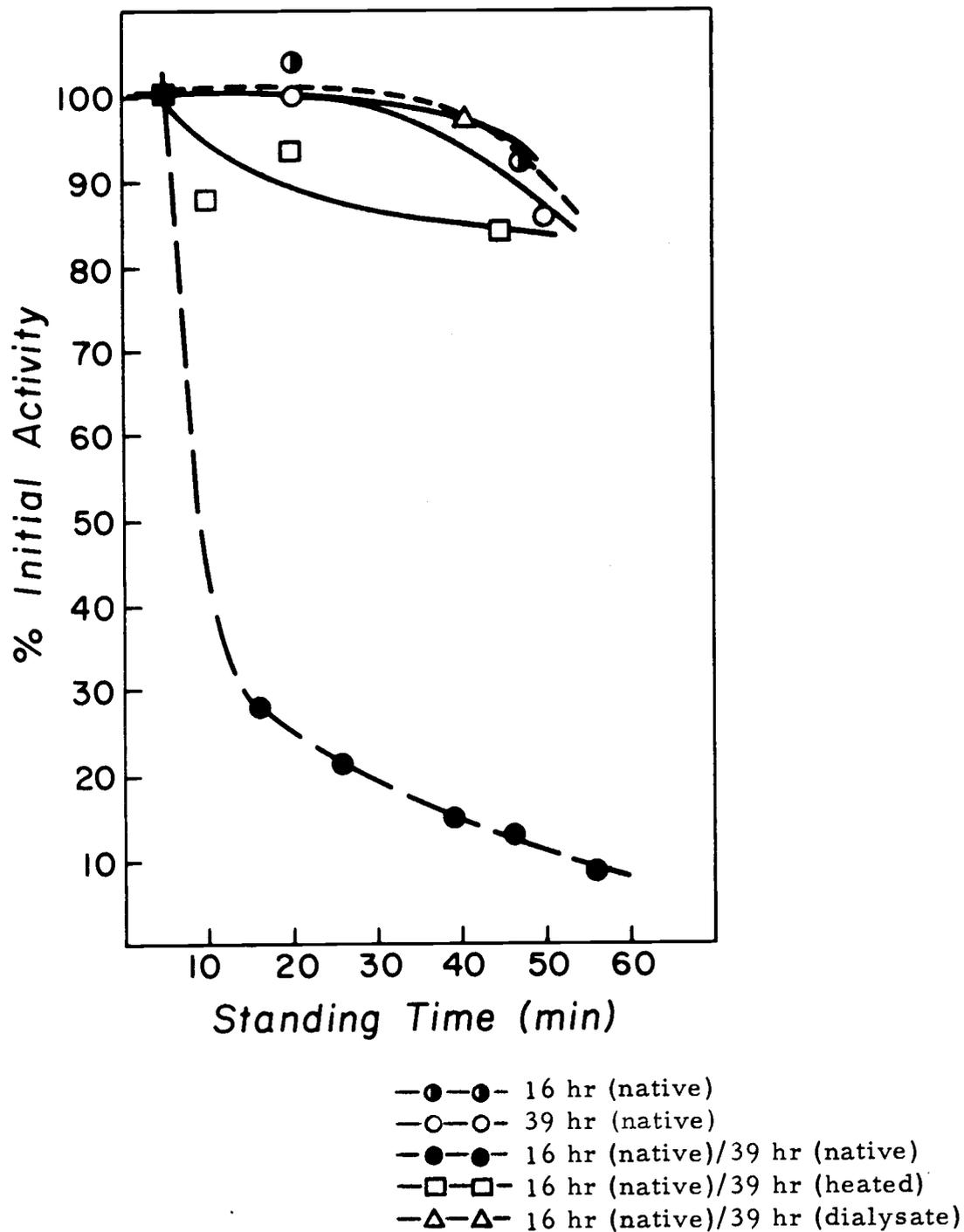


Figure 39. Suppression of squalene synthesizing capacity from MVA-2- ^{14}C in mixed supernatants.

minutes in a boiling water bath, cooled to room temperature, and then mixed with an equal volume of freshly prepared 16 hour supernatant. The 16 hr (native)/39 hr (heat denatured) mixture, when assayed under the same conditions as previously described, exhibited only minor suppression against squalene synthesis. (Between 5 and 50 minutes, 14% activity was lost. This compares with 90% loss in activity for the 16 hr (native)/39 hr (native) mixture.) In a similar experiment, a 15 ml aliquot of 39 hour supernatant was dialyzed against glass distilled water (\sim 400 ml) for several hours. The dialysate was then concentrated to approximately 15 ml on a rotary evaporator (under high vacuum), mixed with 15 ml of freshly prepared 16 hour supernatant, and assayed for squalene synthesizing capacity with MVA-2- ^{14}C as previously described. Aliquots of the 16 hr (native)/39 hr (dialysate) mixture lost less than 4% activity over the first 50 minutes assayed. The results of each of these additional experiments are also included in Figure 39.

These results suggest the presence of a potent inhibitor, possibly a protein, which apparently blocks the flow of carbon between MVA and squalene and is activated sometime after the onset of imbibition. From 16 hours and on, the inhibitor plays a dominant role in suppressing in vitro synthesis of squalene from MVA.

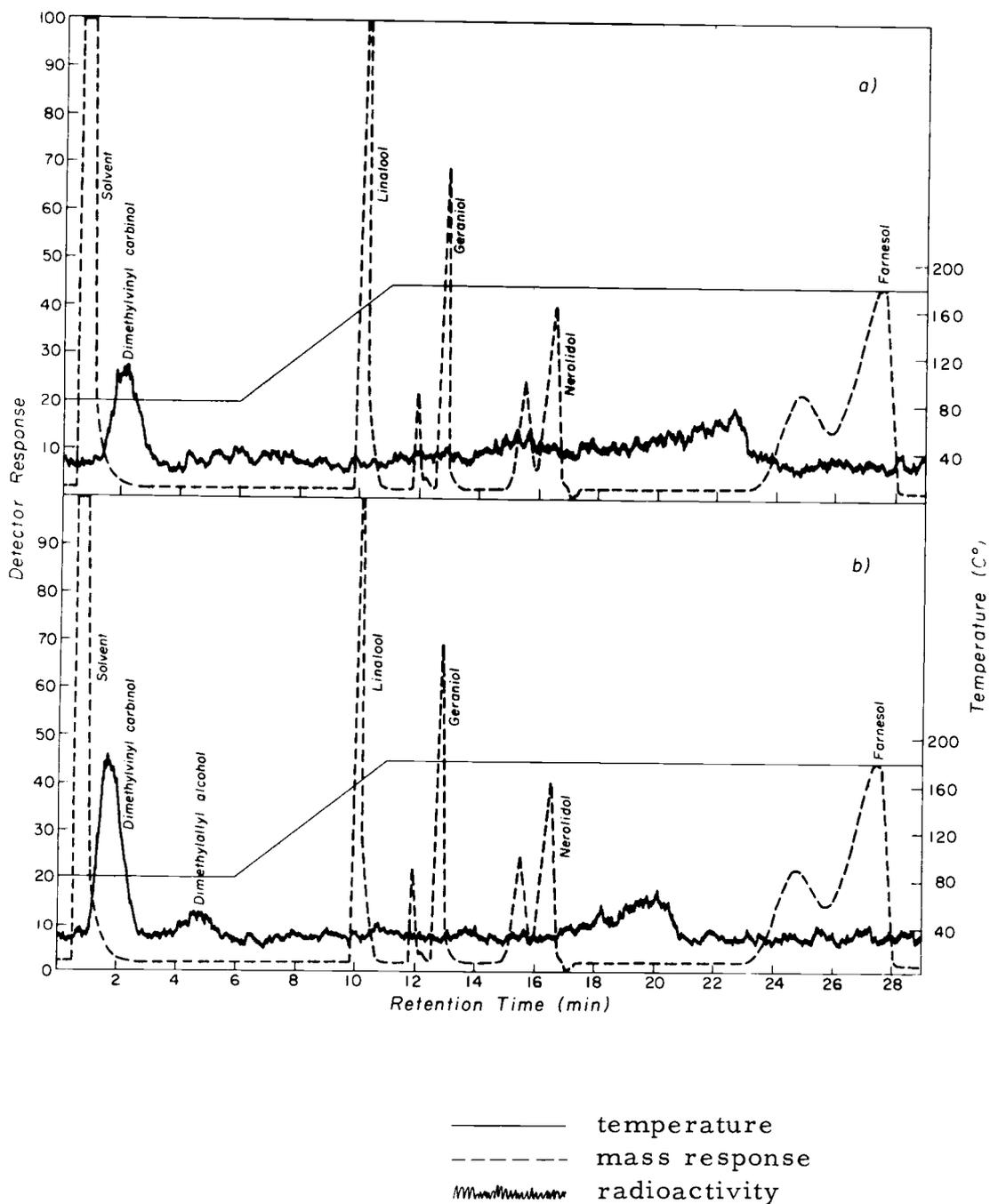


Figure 40. Distribution of ^{14}C label from MVA-2- ^{14}C among the acid-labile prenyl pyrophosphates in incubations of the 40,000 g supernatant at (a) 16 hrs and (b) 39 hrs germination: gas chromatography of the ^{14}C -prenol factions.

Preliminary Identification of the
Suppressed Enzyme in Phase III

It was reasoned that suppression of MVA-2-¹⁴C flow into squalene should result in a concurrent accumulation of label at the site of inhibition along the isoprenoid pathway. Pulse labeling studies, carried out as previously described (page 144), showed accumulation of label in DMAPP and squalene, but not in GPP and FPP. Gas radiochromatographic profiles of the 16 and 39 hour prenol fractions are shown in Figures 40a and 40b, respectively. It may be seen from Table 16, that the 16 hour supernatant, as opposed to the 39 hour supernatant, is 3-4 times more active in converting MVA-2-¹⁴C into squalene. Concurrently, the 39 hour supernatant exhibits a proportionally greater accumulation of DMAPP-¹⁴C, which is interpreted as selective inhibition of the pathway at the prenyltransferase step. The thermolabile factor associated with phase III was therefore tentatively identified as an inhibitor of IPP/DMAPP prenyltransferase.

In Vitro Direct Assays of the Isoprenoid Pathway

By direct assays of related enzymes of a common metabolic pathway, it is tacitly assumed that such activity measurements for each enzyme are comparable by some unknown factor to in vivo conditions. The latter factor presumably accounts for enzyme activity lost during the isolation steps necessary to prepare an in vitro system.

For example, soluble enzymes are frequently assumed to be extracted into an appropriate buffer to the same degree as an active entity. Such enzymes are often compared to one another in terms of their metabolic capacity measured in vitro. It must be kept in mind, however, that in vitro differences may be unrelated to in vivo conditions, where the so called unknown factor for two enzymes is not of comparable magnitude. Even soluble enzymes are not equally stable under the same isolation techniques; conformational changes may render one enzyme less active; an inhibitor, normally compartmentalized, may preferentially bind to one enzyme, etc. For obvious differences in enzymic properties, such comparisons are particularly hazardous. Hence, soluble enzymes are rarely compared with particulate, or membrane bound enzymes, nor are enzymes of widely divergent pH optima compared to one another under the above conditions. These problems in interpreting in vitro data are generally well recognized, although sometimes ignored for convenience.

Of equal importance is the effect of precursor metabolites on the activity of individual enzymes of the same pathway. The flow of carbon through a pathway is not simply the summation of individual enzymic activities, as measured by direct enzymatic assays under zero order kinetics, since such enzymes, in all probability, do not function in an independent fashion. Aside from compartmentation problems, in which the enzyme and substrate may be spatially

segregated, variations in precursor pool sizes during the course of development may prevent such enzymes from acting independently. Hence, metabolites of the pathway may act as positive or negative effectors at one or several sites along the pathway. Either directly, or indirectly, the activity of any enzyme in a common pathway may markedly change the metabolic balance of every other enzyme in the pathway, by causing a rapid redistribution of metabolites. Where applicable, certain manipulations may be made to minimize differences in correlating enzyme activities. In the following paragraphs, correlations among the isoprenoid enzymes are drawn, based on in vitro assays, subject to all of the limitations discussed above. Certain assumptions were made, which will be appropriately noted. Developmental demands on the flow of carbon among isoprenoid intermediates may be more conveniently discussed and correlated with the individual enzyme patterns, if reference is made to the flow diagram of Figure 4.

Assays of MVA kinase, MVAP kinase, MVAPP decarboxylase, IPP isomerase, IPP/DMAPP prenyltransferase and squalene synthetase, each as a function of germination, have been previously described. For purposes of this discussion, the following assumptions are made: (i) that the in vitro assays represent the specific activity of each enzyme in vivo and (ii) between 8 and 12 hours germination, the enzyme activities are in a steady state, neither increasing nor

decreasing in proportion to one another. With regard to the second assumption, it may be seen that the specific activity of each enzyme is relatively constant over this same period. It should be noted that such conditions are temporal, subject to change during subsequent stages of germination. The flow of carbon through the isoprenoid pathway is first considered under the above steady-state conditions. Thereafter, the enzymic changes associated with germination will be examined for their influence on this flow. Such changes appear to mark the onset of new requirements for specific metabolites of the pathway, possibly associated with the biogenesis of physio- and morphologically divergent cellular compartments.

Table 17 summarizes the average specific activity of each enzyme obtained during the steady-state conditions between 8 and 12 hours germination. Relative enzyme activities were also calculated with respect to the least active enzyme, IPP/DMAPP prenyltransferase. It will be recalled that IPP is an important intermediate of the pathway, serving as a precursor of all subsequent condensations leading to higher isoprenoid products (Figure 4). It is therefore quite significant that the enzymic activities under the above conditions are distributed in such a way as to favor the formation of IPP. Furthermore, the balance between IPP and the thermodynamically more favorable DMAPP isomer appears to be mediated through IPP/DMAPP prenyltransferase activity (i. e., the accumulation of IPP and

Table 17. In Vitro Specific Activities of the Common Isoprenoid Enzymes at Steady State Conditions.¹

Enzyme	Specific activity ($\mu\text{moles min}^{-1} \text{mg}^{-1}$)	Relative ratio ²
Mevalonic kinase	0.45 ³	1500
MVAP kinase	0.15	500
MVAPP decarboxylase	0.03	100
IPP isomerase	0.04	133
IPP/DMAPP prenyl-transferase	0.0003	1
Squalene synthetase	0.008	27

¹ Steady state conditions were defined as those enzyme activities measured between 8 and 12 hours germination. The average values are reported.

² Calculated with respect to the specific activity of IPP/DMAPP prenyl-transferase.

³ Estimated from the initial substrate concentration and first order rate constant.

DMAPP, and hence the establishment of equilibrium levels of IPP and DMAPP, is directly related to prenyltransferase activity). In addition to the information given in Table 17, the following characteristics of the isoprenoid pathway are useful in arriving at the above interpretation: (i) the phosphorylation of MVA to MVAP with ATP is, in all probability, a reversible equilibrium in favor of MVAP formation (the K_{eq} is, as of yet, unknown); (ii) the K_{eq} of $MVAP + ATP \rightarrow MVAPP + ADP$ is approximately unity (0.7 - 1.1) (80); (iii) the conversion of MVAPP into IPP with ATP is an irreversible decarboxylation; (iv) the K_{eq} between IPP and DMAPP favors DMAPP by approximately 87:13 (153); and (v) IPP isomerase activity \gg IPP/DMAPP prenyltransferase activity (Table 17).

Since MVA kinase activity exceeds that of MVAP kinase, MVAP may be anticipated to accumulate in excess. This creates a nonequilibrium state between MVAP and MVAPP. In an attempt to reestablish equilibrium conditions, the flow of carbon is driven in the forward direction. If the availability of MVA is limited, equilibrium is not attained, since MVAPP is irreversibly decarboxylated to yield IPP, thereby enhancing the nonequilibrium state between MVAP and MVAPP. If an excess of MVA is available, however, MVAPP will eventually accumulate to a level in equilibrium with MVAP (i. e., MVAP kinase activity $>$ MVAPP decarboxylase). Hence, the carbon flow between MVA, MVAP, and MVAPP may flow in either a forward

direction, where the availability of MVA is limited, or flow in a reverse direction, where a large excess of MVA is available. Since MVAPP decarboxylase activity exceeds that of IPP/DMAPP prenyltransferase by several fold (Table 17), most of the carbon (under limiting conditions) will accumulate between the latter two enzymatic steps. There are only two isoprenoid intermediates, IPP and DMAPP, between these enzymes. Moreover, because of the very high IPP isomerase activity, a very rapid equilibrium between the latter two intermediates should be established. Since IPP/DMAPP prenyltransferase activity is much lower than that of the isomerase, the flow of carbon between IPP and DMAPP may readily reverse, in order to maintain equilibrium conditions, should a disproportionate amount of IPP be metabolized. It is apparent, however, that all isoprenoid products beyond GPP must consume a disproportionate level of IPP, as opposed to DMAPP, based on stoichiometric requirements alone (Table 18). Since such demands create a nonequilibrium state between IPP and DMAPP, carbon flow should pass from DMAPP to IPP in proportion to the demands made on IPP. In essence, catalytic capacities of the first three enzymes, MVA kinase, MVAP kinase and MVAPP decarboxylase, are in such ratios as to create a nonequilibrium condition, thereby driving carbon into IPP. The latter enzymes, IPP isomerase and IPP/DMAPP prenyltransferase, on the other hand, are arranged in such catalytic proportions as to maintain

an equilibrium between IPP and DMAPP, which allows for the most efficient synthesis of higher isoprenoids. Maintenance of a steady supply of IPP is achieved by modulating the proportional ratio of MVAPP decarboxylase, IPP isomerase and IPP/DMAPP prenyltransferase activities.

Table 18. Stoichiometric Requirements for IPP and DMAPP in the Biosynthesis of Common Isoprenoid Intermediates.

Intermediate	Units of	
	IPP	DMAPP
GPP	1	1
FPP	2	1
GGPP	3	1
Kaurene	3	1
Squalene	4	2
Triterpene	4	2
Sterol	4	2
Phytoene	6	2
Carotene	6	2
Plastoquinone (side chain)	8	1
Ubiquinone (side chain)	9	1

Changes among the aforementioned enzymes as a function of germination were compared on a relative basis to one another by normalization. The specific activity of each enzyme at any stage of germination was expressed as a percent of its initial specific activity,

as measured at two hours after imbibition (Figure 41). It is tacitly assumed, that the unknown factor relating an in vitro assay to in vivo activity for a given enzyme does not change with germination (i. e. , the efficiency by which a specific enzyme is extracted and maintained as an active entity is not variable with germination). Since specific activities of the enzymes are normalized with respect to an initial stage of germination, proportional changes in the enzyme balance, or catalytic ratios, may be compared at any stage thereafter. Two hours after the onset of imbibition, this ratio was arbitrarily set at unity.

It is apparent from examining Figure 41, that selective controls are applied during germination which bring about differential changes in the enzyme ratios. It is not clear, however, from specific activity measurements based on total protein, to what extent the isoprenoid enzymes are modulated, as a whole, in relation to biochemical changes normally taking place in the developing cell. In essence, protein measurements may provide an approximate estimate of the cell number at any state of development, but are less meaningful where large protein reserves are known to exist. Since germinating seeds generally contain large pools of storage protein, such measurements provide a convenient reference for comparing common enzyme activities, but may not clearly reflect the onset of enzyme activation. Two marker enzymes, alkaline phosphatase and FDP aldolase (class II) (146), were therefore assayed as a function of germination, to serve as

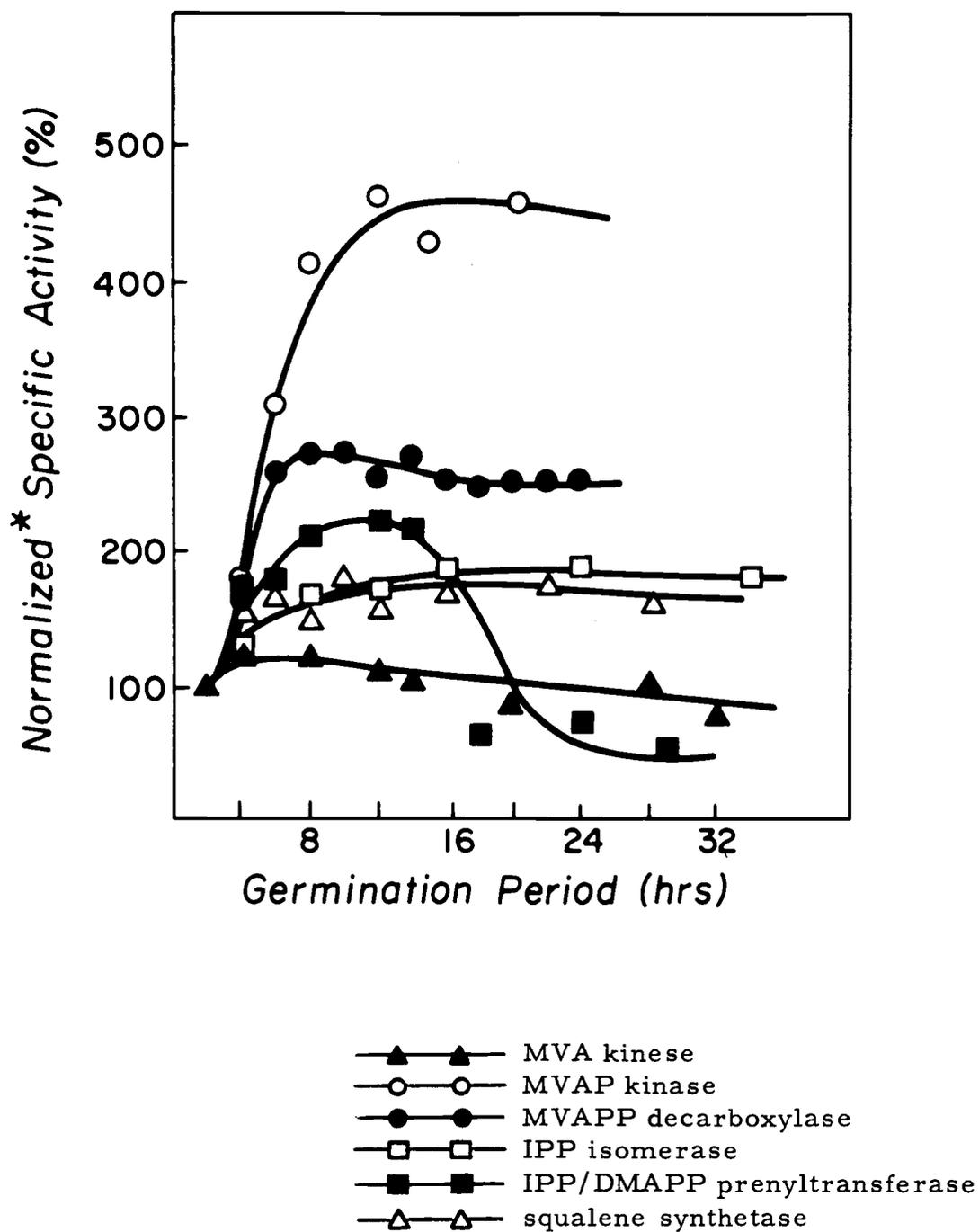


Figure 41. Normalized enzymic patterns of the common isoprenoid enzymes as a function of germination. *Based on the appropriate specific activity of each enzyme at 2 hrs germination.

references against which the enzymic changes of the isoprenoid enzymes could be compared. The former two enzymes were assumed to reflect relative levels of cellular metabolism as a function of germination. The rate of water imbibition was also measured to determine to what extent enzymic changes might relate to water uptake. Figure 42 summarizes the specific activities observed for alkaline phosphatase, FDP aldolase and the rate of water imbibition over the first several hours of germination. The general rate of cellular metabolism, as defined by the marker enzymes, rises approximately two-fold over the first eight hours of germination and falls off gradually thereafter. This initial rise is associated with the uptake of water, although the precise relationship between the two is not clearly defined. For the purposes of this study, only those enzymic changes which deviate from this pattern are of interest. By comparing Figures 41 and 42, it is evident that three of the isoprenoid enzymes exhibit distinctly divergent patterns: MVAP kinase, MVAPP decarboxylase and IPP/DMAPP prenyltransferase. The enzymic patterns of these latter three enzymes, then, must be considered in terms of their effect on the carbon flow, as previously discussed, and how such changes may suggest a shift in the developmental requirements for specific metabolites of the isoprenoid pathway.

Conversion of MVA into IPP as previously described, was shown to be most favorable when the relative ratio of enzyme activities

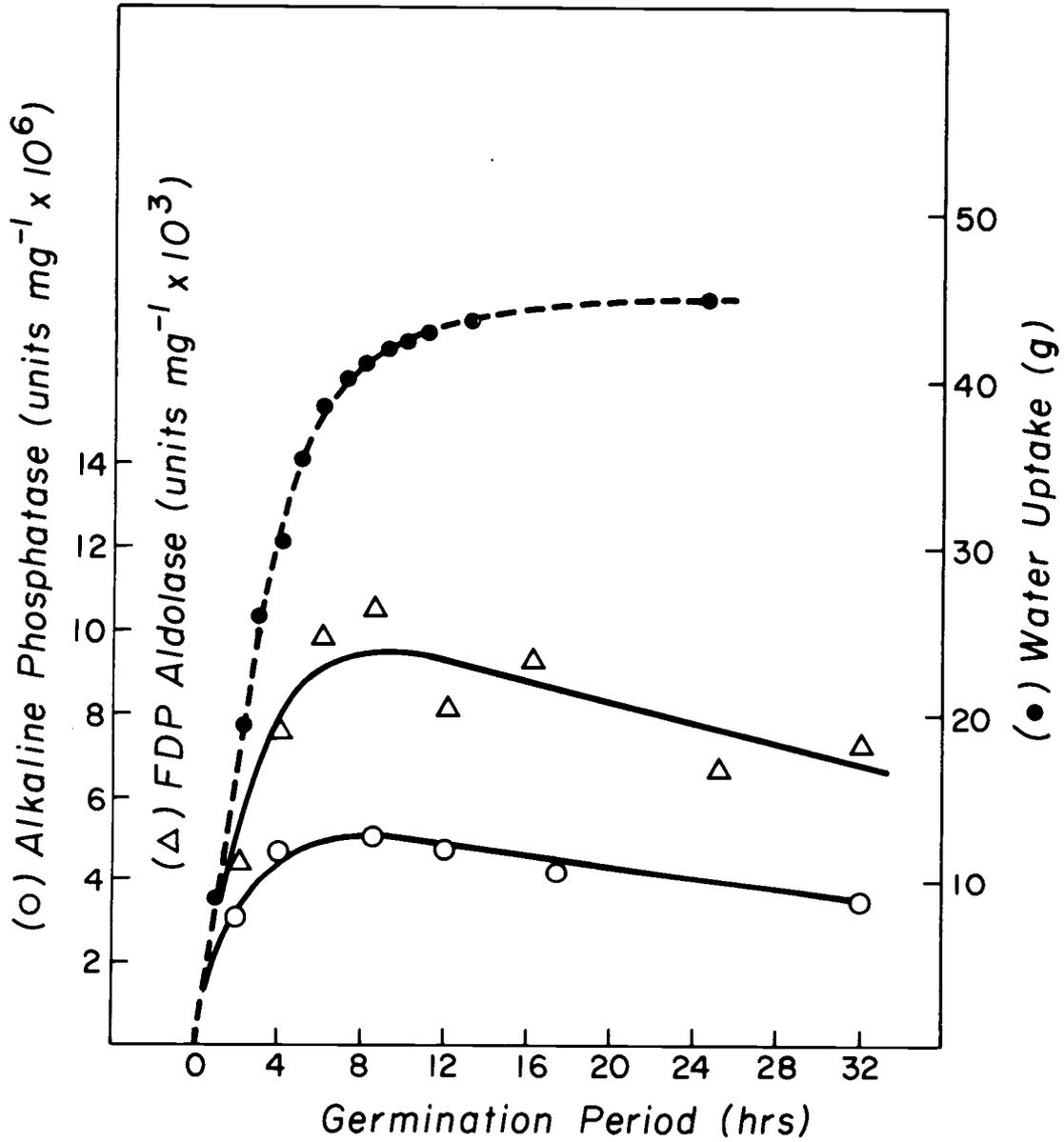


Figure 42. Specific activities of FDP aldolase and alkaline phosphatase and the uptake of water as a function of germination.

is as follows: MVA kinase > MVAP kinase > MVAPP decarboxylase. The rate of IPP formation, on the other hand, is dependent upon MVAPP decarboxylase activity. Thus the initial rise in MVAPP decarboxylase specific activity over the first eight hours of germination allows for an increased rate of IPP formation. Similarly, the corresponding rise in MVAP kinase specific activity over this same period allows for a more rapid turnover of MVAP into MVAPP, thereby insuring a rapid flow of carbon through the first three steps of the pathway. It should be remembered that MVA kinase is at all times markedly greater than MVAP kinase activity. Thus, in the first several hours of germination, the relative capacity of seeds to make IPP rises several fold. During a similar period, the in vivo metabolic capacity of the seeds to make NSF products was observed to rise through a maximum (at \sim 16 hours germination) and declined, thereafter. This latter phenomenon appears to be mediated through a specific prenyltransferase (phase I), which may be seen to rise through a maximum between 8 and 12 hours, after which its specific activity falls off. Hence, the carbon flow would appear to initially pass through IPP and DMAPP and into the triterpene pathway leading to squalene, penta- and tetracyclic products. With the appearance of an inhibitor of prenyltransferase I, the carbon flow through the above pathway is suppressed. A second prenyltransferase (phase II), however, is believed to exist in the seed, which may divert the carbon

flow into an alternate pathway leading to GGPP and higher isoprenoid products of this latter pathway (Figure 4). Before discussing other lines of evidence which favor the existence of two such prenyltransferases, the prenyltransferase pattern shown in Figure 41 must be discussed in more detail. This pattern exhibits certain features which are inconsistent with previous observations. An explanation for these differences is also offered. However, no direct proof of two prenyltransferases has yet been established. The flow of carbon beyond IPP is, therefore, described in what appears to be a consistent model with the experimental results of this study, as well as those by a number of other authors.

It may be recalled that previous studies, in which MVA-2-¹⁴C served as substrate, indicated that phases I and II of the (+) sucrose pattern were dependent upon prenyltransferase activity. Subsequent experiments suggested that two prenyltransferases of different origin may exist in germinating seeds, and that intermixing of what may be designated as prenyltransferase I and II results in the biphasic (+) sucrose pattern. It is likely that intermixing occurs during homogenization and that the prenyltransferases are normally segregated from one another in the intact seed. If the results of the (+) sucrose pattern are normalized in the same manner as described in the previous paragraphs and then compared to prenyltransferase measurements shown in Figure 43, it is immediately apparent that the two assays

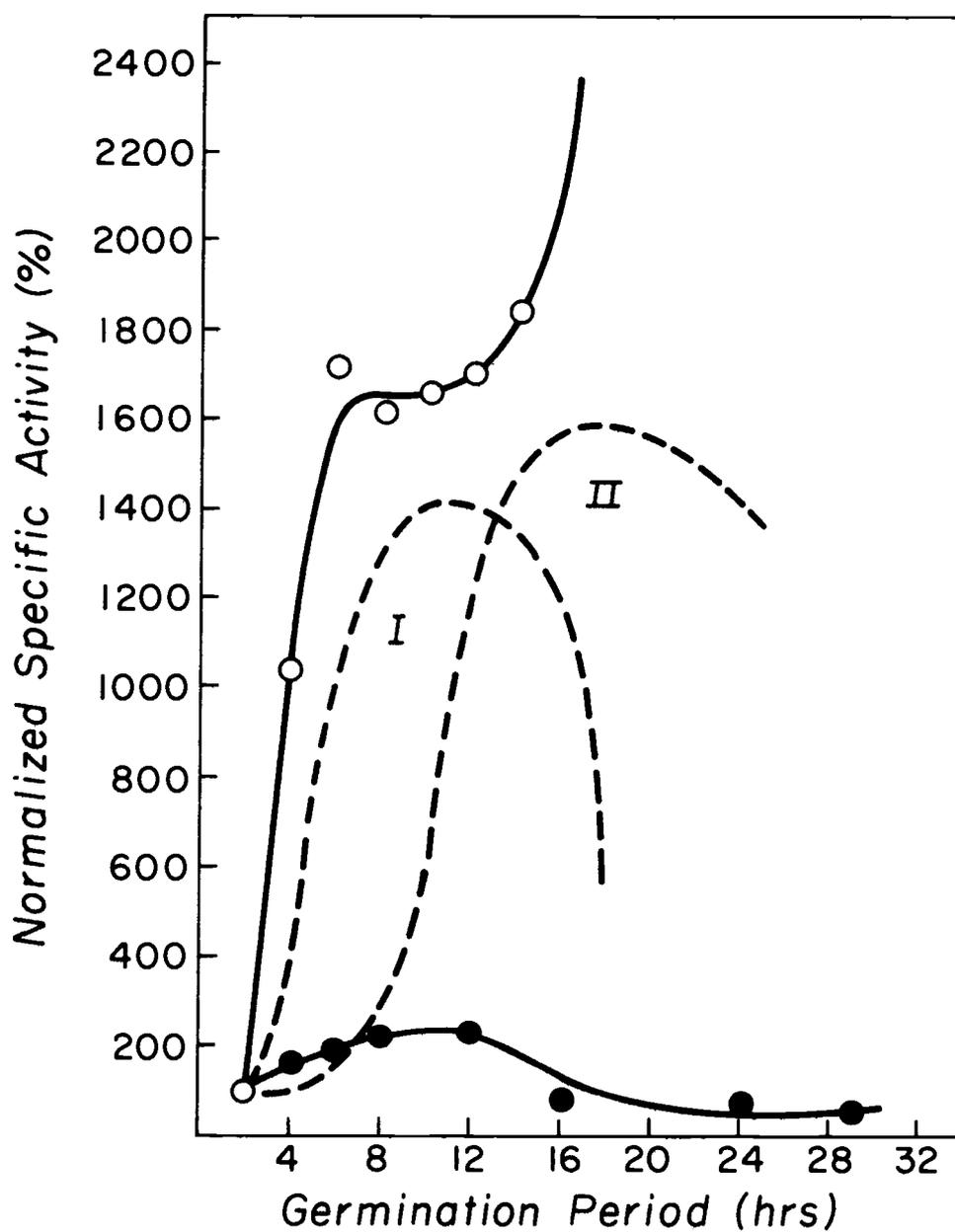


Figure 43. Comparison of direct (●) and indirect (○) assays of IPP/DMAPP prenyltransferase as a function of germination. Dashed lines indicate postulated enzymic patterns of prenyltransferase I and II.

yield considerably different estimates of prenyltransferase activity. These differences are several times greater than what might be attributed to experimental error (i. e., 15-20 fold differences between the two assays are considerably greater than the estimated maximal error in these assays of $\pm 10\%$). The indirect assay of prenyltransferase activity from MVA differs from the direct assay with IPP and DMAPP, in that the former assay is conducted in the presence of MVA, MVAP and MVAPP in addition to the immediate substrates of the prenyltransferase, IPP and DMAPP. It is therefore suggested that one or possibly more of these latter precursors of IPP may activate IPP/DMAPP prenyltransferase (i. e., to serve as positive effectors of prenyltransferase). Quite analogous observations have been made on similar pathways, such as the stimulatory effect of citrate on fatty acid synthesis (108). Monod, Wyman and Changeux have recently reviewed several metabolic pathways and listed a number of recently discovered metabolites, which act as positive effectors, or activators, of specific regulatory enzymes (118). It appears, from the results cited above, that the direct assay of prenyltransferase was not representative of its true metabolic capacity as a function of germination. As a tentative estimate of total prenyltransferase activity, the (+) sucrose pattern will be offered in place of the previous assay. It is noteworthy that the (+) sucrose pattern exhibits a specific activity still less than that of any other enzyme listed in

Table 17 (i. e., between 8 and 12 hours, the turnover capacity is $\sim 0.005 \mu\text{moles min}^{-1} \text{mg}^{-1}$). Included in Figure 43 are the postulated developmental patterns of IPP/DMAAPP prenyltransferase I and II, respectively (dashed curves).

Biochemical and Physiological Changes
Accompanying the Breaking of Dormancy⁷

The breaking of dormancy in seeds and subsequent developmental changes leading to cell division have received considerable attention over the last several years. Three phases of physiological development are known to accompany the transition between dormancy and growth: (i) activation of the dormant seed; (ii) germination; and (iii) outgrowth. Activation of the dormant seed is generally recognized by an initially rapid uptake of water accompanied by a very rapid rise in metabolic activity (respiration, protein synthesis, glycolysis, etc.). Preexisting cytoplasmic- and organelle-associated metabolic functions are reactivated. The second phase (germination) results in a slower uptake of water and respiration over a sustained period of several hours. During this phase the seed prepares all necessary substrates for the biogenesis of new cellular components (i. e.,

⁷In this context, dormancy is defined as a state of metabolic inactivity which precedes a state of metabolic activation and subsequent steps leading to cellular growth and differentiation.

mitochondrial membranes, nuclei, plastids, etc.) (19). The final state of development is usually identified by a steady increase in fresh weight and protrusion of the embryo root through the seed coat.

Several excellent papers and reviews on biochemical changes induced by the breaking of dormancy in seeds may be found elsewhere (19, 112, 142).

Electron micrographs of cotyledons from P. sativum reveal all of the normal subcellular components present in highly differentiated cells (i.e, nucleoli, mitochondria, protoplasts, Golgi bodies, protein bodies, polyribosomal clusters, starch granules, etc.) (169). It is conceivable that P. sativum may develop a highly organized set of isoprenoid enzymes, segregated between the cytoplasm and protoplasts (immature chloroplasts), or similarly related organelles. The regulatory model proposed by Goodwin and Mercer (66) regarding isoprenoid enzyme segregation in etiolated maize seedlings between the cytoplasm and chloroplasts may be considered with respect to developmental requirements induced during the transition phases of seed development. Figure 44 illustrates the potential enzyme pathways which may arise nonsynchronously between the cytoplasm and chloroplast. Isoprenoid requirements associated with the cytoplasm need not arise simultaneously with the needs of the chloroplasts, since each system may function independently of the other. It is, furthermore, rather unlikely that the biosynthetic requirements for each

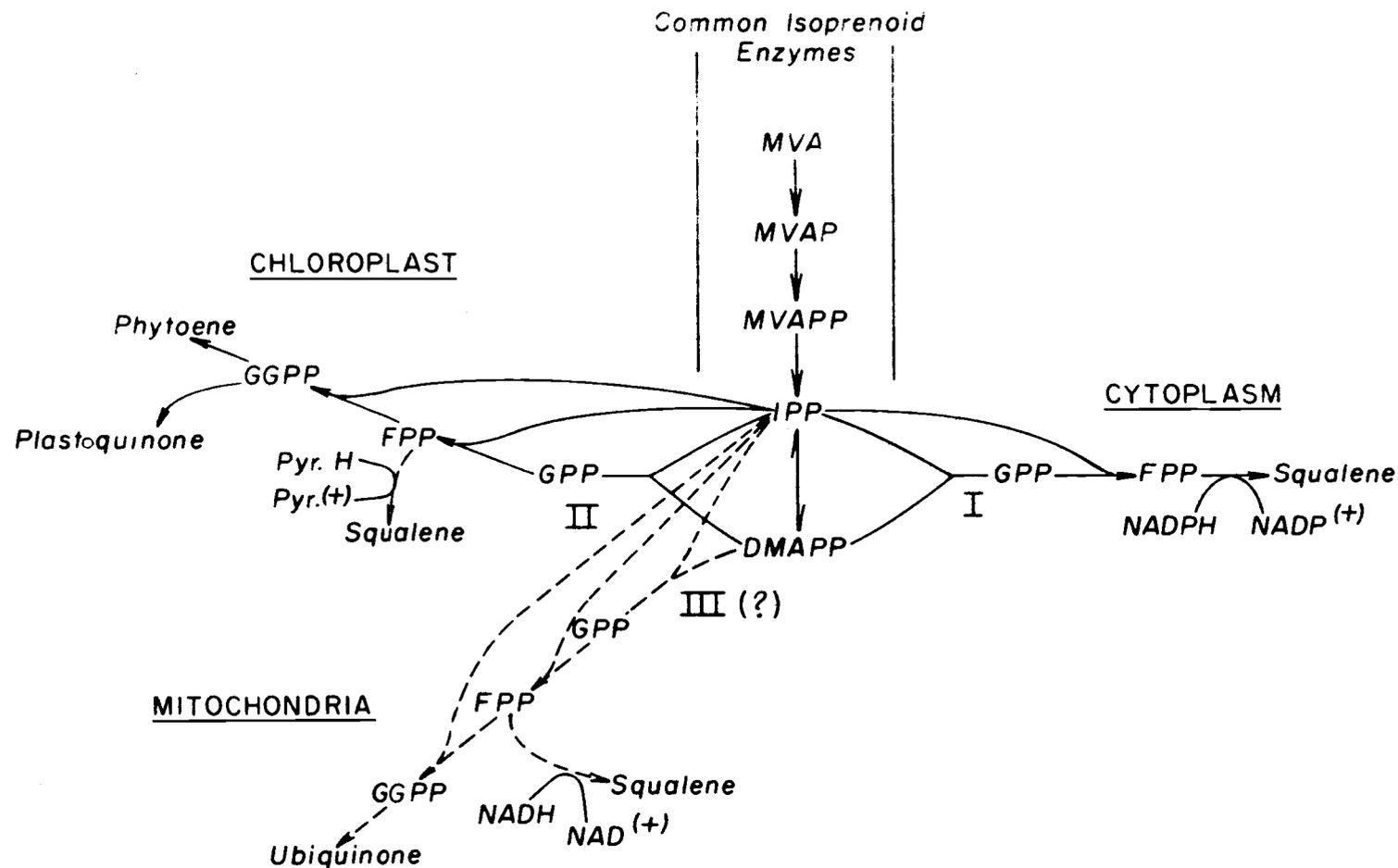


Figure 14. Postulated model of enzyme segregation and compartmentation. Solid lines represent essentially the model proposed by Goodwin and Mercer (66). An equally plausible pathway (dashed lines) may be associated with the mitochondria.

compartment would coincide during development. An efficient method of regulating the flow of carbon through one pathway or the other would be most desirable. By shifting the carbon flow between either pathway according to the needs of the developing seed, an unnecessary waste of energy and substrate may be eliminated.

It is interesting to note that, experimentally, the capacity to make squalene, triterpene and sterol products rises coincident with water uptake during phase I, which may be considered the activation phase. Hence, this metabolic capacity may be associated with pre-existing activity in the maturing seed prior to dormancy. Prenyltransferase I mediates the flow of MVA into NSF products. With the onset of germination, the secondary phase of development, an apparently new prenyltransferase (II) appears in the seed. This second transferase is dependent upon sucrose for successful extraction into the homogenate. A question must therefore arise concerning the origin of this new transferase. Might it arise from a protoplast, or similar organelle, which must prepare, perhaps, an important pigment (plastoquinone, carotenoid, ubiquinone, etc.) for subsequent chloroplast (or mitochondrial) biogenesis? Does the concurrent suppression of prenyltransferase I by a thermolabile factor represent a developmental control of the seed to channel carbon into a new isoprenoid pathway associated with the chloroplast?

SUMMARY

An in vivo analysis of NSF product formation from MVA-2-¹⁴C versus germination in P. sativum confirmed that β -amyrin (triterpene) and β -sitosterol (sterol) are sequentially synthesized at different rates, depending upon the germination stage. Kinetic transitions between squalene, squalene-triterpene, and squalene-triterpene/squalene-sterol formation are discussed in relation to the metabolic steps required for either triterpene or sterol biosynthesis. Metabolically, either product must arise from 2,3-oxidosqualene. Changes in triterpene and sterol formation as a function of germination, however, suggest that the enzymes responsible for such synthesis do not compete for oxidosqualene, but for squalene. A regulatory model based on these observations is proposed. Squalene epoxidase is postulated to combine with specific cyclases as an enzyme aggregate. As an aggregate, the enzyme complex competes for squalene to make the appropriate cyclized product. Regulation and selection of product formation is controlled through association and dissociation of the aggregate.

Methods for the biosynthesis and isolation of MVAP-2-¹⁴C, IPP-4-¹⁴C and FPP-4, 8, 12-¹⁴C from MVA-2-¹⁴C in yeast autolysates, prepared from commercial dry baker's yeast, are described in detail. Comparisons between various biosynthetic methods have been made. Methods of identification, isolation techniques and criteria of

purity are also discussed; those methods most suitable for the isolation and identification of each isoprenoid intermediate have been emphasized.

Individual assays of MVA kinase, MVAP kinase, MVAPP decarboxylase, IPP isomerase, IPP/DMAPP prenyltransferase and squalene synthetase in 40,000 g supernatants of P. sativum, prepared from germinating seeds at selected intervals after the onset of imbibition, suggest that the enzymes are regulated in such a manner as to always favor IPP and DMAPP formation. The enzymes are activated in such proportions as to favor nonequilibrium conditions between MVA and IPP, and equilibrium conditions between IPP and DMAPP, thereby insuring the most efficient synthesis of higher isoprenoid products at all times. A reversal of carbon flow from DMAPP to IPP appears likely as a means of maintaining a sufficient supply of IPP to meet new biosynthetic requirements induced by germination. DMAPP may serve as a storage pool, to meet biosynthetic demands during periods when the endogenous supply of MVA is limited.

IPP/DMAPP prenyltransferase was assayed indirectly in 40,000 g supernatants with MVA-2-¹⁴C and more directly with IPP-4-¹⁴C. Under identical conditions in the preparation of 40,000 g supernatants, the former assay yielded an estimated activity some 15-20 fold greater than by IPP-4-¹⁴C. These results suggest that IPP/DMAPP prenyltransferase is allosterically activated by one, or

more, of the first three isoprenoid intermediates (i. e., MVA, MVAP, and/or MVAPP).

Indirect evidence was also obtained for the presence of two distinct IPP/DMAPP prenyltransferases, tentatively identified as enzymes I and II. Detection of prenyltransferase I is independent of sucrose, while detection of prenyltransferase II is highly dependent upon the inclusion of sucrose (0.45 M) in the homogenizing buffer. The two prenyltransferases are activated sequentially during the course of germination. Prenyltransferase I is postulated to be associated with the cytoplasm, while prenyltransferase II may be associated with an organelle, possibly with immature chloroplasts (protoplasts) or mitochondria. Enzymic changes in the activities of prenyltransferases I and II appear closely correlated with developmental changes in the seed, initiated with the breaking of dormancy.

A thermolabile, large molecular weight factor has been tentatively identified as a specific inhibitor of IPP/DMAPP prenyltransferase. This factor is temporal, appearing at later stages of germination. The action of this factor as a means of diverting the flow of carbon between cytoplasmic and organelle associated pathways is discussed. Suppression of prenyltransferase I activity by this factor, coincident with the rise in prenyltransferase II activity, may suggest that compartmentalized isoprenoid pathways are not completely autonomous, but are suppressed or activated to meet the needs of their

respective environments, thereby conserving considerable energy and substrate.

It is now evident that the isoprenoid pathway is regulated in a complex fashion during development. Efforts must be made to isolate homogeneous organelle and cellular fractions, by which the more complex enzyme patterns may be more clearly defined. Hopefully, such efforts may lead to a better insight into the regulatory controls exerted upon the isoprenoid pathway during cellular growth and differentiation.

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