PARTIAL CHARACTERIZATION OF A CELL - FREE TERPENOID BIOSYNTHETIC SYSTEM FROM SHOOTS OF TULIPA GESNERIANA L. cv. GOLDEN MELODY

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ABSTRACT

PARTIAL CHARACTERIZATION OF A CELL-FREE TERPENOID BIOSYNTHETIC SYSTEM FROM SHOOTS OF TULIPA GESNERIANA L. cv. GOLDEN MELODY

By

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Cell-free extracts which incorporated mevalonate-2-^{14}C into terpenes were obtained from shoot tissue of Tulipa gesneriana L. cv. Golden Melody. Maximal incorporation occurred at pH 6.5 in 0.1 M K-phosphate buffer with an incubation temperature of 35 C. Total incorporation was linear up to 5 mg protein/assay and it could be enhanced by increasing MVA concentration up to 8 x 10^{-8} M. After differential centrifugation, all activity was located in the supernatant.

There was an absolute requirement for a high energy source as well as a divalent cation. In combination with the divalent cations Mg^{++} and Mn^{++}, ATP stimulated the incorporation more than CTP, GTP and UTP. Using ATP as the energy source, Mn^{++} was more stimulatory at low concentrations (1 x 10^{-3} M) than at high concentrations. Mg^{++} was less effective than Mn^{++} and a Mg^{++} + Mn^{++} combination was intermediate.

The products biosynthesized from MVA in the cell-free system were isolated in two fractions. The "neutral"

fraction was isolated and extracted with benzene after incubation and centrifugation. The "acid hydrolyzable" fraction was extracted with benzene after acid hydrolysis of the aqueous "neutral" fraction.

For identification of the neutral terpene products, the optimal conditions are either high protein concentration (18 mg/assay) and a 4 hr incubation period or high protein concentration without centrifugation and a 1 hr incubation period. For identifcation of the acid hydrolyzable products, the optimal conditions are either low protein concentration (5 mg/assay) and a 1 hr incubation period or an enzyme preparation ratio of 1:12 (wt:vol) with a 1 hr incubation period.

The chemicals AMO-1618, CCC, Iodoacetamide and Phosfon D showed little or no inhibitory effect on the incorporation into either fractions.

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AMO-1618	4-Hydroxy-5-isopropyl-2-methylphenyltrimethyl-ammoniumchloride 1-piperidine carboxylate
ATP	Adenosine-5-triphosphate
CCC	(2-Chloroethyl)-trimethylammonium chloride
CTP	Cytidine-5-triphosphate
DBED	Dibenzoylethylenediamine
DMA-PP	3,3-Dimethylallyl-pyrophosphate
F-PP	Farnesyl-pyrophosphate
G-PP	Geranyl-pyrophosphate
GG-PP	Geranylgeranyl-pyrophosphate
GTP	Guanosine-5-triphosphate
I-PP	Isopentenyl-pyrophosphate
MVA	Mevalonic acid
MVA-P	Mevalonate-5-phosphate
MVA-PP	Mevalonate-5-pyrophosphate
Phosfon D	2,4-Dichlorobenzyl-tributylphosphonium cloride
UTP	Uridine-5-triphosphate

PART I

LITERATURE REVIEW

Introduction

In plants the isoprenoid pathway leads to the formation of gibberellins, abscisic acid, sterols, phytol, caretenoids and other naturally occurring compounds (Goodwin,1967). All evidence indicates that they originate from a C-5 isoprenoid intermediate, which is derived from MVA (Goodwin, 1967; Green and Baisted, 1972; Heftmann, 1970). Because of the importance of the terpenoid products in virtually all metabolic processes in plants, cell-free systems have been isolated to study the biosynthesis of these products.

The first cell-free system from higher plants was from tomato plastids (Shneour and Zabin, 1959). MVA was biosynthesized into lycopene and many other unidentified products. Porter and co-workers (review Porter, 1969), using Lycopersicon esculentum, purified a cell-free system and reported the conversion of MVA into carotenes and related compounds. They also identified the intermediate products biosynthesized from MVA into carotenes.

Gibberellins, as a plant hormone, play an important role in growth and development of higher plants (Lang, 1957, 1965, 1970). Starting in 1965, West, Graebe and

co-workers investigated the biosynthesis of gibberellins and their precursors in cell-free extracts isolated from Echinocystis macrocarpa (Graebe, et al., 1965; Dennis, et al., 1965; Dennis and West, 1967; Upper and West, 1967; Oster and West, 1968; West, et al., 1968; Murphy and West, 1969; West and Upper, 1969) and from Ricinus communis (Robinson and West, 1969a, 1969b). These studies provided much information concerning the characterization of the various end products as well as the properties of cell-free systems, per se. Kaurene, a gibberellin precursor, biosynthesized from MVA in cell free extracts from Pisum sativum, was reported by Moore and co-workers (Anderson and Moore, 1967; Coolbaugh and Moore, 1969, 1971a, 1971b; Moore, et al., 1972). Graebe, MacMillan and co-workers identified the intermediate products in gibberellin biosynthesis in cell-free extracts isolated from Cucurbita pepo (Graebe, 1970; Graebe, et al., 1972; Bowen, et al., 1972).

In the bulbous iris, gibberellin-like substances were reported (Aung, et al., 1969a; Rodrigues Pereira, 1965). Staby, et al. (1970, 1972b, 1973) isolated a cell-free system from iris shoots in an effort to investigate the biosynthesis of the gibberellin-like substances. They reported the presence of non radioactive sterols (Staby, et al., 1972b) and ^{14}C-farnesol, geraniol and squalene synthesized from MVA-2-^{14}C (Staby, et al., 1973),

but found no evidence of kaurene biosynthesis. In tulips, gibberellin-like substances have been reported and tentatively identified (Aung and De Hertogh, 1967, 1968; Aung, et al., 1969a, 1969b, 1970; De Hertogh, et al., 1970; Einert, et al., 1972).

Some Characteristics of Cell-Free Terpenoid Systems from Higher Plants

Substrates

The most common substrate used for the biosynthesis of terpenes in cell-free systems from higher plants is MVA. Most studies assumed that any products arising from MVA were terpenes (Green and Baisted, 1972; Pollard, et al., 1966).

MVA has beed used in the form of free acid (George-Nascimento and Cori, 1971a; Pollard, et al., 1966; Potty and Bruemmer, 1970b; Rogers, et al., 1966), lactone (Murphy and West, 1969), as a sodium salt from the lactone (Coolbaugh and Moore, 1969, 1971a, 1971b; Moore, et al., 1972), and as a potassium salt from the lactone (Garcia-Perigrin, et al., 1972; Graebe, 1968, 1970; Graebe, et al., 1972). MVA has also been used as a plain sodium salt (Baisted, 1971; Green and Baisted, 1970, 1971, 1972), as a plain potassium salt (Loomis and Battaille, 1963) and as a DBED salt (Dennis, et al., 1965; Jungalwala and Porter, 1967; Oster and West, 1968; Robinson and West, 1969a, 1969b; Staby and De Hertogh, 1970; Staby, et al., 1972a, 1972b, 1973).

The most common labeled form was MVA-2-^{14}C. MVA-1-^{14}C was only used for the enzyme 5-pyrophosphomevalonate decarboxylase to measure $^{14}CO_2$ evolution (Green and Baisted, 1972; Potty and Bruemmer, 1970c).

Salts of the lactone have been nearly exclusively used for studying the kaurene biosynthesis (Coolbaugh and Moore, 1969, 1971a, 1971b; Moore, et al., 1972; Graebe, 1968, 1970; Graebe, et al., 1972).

Buffers and pH

For the extraction and incubation media Tris and phosphate buffers have been the most commonly used.

With Tris the optimum has been reported as low as pH 6.2 with extracts of Cucurbita pepo (Loomis and Battaille, 1963) and as high as pH 8.2, using Pisum sativum (Pollard, et al., 1966). Staby, et al. (1972a) reported that pH 7.7 was optimal for maximal incorporation of MVA-2-^{14}C into neutral terpenes by "Wedgwood" iris.

Using phosphate buffer, the optimum has been as low as pH 6.5 in Citrus sinensis cell-free system (Potty and Bruemmer, 1970a) and as high as pH 8.0 in extracts of petals of a hybrid tea rose (Dunphy and Allcock, 1972).

The optimum pH reported for the specific enzymes involved in the terpenoid biosynthetic pathway are:

A. Mevalonic kinase and phosphomevalonate kinase. Loomis and Battaille (1963) reported that pH 5.7 was optimal for these enzymes in Cucurbita pepo. Rogers,

et al. (1966) working with a cell-free system from Phaseolus vulgaris, found that pH 5.5 was optimal outside the chloroplasts and pH 7.5 inside the chloroplasts. In Agave americana (Garcia-Perigrin, et al., 1972) and in Pisum sativum (Green and Baisted, 1972) the optimum is pH 7.3 - 7.5. Optimal phosphorylation of MVA into the products MVA-P and MVA-PP at pH 6.5 was reported for Citrus sinensis (Potty and Bruemmer, 1970a).

B. Pyrophosphomevalonate decarboxylase.

Popjak (1969) reported an optimum pH 5.1 in liver tissue. Optimal conversion of MVA-5-PP into I-PP took place at pH 7.0 in Citrus sinensis (Potty and Bruemmer, 1970c).

C. Isopentenyl pyrophosphate isomerase.

Potty and Bruemmer (1970c) found that pH 7.0 was optimal in Citrus sinensis. Popjak (1969) reported pH 6.0 for liver tissue.

D. Squalene synthetase.

The optimal pH in Pisum sativum for squalene synthetase was pH 7.4 (Green and Baisted, 1972).

The pH also has effect on product formation. Graebe (1968), working with Pisum sativum fruit, reported that for both squalene and phytoene production the optimal pH was between pH 6.5 - 7.5, while kaurene production was optimal at pH 6.0. Above pH 7.5 squalene and kaurene production rapidly decreased, whereas phytoene production was relatively unchanged at pH 8.6. Kaurene production was optimal at pH 7.1 in Pisum sativum (Coolbaugh and

Moore, 1969) and at pH 7.0 in Ricinus communis (Robinson and West, 1969b).

Incubation Time

Incubation periods used have varied from a few minutes to many hours. Increases in products formed from MVA, in extracts of various plant species were approximately linear up to 3 hours for geranylphosphate (Vrkoc and Loomis, 1969), 1 hour for kaurene (Coolbaugh and Moore, 1971b), 12 minutes for kaurene (Robinson and West, 1969b), 5 hours for phytoene (Shah, et al., 1968) and 1 hour for squalene (Green and Baisted, 1972). The increase in allylic and prenyl phosphate products was linear up to 150 minutes (George-Nascimento and Cori, 1971a). The conversion of I-PP into GG-PP (Porter, 1969) as well as the conversion of I-PP and F-PP into phytoene (Jungalwala and Porter, 1967) was approximately linear for 5 hours.

Green and Baisted (1972), using germinating pea seeds, showed that all enzymes required for the conversion of MVA into squalene were present in the supernatant of homogenized germinating pea seeds. Incubation times varied from 2 minutes for mevalonate kinase and isopentenyl pyrophosphate isomerase up to 25 minutes for trans-prenyltransferase.

Incubation Temperature

The most frequently used incubation temperature for the biosynthesis of terpenes from MVA was 30 C with a range from 23 C (Pollard, et al., 1966) to 38 C (Porter, 1969). Temperatures of 38 C were optimal to convert MVA into GG-PP (Porter, 1969). It also inactivated the enzyme system for the conversion of GG-PP into phytoene in tomato plastids. Staby, et al. (1973) found maximal incorporation of MVA-2-^{14}C into neutral terpenes at 33 C and nearly no incorporation at temperatures of 10 C and 50 C using extracts from iris shoots.

Energy Source

In most cell-free system studied, ATP was used as an energy source for the conversion of MVA into terpenes. ATP was more effective at optimal concentrations than the other triphosphates CTP, GTP, ITP and UTP, on mole per mole base (Loomis and Battaille, 1963; Potty and Bruemmer, 1970a). Pollard, et al. (1966) reported that CTP could completely substitute for ATP using Pisum sativum extracts. An ATP generating system using phosphoenol-pyruvate and pyruvate kinase could partly substitute for ATP in pea fruit cell-free system (Graebe, 1968).

The concentration of ATP used per assay influenced the activation of the system. Loomis and Battaille (1963) reported that excess ATP resulted in complexes between the

added ATP and the divalent cations. They calculated that the ratio of ATP:Mn^{++} was between 2 and 4, while the ratio ATP:Mg^{++} was between 1 and 2. Beytia, et al. (1969) reported a ratio of 1.67 for ATP/divalent cation for maximal incorporation of MVA into terpenes in Pinus radiata seedlings.

An energy source, like ATP, is required only in the converion of MVA into products up to I-PP (Green and Baisted, 1972; Porter, 1969).

Divalent Cations

The effects of the divalent cations Mn^{++} and Mg^{++} on the quality and quantity of terpenes biosynthesized in cell-free systems are well documented (Beytia, et al., 1969; Coolbaugh and Moore, 1969; George-Nascimento and Cori, 1971a, 1971b; Graebe, 1968; Jungalwala and Porter, 1967; Loomis and Battaille, 1963; Porter, 1969; Robinson and West, 1969a; Vrkoc and Loomis, 1969).

The total amount of terpenes produced from MVA was influenced by the concentrations of the divalent cations. At low concentration Mn^{++} promoted incorporation more than Mg^{++}, while Mn^{++} was more inhibitory at higher concentrations (Beytia, et al., 1969; Coolbaugh and Moore, 1969; Graebe, 1968; Jungalwala and Porter, 1967; Loomis and Battaille, 1963; Tchen, 1958).

Mn^{++} and Mg^{++} also influenced the nature of the terpenes biosynthesized. The addition of Mn^{++} stimulated

phytoene production in pea fruit (Graebe, 1968) and the phosphorylation of geraniol in peppermint (Vrkoc and Loomis, 1969). Mn^{++} promoted the conversion of MVA-2-^{14}C into kaurene (Coolbaugh and Moore, 1969), the conversion of I-PP and DMA-PP into GG-PP in tomato (Porter, 1969) and the conversion of F-PP into nerolidol in Pinus radiata (George-Nascimento, et al., 1971b). Mg^{++} stimulated the formation of both kaurene and squalene in pea fruit (Graebe, 1968). The conversions of MVA into I-PP (Porter, 1969) and GG-PP into phytoene (Jungalwala and Porter, 1967) in tomato were affected by Mg^{++}. George-Nascimento, et al. (1971b) reported that pH and Mg^{++} interacted. At pH 8.4, more nerolidol and other farnesols were formed than under the same conditions at pH 7.4 in Pinus radiata. Combinations of Mn^{++} and Mg^{++} have been used. Graebe (1968), using a higher concentration of Mg^{++} than Mn^{++}, detected kaurene, squalene and phytoene. Jungalwala and Porter (1967) concluded that Mg^{++} and Mn^{++} were required for the condensation of I-PP and F-PP and Mg^{++} necessary for the subsequent formation of phytoene in tomato.

Inhibitors

Certain chemicals inhibit various steps in terpene biosynthesis in many cell-free extracts.

Iodoacetamide inhibited I-PP isomerase (Agranoff, et al., 1960) and geraniol dehydrogenase (Potty and Bruemmer, 1970b) in yeast and orange extracts, respectively.

The addition of iodoacetamide reduced the phytoene production by nearly 100% in tomato plastids (Jungalwala and Porter, 1967), MVA kinase activity by 40% in pumpkin (Loomis and Battaille, 1963), neutral terpene production in cell-free extracts of pea by 66% (Pollard, et al., 1966) and of iris by 91% (Staby, et al., 1973). Oster and West (1968) reported an increase in MVA phosphorylation, while Potty and Bruemmer (1970a) showed a decrease in MVA phosphorylation using Echinocystis macrocarpa and Citrus sinensis cell-free systems, respectively.

AMO-1618 inhibited kaurene biosynthesis by preventing the cyclization of GG-PP to copalyl-PP (Dennis, et al., 1965; Graebe, 1968; Oster and West, 1968; Robinson and West, 1969b). AMO-1618 had little or no effect on squalene and phytoene production from MVA in pea fruit cell-free extracts (Graebe, 1968) and on neutral terpenes biosynthesized in an iris shoot cell-free system (Staby, et al., 1973).

Phosfon D inhibited kaurene biosynthesis (Dennis, et al., 1965; Robinson and West, 1969b). Inhibition also occurred in the formation of diterpene hydrocarbons from GG-PP (Robinson and West, 1969b) and in the formation of neutral terpenes from MVA (Staby, et al., 1973), using Echinocystis macrocarpa, Ricinus communis and Iris hollandica cell-free extracts, respectively. Shechter and West (1969) reported the inhibition of the conversion of ent-labda-8,

(17),13-dien-15-yl pyrophosphate into ent-kaurene in cell-free extracts from Fusarium moniliforme.

CCC inhibited the biosynthesis of kaurene and other diterpene hydrocarbons in Ricinus communis (Robinson and West, 1969b). No inhibition occurred in kaurene biosynthesis in the cell-free extracts from Echinocystis macrocarpa (Dennis, et al., 1965). CCC had little or no effect on the formation of neutral terpenes in cell-free extracts from iris shoots (Staby, et al., 1973).

Localization of Terpene Biosynthesizing Enzymes

Activities of kaurene synthetase in Pisum sativum (Coolbaugh and Moore, 1971a) and in Ricinus communis (Robinson and West, 1969b) cell-free extracts were in the 105,000 x g supernatant solution. Squalene enzymes are also located in this soluble fraction (i.e. Graebe, 1967; Green and Baisted, 1972; Jungalwala and Porter, 1967).

Graebe (1968) stated: ". . . .even though all the enzymes needed to catalyse the sequence from mevalonate to kaurene, squalene and phytoene were located in the particle-free supernatant of the present system, they may partly have originated from various sub-structures of the intact cell. . . ." He also stated: ". . .The characterization was done with the knowledge that the system was both crude and complicated."

Enzymes necessary for the incorporation of MVA into kaurene (Graebe, 1968), phytoene (Graebe, 1968;

Jungalwala and Porter, 1967), squalene (Graebe, 1968), phosphorylated MVA (Rogers, et al., 1966) and cyclic and acyclic carotenes (Porter, 1969) were all reported in the cell-free extracts of chloroplasts from different plant sources. MVA was converted into kaurene in Echinocystis macrocarpa endosperm (Murphy and West, 1969; Oster and West, 1968). In cell-free extracts of Cucurbita pepo endosperm, MVA was converted into ent-kaurene, ent-kaur-16-en-19-oic acid, ent-7-hydroxy-kaur-en-19-oic acid, ent-gibberell-16-en-7 al-19-oic acid (Bowen, et al., 1972; Graebe, et al., 1972). The soluble fractions of seedlings of Cucurbita pepo (Loomis and Battaille, 1963), of Pisum sativum (Pollard, et al., 1966), of Ricinus communis (Robinson and West, 1969a, 1969b), of cotelydons of Pisum sativum (Coolbaugh and Moore, 1971; Moore, et al., 1972), and of the seedcoat of Pisum sativum (Coolbaugh and Moore, 1971) and of the shoot of Iris hollandica (Staby, et al., 1972) contained the enzymes involved in the biosynthesis of terpenoid products from MVA.

Protein Concentration

In most cell-free systems from higher plants a linear increase in terpenes biosynthesized, up to a definite protein level, has been reported with increasing protein concentration (i.e. Potty and Bruemmer, 1970a; Robinson and West, 1969b).

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PART II

INTRODUCTION

In higher plants, the isoprenoid pathway leads to the formation of gibberellins, abscisic acid, sterols, caretenoids and other naturally occurring terpenoids (Goodwin, 1967). Some of these substances are plant hormones while others are involved in processes such as photosynthesis and membrane permeability (Goodwin, 1967). All evidence indicates that they originate from a C-5 isoprenoid intermediate, which is derived from mevalonic acid (Goodwin, 1967; Green and Baisted, 1972; Heftmann, 1970).

One group of the naturally occurring plant hormones is the gibberellins. They regulate a wide range of physiological processes in higher plants (Lang, 1957, 1965). Because of their importance for the growth and development of higher plants, cell-free systems have been isolated from different plant tissue to investigate the biosynthesis of gibberellins or their precursors. Kaurene, a gibberellin precursor, has been biosynthesized from mevalonic acid in cell-free extracts from Pisum sativum (Coolbaugh and Moore, 1969), Cucurbita pepo (Graebe et al., 1972), Echinocystis macrocarpa (West and Upper, 1969) and Ricinus communis (Robinson and West, 1969a, 1969b).

Aung, et al. (1969) reported the presence of gibberellin-like substances in several bulbous crops. Staby et al. (1970, 1972, 1973), using cell-free extracts from shoots of Iris hollandica, could not obtain evidence for production of kaurene from mevalonic acid. The products they identified were stigmasterol, campesterol, octacosanol, β-sitosterol, which were non-radioactive (Staby and De Hertogh, 1972) and farnesol, geraniol and squalene, which were radioactive (Staby, et al., 1973).

In tulips, gibberellin-like substances have been reported in many tissues of the bulb (Aung et al., 1967, 1970). Einert et al. (1972) found that shoot tissue contained the highest levels of extractable gibberellin-like substances.

Because of the high amount of extractable gibberellin-like substances from shoot tissue this study was undertaken to begin characterising the terpenoid biosynthetic system using tulip shoots as a source of the enzymes.

MATERIALS AND METHODS

Tulip bulbs, Tulipa gesneriana L. cv Golden Melody, were received from the Netherlands in late August 1971 and 1972. Upon arrival, the bulbs were stored and handled as outlined in the Holland Bulb Forcer's Guide (De Hertogh, 1970). All chemicals used were reagent grade.

The nucleotide triphosphates ATP, CTP, GTP, UTP as sodium salts and chloramphenicol were obtained from Sigma Chemical Co., St. Louis, Missouri. Bovine serum albumin was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. MVA-2-^{14}C (N'N'-dibenzoylethylenediamine salt) was purchased from New England Nuclear Corp., Boston, Massachusetts. Depending on the sample lot, the specific activity varied between 6.3 and 7.1 mC/mmole.

Standard preparation and incubation of cell-free extracts. Shoot tissue (scape plus floral organs) of tulip bulbs was homogenized at 0-2 C for 2 minutes at top speed in a Lourdes blendor using 0.1 M K-phosphate buffer at pH 6.5, containing 50 ug/ml chloramphenicol. Ratio of shoots to buffer was 1;3 (wt:vol). The homogenates were filtered through 2 layers of cheesecloth and centrifuged at 15,000 x g for 30 minutes.

The standard incubation medium consisted of 2.5 ml of the supernatant solution, 1.0 ml of K-phosphate buffer pH 6.5, 1 x 10^{-5} moles each of $MnCl_2$, $MgCl_2$ and ATP, and 1.6 x 10^{-8} moles of MVA-2-^{14}C, in a total volume of 4 ml.

The mixture was incubated for 60 minutes at 35 C in a waterbath gyrator at 150 rpm.

Results on the incorporation of MVA-2-^{14}C into terpenes are expressed as the complete reaction mixture less the minus ATP (control). All studies reported are the means of 2 or more duplicated experiments.

In certain studies the preparation and standard incubation medium was modified. Standard medium was modified for the protein concentration, cofactor requirements, pH, and substrate concentration study. Standard incubation time was modified for the incubation time course study.

Extraction of radioactive reaction products. Incubations of cell-free extracts were terminated by adding 4 ml of acetone. The precipitate was separated by centrifugation at 30,000 x g for 10 minutes. The pellet was resuspended and washed 2 times with 2 ml of acetone. The acetone washings were combined with the original supernatant and subsequently extracted 2 times with 5 ml benzene. This fraction is called the "neutral" terpene fraction.

The benzene extracted aqueous mixture was then treated with 0.05 ml 5 N HCl, incubated at 60 C for 60 minutes in a waterbath, and neutralized with 0.05 ml 5 N KOH. The acid hydrolyzed products were extracted 2 times with 5 ml benzene. The fraction iscalled the "acid hydrolyzable" terpene fraction.

Radioactive monitoring. The isolated fractions were evaporated to dryness in vacuo at 45 C. Each sample was then dissolved in 15 ml scintillation fluor, which consisted of 100 mg 1,4-bis-(2- 4-methyl-5-phenylaxozolyl)-benzene and 4 g 2,5-diphenyl-oxazole in 1 liter of toluene. All samples were counted in a Beckman LS-100 C with a preset error of 1 percent (40,000 cpm). Maximum time for counting was 10 minutes.

Protein determination. Protein determination was made on aliquots of each enzyme preparation by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

RESULTS

Effect of pH. The pH optimum for total incorporation of MVA-2-^{14}C into terpenes is 6.5 (Figure 1). Both the "neutral" and "acid hydrolyzable" fraction showed the same pH optimum (Figure 1). In this study, the tissue was homogenized and then incubated using the same pH buffer. If changes occurred, the pH was adjusted to the required pH prior to incubation.

Incubation temperature. The optimal temperature for total incorporation as well as for incorporation into the "neutral" fraction was 35 C (Figure 2). The "acid hydrolyzable" fraction showed no distinct optimum.

Protein concentration. The protein concentration used ranged from 0 to 20 mg (Figure 3). The amount of total incorporation of MVA-2-^{14}C into terpenes was approximately linear up to 5 mg protein. At higher concentrations of protein no increase in total incorporation was observed. At concentrations less than 4 mg protein nearly all of the activity was detected in the "acid hydrolyzable" fraction. At concentrations higher than 4 mg protein there was an

Figure 1.--Effect of pH of PO_4 buffer on the incorporation of MVA-2-^{14}C into terpenes.

Standard extraction and incubation used as described in experimental, except for pH indicated.

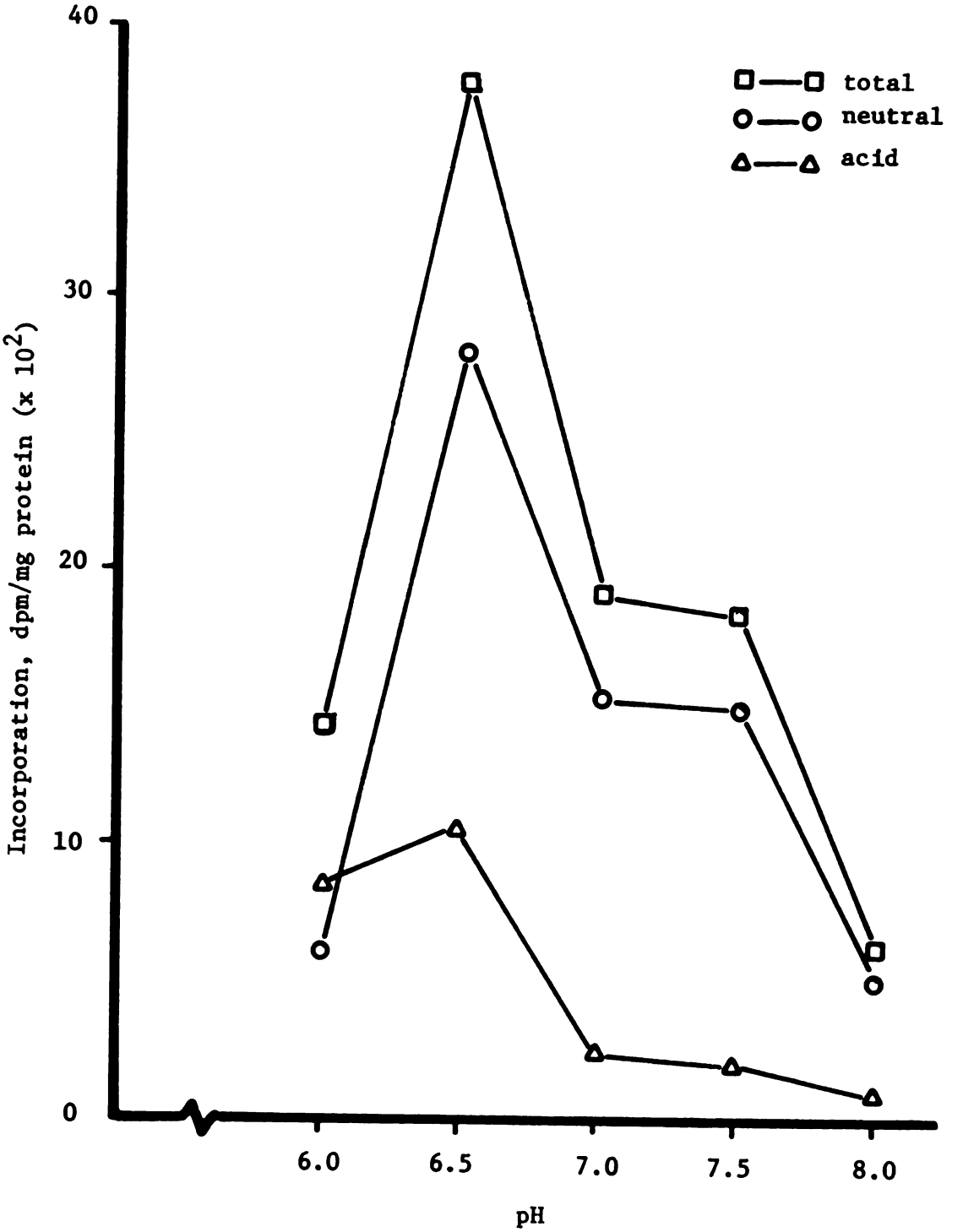

40
30
20
10
0
Incorporation, dpm/mg protein (x 10^2)
total
neutral
acid
6.0
6.5
7.0
7.5
8.0
pH

Figure 2.--Effect of incubation temperature on the incorporation of MVA-2-^{14}C into terpenes.

Standard extraction and incubation used as described in experimental, except for temperatures indicated.

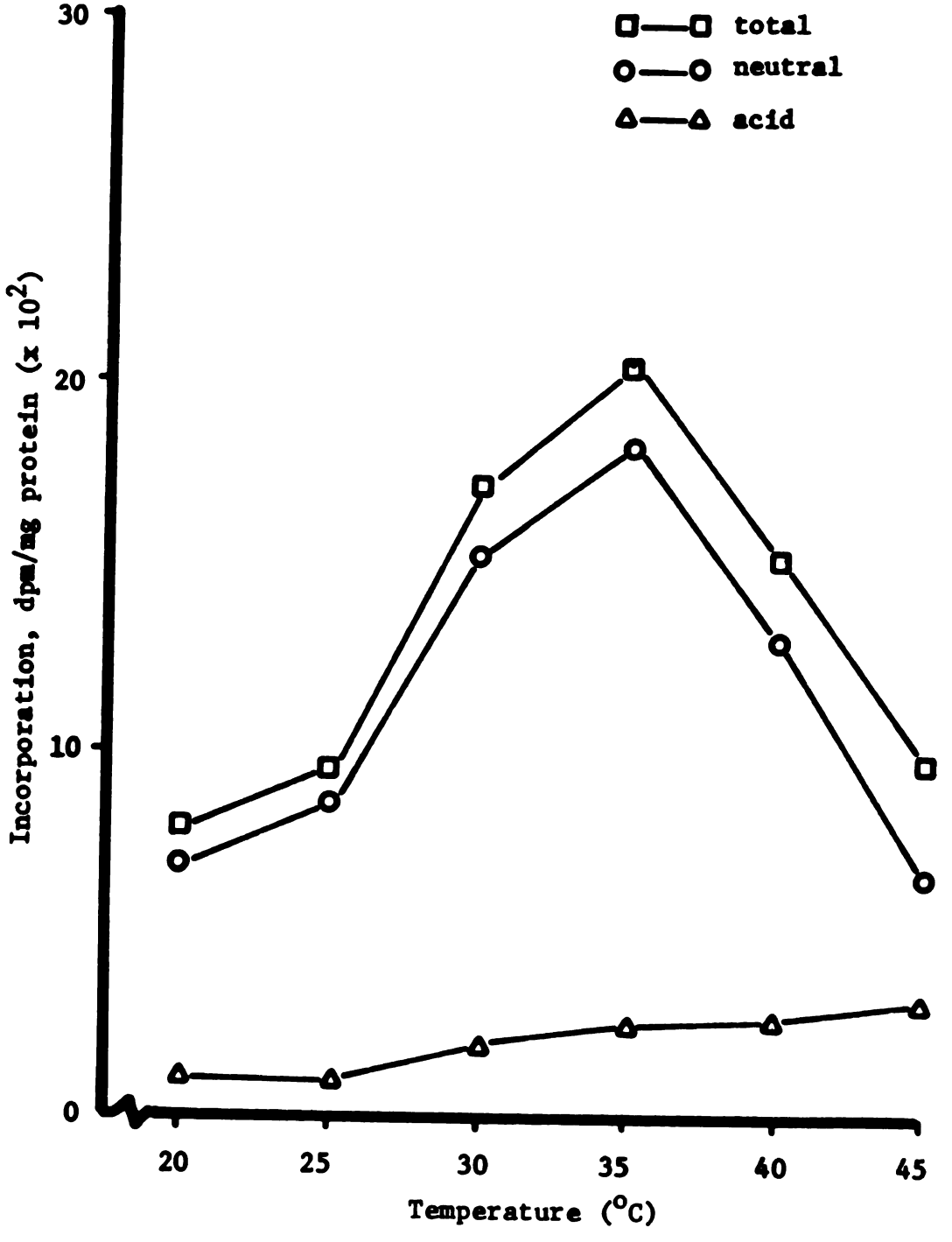
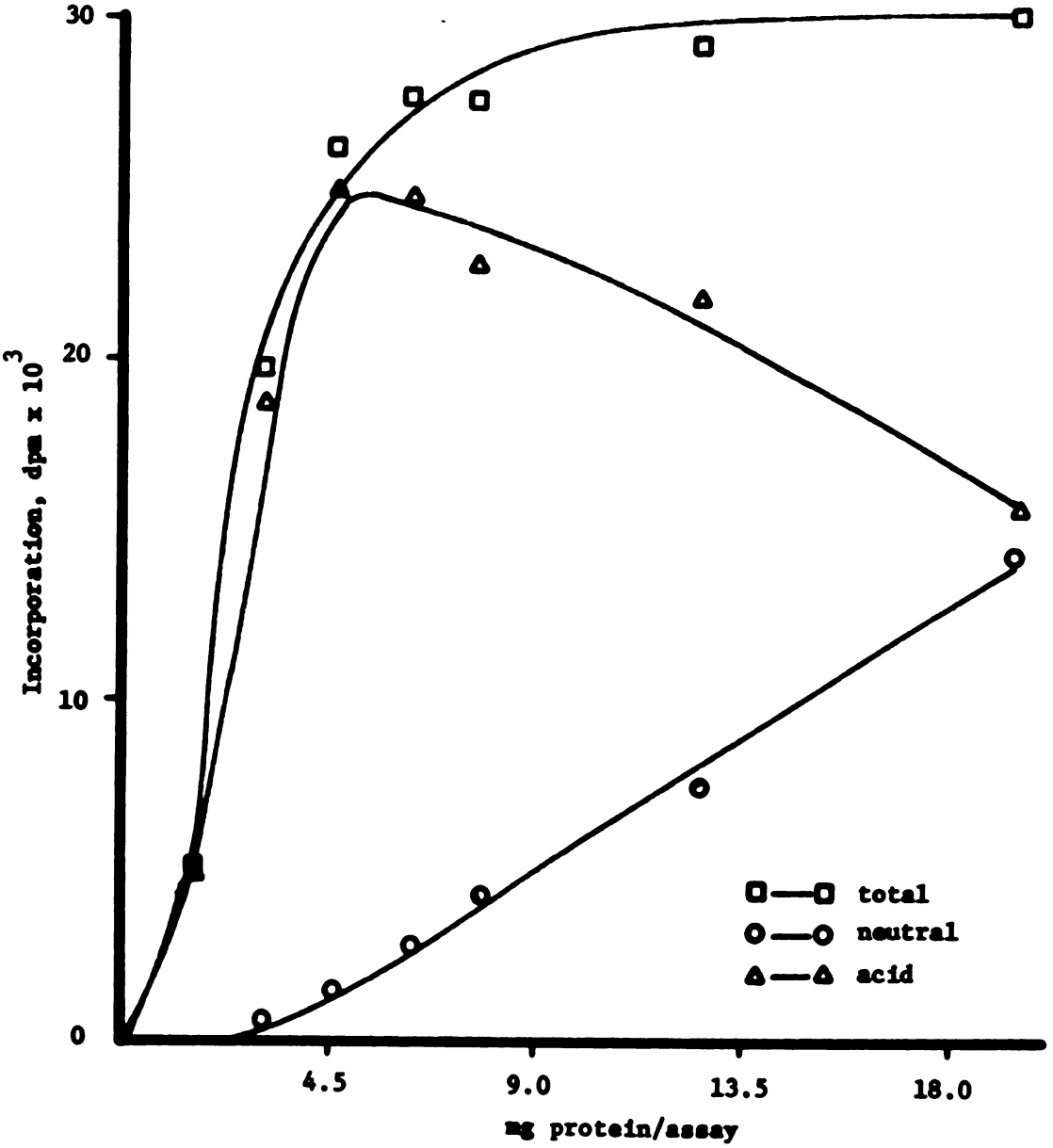

total
neutral
acid
30
20
10
0
Incorporation, dpm/mg protein (x 10^2)
20
25
30
35
40
45
Temperature (^oC)

Figure 3.--Effect of protein concentration on the incorporation of MVA-2-^{14}C into terpenes.

Standard extraction and incubation used as described in experimental, except for protein concentration indicated.


30
20
10
0
Incorporation, dpm x 10^3
4.5
9.0
13.5
18.0
mg protein/assay
total
neutral
acid

increase in the "neutral" fraction which correlated with the decrease in the "acid hydrolyzable" fraction.

Incubation time. To provide additional information on the relationship of the "acid hydrolyzable" to "neutral" fraction, low (5 mg) and high (18 mg) concentrations of protein were utilized.

With the low concentration, most incorporation occurred in the "acid hydrolyzable" fraction up to 60 minutes (Figure 4). After 60 minutes, there was an approximate linear increase in the "neutral" fraction, while the rate of incorporation into the "acid hydrolyzable" fraction was decreasing.

With a high concentration of protein, activity in the "neutral" fraction increased linearly for 120 minutes and then at a lower rate from 120 to 240 minutes (Figure 5). In the "acid hydrolyzable" fraction there was a rapid increase in incorporation for the first 60 minutes and then no change. Total incorporation was approximately linear for 60 minutes and increased at a lower rate from 60 to 240 minutes. The total incorporation of MVA-2-^{14}C into terpenes was less with the high concentration of protein than with low concentration of protein (Figure 4).

Cofactor requirements. The cofactors investigated in this study were ATP, CTP, GTP, UTP, with or without the divalent cations Mg^{++} and Mn^{++}. The concentrations of the nucleotide triphosphates used was 1×10^{-5} moles. Standard

Figure 4.--Effect of protein (5 mg protein) and time on the incorporation of MVA-2-^{14}C into terpenes.

Standard extraction and incubation used as described in experimental, except for protein and incubation time indicated.

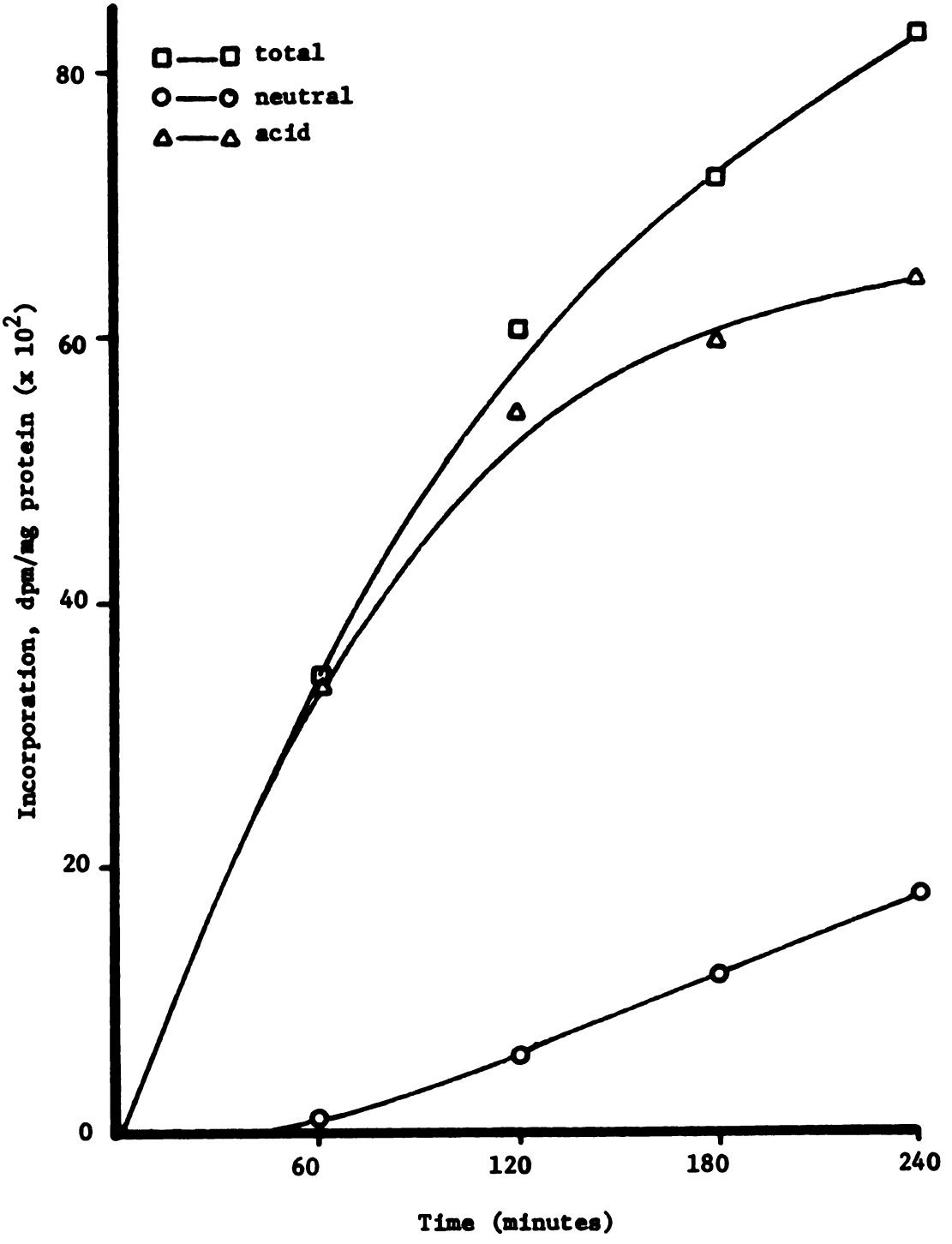
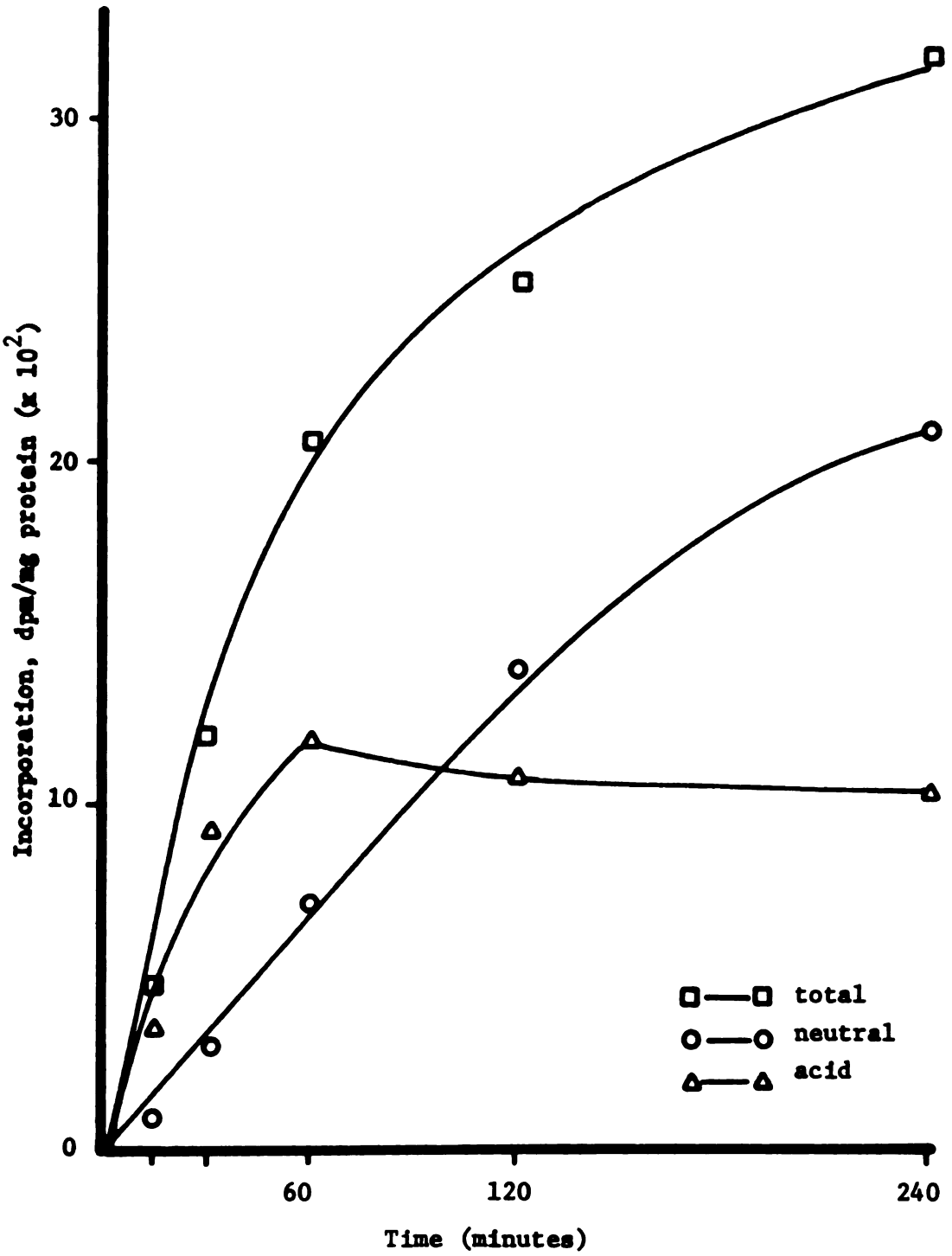

total
neutral
acid
80
60
40
20
0
60
120
180
240
Incorporation, dpm/mg protein (x 10²)
Time (minutes)

Figure 5.--Effect of protein (18 mg protein) and time on the incorporation of MVA-2-^{14}C into terpenes.

Standard extraction and incubation used as described in experimental, except for protein and incubation time indicated.


30
20
10
0
Incorporation, dpm/mg protein (x 10²)
60
120
240
Time (minutes)
total
neutral
acid

incubation and extraction was used as described in experimental except for the nucleotide triphosphates and the divalent cations. Total incorporation was stimulated by the nucleotide triphosphates with the divalent cations. ATP with Mg^{++} and Mn^{++} stimulated the incorporation more than CTP, GTP and UTP (Table 1). The nucleotide triphosphates without the divalent cations did not show any stimulating effect on the incorporation of MVA-2-^{14}C into terpenes. GTP with Mg^{++} and Mn^{++} did not stimulate high levels of incorporation into the "neutral" fraction compared to the other triphosphates. Using ATP, CTP, GTP, UTP without Mg^{++} and Mn^{++}, the "neutral" fraction did not show any difference in activity between the nucleotide triphosphates. In the "acid hydrolyzable" fraction, the combination of ATP with Mg^{++} and Mn^{++} stimulated the incorporation of MVA-2-^{14}C into terpenes more than CTP, GTP and UTP with Mg^{++} and Mn^{++}. Without the cations, ATP showed a slight inhibitory effect in the "acid hydrolyzable" fraction compared to CTP, GTP and UTP. There was little or no difference between the control and the Mg^{++} and Mn^{++} fractions.

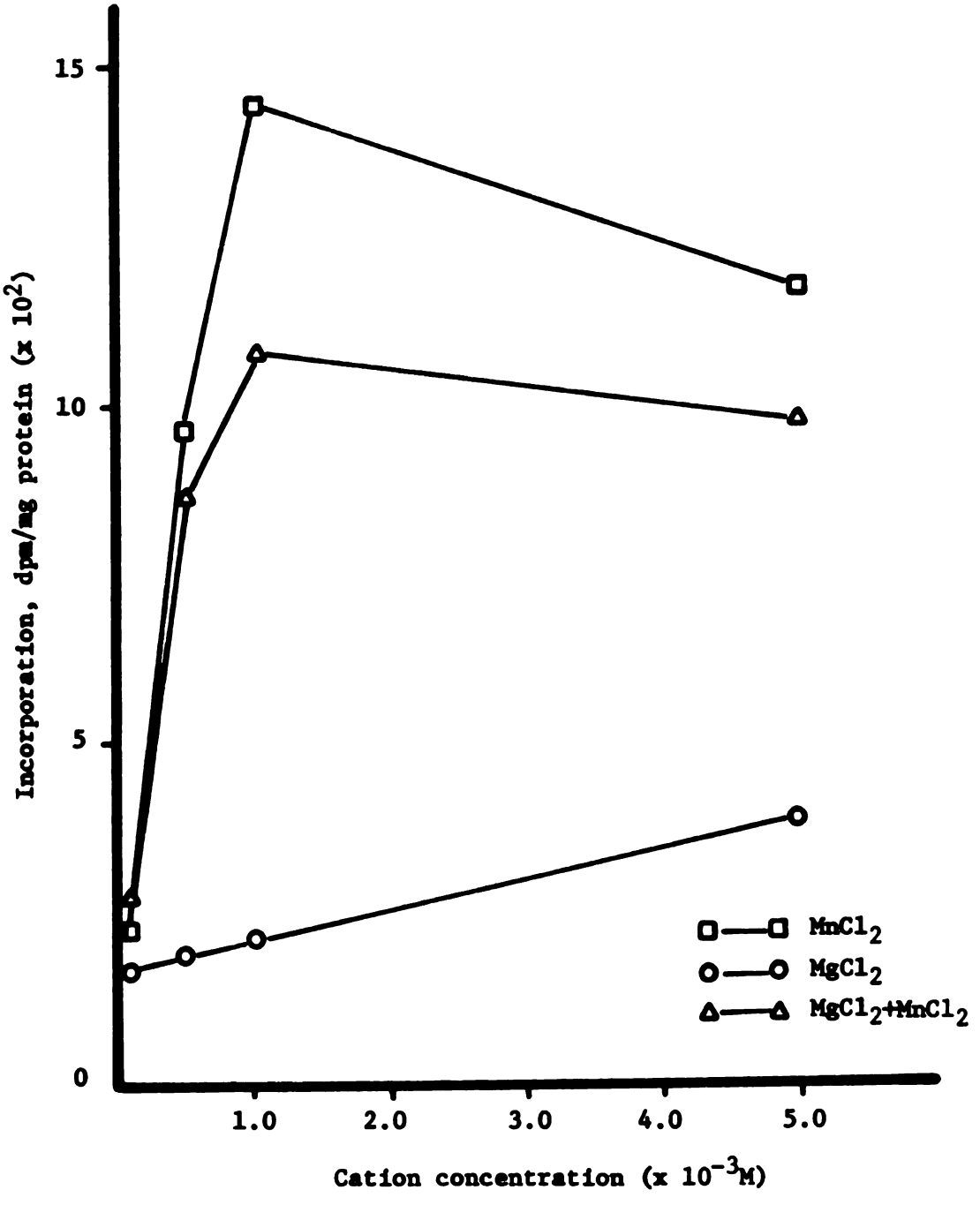
Mn^{++} stimulated the total incorporation more than Mg^{++} or the combination of Mg^{++} + Mn^{++} (Figure 6). Higher concentrations of Mn^{++} were inhibitory. The combination of Mg^{++} + Mn^{++} was less active than Mn^{++} alone. High concentrations of the combination were less effective. The

TABLE 1.--Influence of the nucleotide triphosphates and divalent cations on the incorporation of MVA-2-^{14}C into terpenes.

Incubation medium	Incorporation (dpm/mg protein)		
	Neutral fraction	Acid hydrolyzable fraction	Total
None	207	264	471
Mg^{++} + Mn^{++}	212	256	468
ATP	192	132	324
CTP	196	214	410
GTP	199	235	434
UTP	182	237	419
ATP + Mg^{++} + Mn^{++}	670	973	1643
CTP + Mg^{++} + Mn^{++}	611	393	1004
GTP + Mg^{++} + Mn^{++}	372	419	791
UTP + Mg^{++} + Mn^{++}	625	476	1101

Figure 6.--Effect of $MgCl_2$ and $MnCl_2$, alone and in combination, on the incorporation of MVA-2-^{14}C into terpenes.

Standard extraction and incubation used as described in experimental, except for cation concentration indicated.


15
10
5
0
Incorporation, dpm/mg protein (x 10^2)
1.0
2.0
3.0
4.0
5.0
Cation concentration (x 10^{-3}M)
$MnCl_2$
$MgCl_2$
$MgCl_2$+$MnCl_2$

divalent cation Mg^{++} produced a linear increase in the total incorporation of MVA-2-^{14}C into terpenes with increasing concentrations. Increasing concentrations of Mg^{++} and Mg^{++} + Mn^{++} promoted activity in the "neutral" fraction while Mn^{++} showed an optimum at 1.0×10^{-3} M (Table 2). The "acid hydrolyzable" fraction showed an optimum at 1.0×10^{-3} M for Mn^{++} and Mg^{++} + Mn^{++}. Mg^{++} produced an optimum at 5.0×10^{-3} M.

Substrate concentration. Incorporation into the total as well as the "neutral" and "acid hydrolyzable" fractions, was linear with increases in the substrate concentration up to 8×10^{-8} M MVA-2-^{14}C (Figure 7).

Localization of enzyme activity. All enzyme activity was located in the soluble fraction of the system (Appendix, Table 4). There was loss of activity upon centrifugation at 100,000 x g.

DISCUSSION

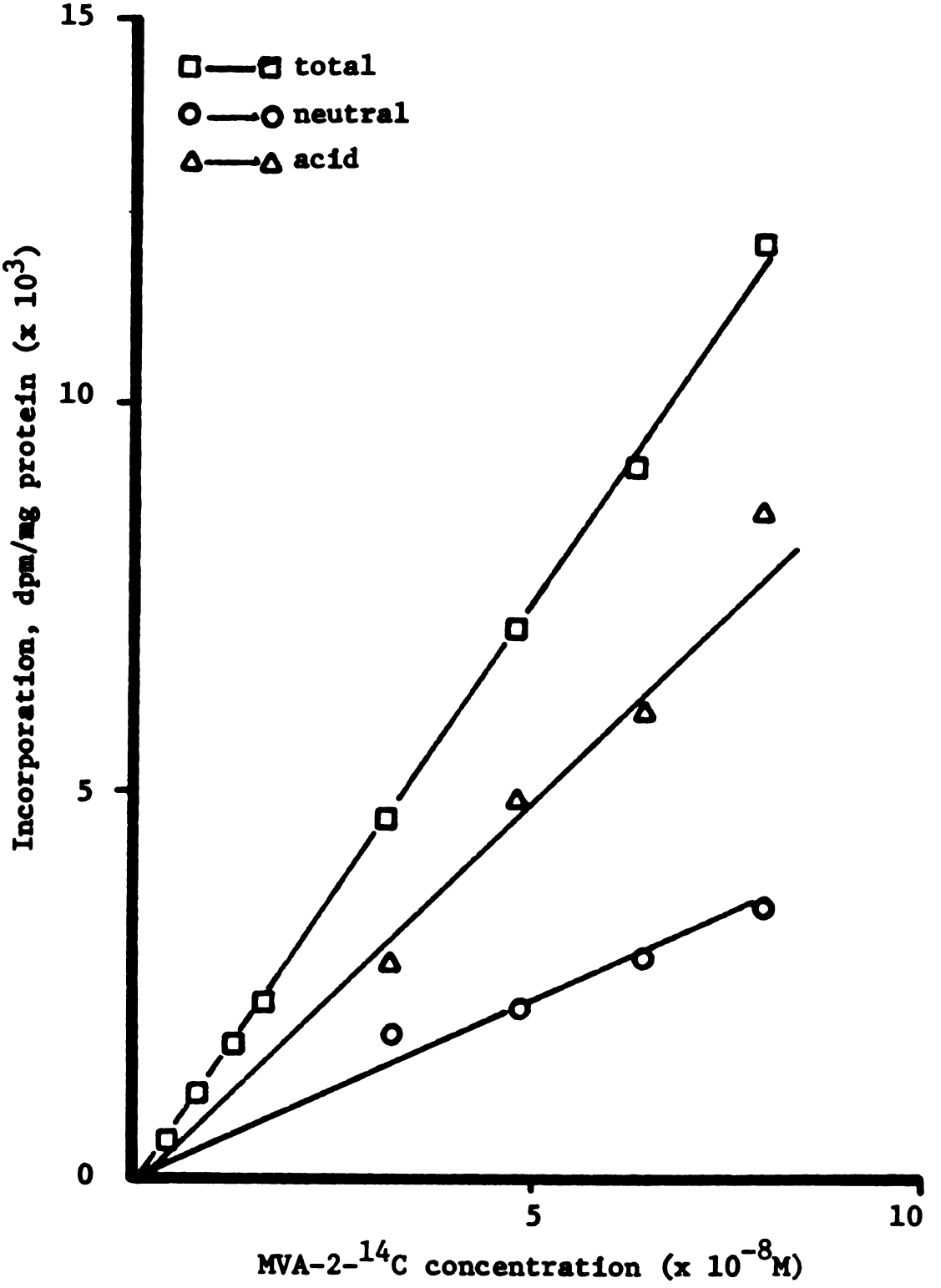
Some characteristics of the cell-free terpenoid biosynthetic system from tulip shoots have been established. As Graebe (1968) stated: "The characterization was done with the knowledge that the system was both crude and complicated." Purification and identification of enzymes involved in the biosynthetic pathway of MVA-2-^{14}C into terpenes were not investigated, so all the results are for the general system.

TABLE 2.--Influence of the divalent cations Mg^{++} and Mn^{++} and the combination Mg^{++} + Mn^{++} on the incorporation of MVA-2-^{14}C into terpenes.

Divalent cation	Concentration x 10^{-3} M	Incorporation (dpm/mg protein)		
		Neutral fraction	Acid hydrolyzable fraction	Total
Mg^{++}	0.1	57	116	173
	0.5	63	122	185
	1.0	64	153	217
	5.0	107	291	398
	10.0	361	215	576
Mn^{++}	0.1	66	148	214
	0.5	361	608	969
	1.0	760	844	1444
	5.0	740	452	1192
	10.0	731	140	871
Mg^{++} + Mn^{++}	0.1	78	194	272
	0.5	286	522	808
	1.0	495	600	1095
	5.0	508	482	990
	10.0	655	190	845

Figure 7.--Effect of MVA-2-^{14}C concentration on the incorporation of MVA-2-^{14}C into terpenes.

Standard extraction and incubation used as described in experimental, except for MVA-2-^{14}C concentration indicated.


15
10
5
0
total
neutral
acid
Incorporation, dpm/mg protein (x 10^3)
5
10
MVA-2-^{14}C concentration (x 10^{-8}M)

Using phosphate buffer, the optimal pH for the system was pH 6.5 (Figure 1). Potty and Bruemmer (1970a) reported a pH 6.5 for the maximal phosphorylation of MVA in Citrus sinensis. The commonly used pH is between pH 7.0 - 7.5 (Coolbaugh and Moore, 1969, 1971; Green and Baisted, 1972; Rogers et al., 1966). Graebe et al. (1972) reported that pH 8.0 was optimal for the conversion of MVA into kaurene and related compounds in Cucurbita pepo endosperm. At this pH the tulip system showed very little activity. Staby (1970), using Iris hollandica shoot tissue, obtained a 85 percent inhibition in the conversion of MVA-2-^{14}C into neutral terpenes with a phosphate buffer at pH 7.7, when compared with a Tris buffer.

The optimal incubation temperature for the terpenoid biosynthetic system was 35 C (Figure 2). These results agree with those of Potty and Bruemmer (1970a, 1970b), Rogers et al. (1966) and Porter (1969). Our results differ from those of Green and Baisted (1972) who found a maximum temperature of 24 C for the conversion of MVA-2-^{14}C into products of the squalene biosynthetic pathway of Pisum sativum.

The total incorporation of MVA-2-^{14}C into terpenes was linear up to 5 mg protein (Figure 3). At higher concentrations no marked increase in total incorporation was detected. The total incorporation was not proportional to the amount of enzyme protein added. George-Nascimento

et al. (1971) using Citrus sinensis and Garcia-Perigrin et al. (1972) using Agave americana reported the rate of product formation was not strictly proportional to the amount of protein added. They suggested the presence of an inhibitor which strongly interferes with the reaction when its concentration reached a definite level. On the other hand, Coolbaugh and Moore (1969), Jungalwala and Porter (1967), Potty and Bruemmer (1970a) reported a proportional increase in product formation from MVA-2-^{14}C with an increase of protein from Pisum sativum, Lycopersicon esculentum, Citrus sinensis, respectively.

An incubation time study showed that the amount of protein utilized influenced the total incorporation. At low concentration of protein (Figure 4) the incorporation after 60 minutes is the same as after 240 minutes at high concentration (Figure 5). The increase in product formation is linear up to about 60 minutes and increases at a lower rate from 60 to 240 minutes. At high concentration of protein there appears to be an inhibitory effect of the protein. Potty and Bruemmer (1970a) reported an increase in product formation up to 270 minutes with a decrease at longer incubation times. The results obtained in this study, showed that even after 240 minutes, there is still an increase in product formation.

There is an absolute requirement for a nucleotide triphosphate and a divalent cation (Figure 6; Tables 1 and 2). ATP combined with Mg^{++} and Mn^{++} stimulated the incorporation more than CTP, GTP and UTP. These data agree with those of Potty and Bruemmer (1970), using Citrus sinensis. They also reported that GTP was less effective. This is not consistent with the results of Pollard et al. (1966) and Tchen (1958) using cell-free extracts from pea and yeast, respectively. Pollard et al. (1966) reported that CTP could completely substitute for ATP, while CTP, GTP and UTP could replace ATP in the yeast system. The nucleotide triphosphates without divalent cations showed a slight inhibitory effect (Table 1). Loomis and Battaille (1963) reported that excess of ATP resulted in complexes between the added ATP and the divalent cations. In our case it may be due to complex formation between the nucleotide triphosphates and the endogenous cations in the supernatant. There was no difference in incorporation between the control (crude extract) and the added divalent cations (Table 1). The data of the crude extracts and the divalent cations are similar in nature to those achieved with pH 8.0 phosphate buffer (Figure 1). At pH 8.0 the divalent cations are partly precipitated and complex formation results between ATP and some cations left in the supernatant. Mn^{++} had a greater effect on the incorporation of MVA-2-^{14}C into

terpenes at low concentration than Mg^{++} (Figure 6). Similar effects are reported by Beytia et al. (1969), Coolbaugh and Moore (1969), Graebe (1968), Loomis and Battaille (1963) in Pinus radiata, Pisum sativum, Cucurbita pepo, respectively. Loomis and Battaille (1963) found an inhibitory effect with high concentrations of Mn^{++} which was due to the formation of a complex between ATP and Mn^{++}. The increase in product formation is linear with increase of Mg^{++}concentration (Figure 6). Pollard et al. (1966) reported that Mg^{++} was required in the conversion of MVA into triterpenes. Beytia et al. (1966), Tchen (1958) and Staby et al. (1973) showed a nearly linear increase in product formation with increasing the concentration of Mg^{++}. Loomis and Battaille (1963) reported a ratio ATP:Mg^{++} between 1 and 2 for maximal phosphorylation. The data show that the ratio may be even less than 1. The ATP concentration was 1×10^{-5} moles while the concentration Mg up to 5×10^{-3} M showed an increase in the "acid hydrolyzable" fraction. The Mg^{++} + Mn^{++} combination is less active than Mn^{++}. Beytia et al. (1969), Graebe (1968), Jungalwala and Porter (1967), Robinson and West (1969b), using Pinus radiata, Pisum sativum, Lycopersicon esculentum, Ricinus communis, respectively, reported the requirements for both divalent cations. Mg^{++} and Mn^{++} influenced the products biosynthesized from MVA (Graebe, 1968). Our results show the preference of Mn^{++} for the incorporation

of MVA into terpenes, but as no product identification has been carried out, it is not possible to conclude that only Mn^{++} is required for the system.

The increase in products biosynthesized from MVA-2-^{14}C was linear up to 8 x 10^{-8}M when incubated for 60 minutes at 35 C (Figure 7). Staby et al. (1973) reported a linear increase up to 9.6 x 10^{-8}M, using Iris hollandica.

The products biosynthesized from MVA-2-^{14}C in cell-free extracts from tulip shoots were isolated in two fractions. The "neutral" fraction was the lipid fraction extractable with benzene and probably contains prenols and rearranged alcohols. The "acid hydrolyzable" fraction probably consisted of released allylic pyrophosphates. Goodman and Popjak (1960) showed that the rearrangement during acid hydrolysis of allylic pyrophosphates is a useful characteristic in the identification of this class of substances. Our results suggested ^{14}C-pyrophosphate intermediates were present. Acid hydrolysis increased the amount of extractable radioactivity from the "neutral" fraction. Beytia et al. (1969), George-Nascimento and Cori (1971), Oster and West (1968) reported an increase in products with regard to qualitative effects of acid hydrolysis.

Results from the pH and incubation temperature studies showed more incorporation into the "neutral" fraction than into the "acid hydrolyzable" fraction (Figures 1 and 2). The results of the protein concentration study showed that up to 4 mg protein nearly all activity was detected in the "acid hydrolyzable" fraction. At concentrations higher than 4 mg protein there was an increase in the "neutral fraction correlated with a decrease in the "acid hydrolyzable" fraction (Figure 3). Pollard et al. (1966), using Pisum sativum, concluded that the "neutral" fraction II (acid hydrolyzable) was the precursor for the "neutral" fraction I (neutral fraction). Some of our data agree with their hypothesis of a precursor fraction. The pH and incubation study data showed more incorporation of MVA-2-^{14}C into the "neutral" fraction compared to the "acid hydrolyzable" fraction (Figures 1 and 2). With continued morphological development of the shoot there is a change from the "neutral" fraction to the "acid hydrolyzable" fraction. This suggests that there is probably an increase in allylic pyrophosphates and/or phosphates and a decrease in prenols. Thus the "neutral" fraction may be the precursor for the "acid hydrolyzable" fraction. The data of the localization study also show an increase in incorporation in the "acid hydrolyzable" fraction, which is correlated with a decrease in the "neutral" fraction (Appendix, Table 4). On the other hand, the results of the time course study

with 5 mg protein show an increase in the "acid hydrolyzable" fraction correlated to an increase in the "neutral" fraction (Figure 4).

ATP combined with Mg^{++} + Mn^{++} promoted incorporation into the "acid hydrolyzable" fraction (Table 1). Comparing the "neutral" fraction there is little or no difference between ATP, CTP and UTP. The increase in the "acid hydrolyzable" fraction using ATP showed that phosphorylation was stimulated more by ATP with Mg^{++} + Mn^{++} than by CTP, GTP and UTP. Beytia et al. (1969) found similar results in Pinus radiata. Using the individual nucleotide triphosphates, little or no effect was detected in the "neutral" fraction compared to the control (Table 1). The individual cations utilized showed that Mn^{++} had a more stimulatory effect than Mg^{++} or the combination (Table 2). Concentration of Mn^{++} up to 1×10^{-3}M promoted incorporation into the "acid hydrolyzable" fraction more than into the "neutral" fraction. At high concentrations this was reversed. Mn^{++} has a strong stimulatory effect on both fractions, and is probably due to the effect of Mn^{++} on several enzymes in the system. Mg^{++} showed less stimulating effect than Mn^{++}. The increase in incorporation of MVA-2-^{14}C into the "neutral" as well as into the "acid hydrolyzable" fraction is very gradual. Our data confirmed the results obtained by Beytia et al. (1969). The effects of Mg^{++} + Mn^{++} indicate that the system is more affected by Mn^{++} than Mg^{++}.

Future research is needed to identify the products biosynthesized from MVA-2-^{14}C and to study the changes from "neutral" into "acid hydrolyzable" fraction or vice versa.

APPENDIX

Preparation of Enzyme Extracts

The following experiment was set up to determine the effects of the ratio plant tissue to buffer for isolation of the cell-free system to study the incorporation of MVA-2-^{14}C into the "neutral" and into the "acid hydrolyzable" fraction.

Standard extraction and incubation were used as described in experimental.

By decreasing the ratio a decrease was observed in the activity in both the "neutral" and "acid hydrolyzable" fractions (Table 3). There was a greater decrease in the "neutral" fraction than in the "acid hydrolyzable" fraction. The results indicated that the most favorable ratio was 1:3 (wt:vol) when both "neutral" and "acid hydrolyzable" fractions were to be studied. On the other hand, if only the "acid hydrolyzable" fraction was to be studied the 1:12 ratio reduced the activity in the "neutral" fraction.

TABLE 3.--Influence of tissue to buffer ratio on the incorporation of MVA-2-^{14}C into terpenes.

Preparation. Ratio (wt:vol)	Incorporation in dpm		
	Neutral fraction	Acid hydrolyzable fraction	Total
1: 3	6549	17312	23861
1: 6	1576	14169	15745
1: 9	1443	12168	13611
1:12	614	10888	11502

Effects of Centrifugation on Isolation of Enzyme Activity

Relative centrifugal forces of 15,000 x g and 100,000 x g were used to prepare the various centrifugation fractions. The centrifuges used were a Sorvall RC-2-B and a Beckmann L 2-658 ultra centrifuge.

Standard assays were run as described in experimental. The pellet was resuspended in the appropriate amounts of buffer after each centrifugation.

All enzyme activity was located in the supernatant after each centrifugation. No activity was detected in the pellet fraction after resuspension and assaying under the same conditions (Table 4). With the increase of the centrifugal force there was a shift in activity from the "neutral" fraction to the "acid hydrolyzable" fraction, indicating that some components necessary for the biosynthesis of the neutral terpenes may be removed.

TABLE 4.--Distribution of enzymatic activity after various centrifugation treatments.

Centrifugation treatment		Incorporation in dpm		
g Force	Time (min.)	Neutral fraction	Acid hydrolyzable fraction	Total
None (crude)	0	7988	4922	12910
Supernatant				
15,000	30	7208	7643	14851
100,000	60	1887	7710	9597
Pellet				
15,000	30	-	-	-
100,000	60	-	-	-

Effects of Inhibitors

The chemicals AMO-1618, CCC, Iodoacetamide and Phosfon D were tested to determine their effects on the incorporation of MVA-2-^{14}C into terpenes. The general effects of these compounds on the biosynthetic pathway in different plant tissues, are summarized in the literature review.

Standard extraction and incubation were used as described in experimental. Concentration used for each chemical was 1×10^{-3} M.

When compared to the standard complete incubation system little or no inhibitory effects were observed on the incorporation of activity into the "neutral" and into the "acid hydrolyzable" fractions (Table 5).

TABLE 5.--Effects of various chemicals on the incorporation of MVA-2-^{14}C into terpenes.

Chemical	Incorporation in dpm			Total dpm/mg Protein
	Neutral fraction	Acid hydrolyzable fraction	Total	
None	5809	9494	15303	1074
AMO-1618	5395	9266	14661	1029
CCC	5840	9475	15315	1075
Iodoacetamide	5559	8054	13613	955
Phosfon D	5223	10366	15589	1094