TERPENE BIOSYNTHESIS IN CELL-FREE SYSTEMS FROM WEDGWOOD IRIS AND TULIPA GESNERIANA L. AND ITS RELATION TO BULB GROWTH AND DEVELOPMENT

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
GEORGE LESTER STABY
1970

This is to certify that the

thesis entitled

TERPENE BIOSYNTHESIS IN CELL-FREE SYSTEMS FROM WEDGWOOD IRIS AND TULIPA GESNERIANA L. AND ITS RELATION TO BULB GROWTH AND DEVELOPMENT

presented by

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has been accepted towards fulfillment of the requirements for

Doctorate **degree in** Horticulture

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Date September 14, 1970

ABSTRACT

TERPENE BIOSYNTHESIS IN CELL-FREE SYSTEMS FROM WEDGWOOD IRIS AND TULIPA GESNERIANA L. AND ITS RELATION TO BULB GROWTH AND DEVELOPMENT

By

George Lester Staby

This thesis reports the results of two studies. First, a cell-free system for the biosynthesis of terpenes from extracts of Wedgwood Iris was characterized. Second, the relation of the activity of certain terpene-synthesizing enzymes using cell-free extracts of Tulipa Gesneriana L. cvs. Ralph and Elmus and Wedgwood Iris to the growth and development of these bulbs during normal forcing treatments was investigated.

Extracts of precooled Wedgwood Iris shoots incorporated approximately 3.0×10^{-9} moles D-mevalonate (MVA)-$2^{-14}C$/hour/mg protein into neutral terpenes. The system had an absolute requirement for ATP and Mn^{++} and Mg^{++} were stimulatory. Mn^{++} was more stimulatory at low (1.25×10^{-3}M) concentrations but inhibitory at high (5.0×10^{-3}M) concentrations compared to Mg^{++}. The optimum temperature for maximal MVA incorporation was approximately 33°C.

Anaerobic conditions slightly stimulated MVA incorporation while the addition of NAD, NADP and/or NADPH slightly inhibited. Sodium fluoride, niacinamide, streptomycin, chloramphenicol, CCC, SK&F 7997-A_3 and AMO 1618 had little or no effect on the total amount of MVA incorporated. Phosfon D and iodoacetamide were highly inhibitory.

Acetate was not incorporated into neutral terpenes even in the presence of reduced pyridine nucleotides. Using MVA-1-^{14}C as the substrate resulted in an immediate release of $^{14}CO_2$. Small but detectable levels of $^{14}CO_2$ were also obtained when MVA-2-^{14}C was used as the substrate, but only after a lag period of approximately 45 minutes.

At least three radioactive neutral terpenes were biosynthesized in iris extracts from MVA-2-^{14}C. Unequivocal identification of these ^{14}C products was not made. Data suggested that one product was prenol-like, possibly farnesol or geranylgeraniol. The biosynthesis of this prenol-like product required soluble enzymes and either Mg^{++} and/or Mn^{++} served as cofactors. The biosynthesis of a more non-polar ^{14}C product required Mn^{++} and microsomes. AMO 1618 inhibited the biosynthesis of this non-polar product.

Some of the prenol-like product was non-covalently associated with TCA precipitable protein. Saponification released some of this radioactivity from the protein and also chemically altered this prenol-like product. This

chemically altered product was more polar than the product prior to saponification.

Campesterol, stigmasterol and β-sitosterol were identified in the iris extracts, however, little or none of these sterols were biosynthesized from MVA-2-^{14}C *in vitro*.

Activity of certain terpene-synthesizing enzymes was related to morphological, physiological and biochemical developments in tulip and iris. With both species, enzyme activity was found to correlate with reported changes in the levels of extractable gibberellin-like substances during low temperature treatments. Also, enzyme activity in tulip was correlated with pollen development and with flower initiation and development and respiration in iris.

Stamen tissue in tulip accounted for 95% of the total enzyme activity with the remaining activity in fleshy scales. With iris, shoot, scale and root tissue provided 73, 18 and 9%, respectively.

Farnesol was tentatively identified as one of the neutral terpenes biosynthesized in the tulip cell-free system.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

1970

ACKNOWLEDGMENTS

Numerous people have supported me and my efforts in a variety of ways.

To Dr. A. A. De Hertogh, my major professor--for support, suggestions and especially for allowing me to do original research;

To Drs. M. J. Bukovac, D. R. Dilley, I. W. Knobloch and C. J. Pollard, my committee members--for their teachings and friendship;

To Dr. A. L. Kenworthy--for being a graduate student's best friend;

To Dr. L. H. Aung--for his advice, encouragement and friendship;

To Drs. A. A. Alpi, T. E. Ferrari and T. J. Johnson--for being helpful in a variety of ways;

To Mr. N. A. Blakely--for his relentless help;

To Drs. P. Markakis, S. K. Ries, C. C. Sweeley and W. W. Wells--for the use of their equipment and for their advice;

To my wife Kathy and to my mother and father--for solving the problems that nobody else could--

Thank You.

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AMO 1618	2-isopropyl-4-(trimethylammonium chloride)-5-methyl phenyl piperidine-1-carboxylate
ATP	Adenosine-5-Triphosphate
BSA	Bovine Serum Albumin
CCC	(2-Chloroethyl)-trimethylammonium chloride
DBED	Dibenzoylethylenediamine
diMeallyl-PP	3,3-Dimethylallyl-Pyrophosphate
EDTA	Ethylenediamine Tetraacetic Acid Tetrasodium Salt
F-PP	Farnesyl-Pyrophosphate
GLC	Gas Liquid Chromatography
G-PP	Geranyl-Pyrophosphate
GG-PP	Geranylgeranyl-Pyrophosphate
I-P	Isopentenyl-Phosphate
I-PP	Isopentenyl-Pyrophosphate
MVA	Mevalonic Acid
MVA-P	Mevalonate-5-Phosphate
MVA-PP	Mevalonate-5-Pyrophosphate
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide, reduced form
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, reduced form

Phosfon D	Tributyl-2,4-Dichlorobenzylphosphonium Chloride
Phosfon S	Tributyl-2,4-Dichlorobenzylammonium Chloride
POPOP	1,4-bis-(2-(4-Methyl-5-Phenyloxazolyl))-Benzene
PPO	2,5-Diphenyloxazole
SK&F 7997-A_3	Tris-(2-Diethylaminoethyl)-phosphate trihydrochloride
TCA	Trichloroacetic Acid
TLC	Thin-Layer Chromatography
TMSi	Trimethylsilyl
Tris	Tris (Hydroxymethyl) Aminomethane
Triton-X 100	p-isooctylpolyoxyethylenephenol polymer

GENERAL INTRODUCTION

Spring-flowering bulbs require specific temperature sequences to ensure proper growth and development. The temperature sequences and the resulting effects differ depending on the bulb species and/or cultivar and whether the bulb is to be used for forcing or bulb production. For example, warm (17-23°C) followed by low (5-9°C) temperatures were necessary for flower initiation and development with tulip* (Luyten et al., 1926), while a high (35°C)-low-warm temperature sequence was optimum for flowering of iris* (Blaauw, 1941; Hartsema and Luyten, 1955).

In bulbs, flower initiation and development and floral stalk elongation were shown to be partially influenced by low temperatures (Blaauw, 1941; Hartsema and Luyten, 1955; Luyten et al., 1926; Rodrigues Pereira, 1962). Gibberellins have been effective in either replacing or supplementing low temperature requirements for flowering and elongation growth with many plant species (McComb, 1967; Lang, 1957, 1965; Suge and Rappaport, 1968; Wittwer and Bukovac, 1957). Therefore, the isolation of

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*Unless otherwise noted, the names tulip and iris refer to Tulipa Gesneriana L. cvs. and Wedgwood Iris, respectively.

extractable gibberellin-like substances (GAs) in bulbs (Aung and De Hertogh, 1967, 1968; Aung et al., 1969a, 1969b, 1970; Barendse et al., 1970; Einert et al., 1970a, 1970b; Halevy, 1970; Rodrigues Pereira, 1965) and the acceleration of iris flowering by exogenously applied gibberellic acid (Halevy and Shoub, 1964a), suggested that gibberellins were also involved in bulb flowering and/or floral stalk elongation. GAs levels were reported to change in tulip (Aung and De Hertogh, 1967; Aung et al., 1969b) and iris (Rodrigues Pereira, 1964) during forcing treatments. These changes in GAs levels have been related to flowering in iris (Rodrigues Pereira, 1964, 1966) and to floral stalk elongation in tulip (Aung et al., 1969b).

The present study was undertaken to determine: (1) Are changes in the quantity of GAs in tulip and iris during forcing treatments a response to changes in their biosynthetic rates in situ? (2) What tissues biosynthesize gibberellins in tulip and iris?

Cell-free systems which incorporate MVA-2-^{14}C into terpenes, the class of compounds which include gibberellins, have been demonstrated with several higher plant tissues (i.e., Anderson and Moore, 1967; Graebe, 1968; Upper and West, 1967). The use of a similar system with bulb tissue could be of assistance in determining: (1) the relative activities of terpene-synthesizing enzymes during

forcing, and (2) which tissues contain these enzymes. The results obtained could then be correlated with known levels of GAs and with other morphological, physiological and biochemical developments of iris and tulip. A similar study has been reported (Coolbaugh and Moore, 1969).

At the onset of this study, it was found that the iris cell-free system was very active in biosynthesizing terpenes. As a result of this finding, a second study was initiated to characterize this iris cell-free system with the purpose of obtaining more information on the biosynthesis of terpenes in plants. Part I of this dissertation concerns this second study.

Part II of this dissertation concerns experiments with cell-free extracts of Tulipa Gesneriana L. cvs. Ralph and Elmus and Wedgwood Iris which attempted to determine: (1) the relative activities of terpene-synthesizing enzymes during normal forcing treatments, and (2) which tissues contained these enzymes.

PART I

INTRODUCTION

Widely distributed throughout the plant and animal kingdom there exists a group of compounds known collectively as terpenes. Haagen-Smit (1948) defined terpenes as "all compounds which have a distinct architectural and chemical relation to the simple C_5H_8 (isoprene) molecule . . ." Classically, terpenes are grouped as follows:

hemiterpenes	C_5H_8
monoterpenes	$C_{10}H_{16}$
sesquiterpenes	$C_{15}H_{24}$
diterpenes	$C_{20}H_{32}$
triterpenes	$C_{30}H_{48}$
tetraterpenes	$C_{40}H_{64}$
polyterpenes	$(C_6H_8)_x$

Examples of a few important terpenes found in plants are: gibberellins, abscisic acid, steroids, phytol, carotenes and rubber. It is also possible that the side-chain of zeatin is a terpene.

Much of the fundamental knowledge on the biogenesis of terpenes was due to the efforts of Ruzicka (1953) and his co-workers. From his and other investigations (see references cited by Bloch, 1965; and Heftmann, 1970), it was determined that all terpenes had common biosynthetic intermediates.

In Bloch's (1965) review of cholersterol biosynthesis in animals, he referred to the work of Bucher (1953) for the preparation of liver homogenates as a "technique which proved invaluable in all subsequent studies on sterol biogenesis." It was from Bucher's (1953) efforts that liver cell-free systems were developed which were capable of biosynthesizing cholesterol and other terpenes.

Similar cell-free systems for the biosynthesis of terpenes have been prepared from plant tissues. From these studies, much information has been obtained on terpene biogenesis in plants. The present study examined the characteristics of a cell-free system using Wedgwood Iris as a source of enzymes for the biosynthesis of terpenes. It was thought that with the information gained from this study, future experiments using the iris cell-free system could be developed to better understand terpene biogenesis in plants.

LITERATURE REVIEW

The review of literature on cell-free systems* is divided into two sections: (A) an historical review of cell-free systems, and (B) a summary of certain characteristics of cell-free systems.

History of Plant Cell-Free Systems Which Biosynthesize Terpenes

In 1959, seven years after Rabinovitz and Greenberg (1952) developed the first cell-free system using fetal rat liver homogenates for the biosynthesis of cholesterol, a cell-free system for the biosynthesis of terpenes was obtained from plant extracts. In this study, Shneour and Zabin (1959) used Lycopersicon esculentum (tomato) plastids as a source of enzymes for the biosynthesis of lycopene from MVA. Various unidentifiable terpenes were also produced from this cell-free system, including digitonin precipitable terpenes (possibly sterols).

Subsequently, a paper by Braithwaite and Goodwin (1960) described the incorporation of acetate and MVA into β-carotene by cell-free extracts of Daucus Carota L. (carrot) roots.

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*Unless otherwise noted, 'cell-free systems' are in reference to systems capable of biosynthesizing terpenes using enzymes extracted from higher plants.

In the following year, Decker and Uehleke (1961) reported the formation of β-carotene from lycopene using chloroplastic fractions of tomato fruits. Also, Henning et al. (1961) reported the incorporation of I-PP into rubber using latex from Hevea brasiliensis (rubber) while Anderson and Porter (1961) used cell-free extracts from carrot for the biosynthesis of phytoene from MVA.

In 1962, Anderson and Porter showed that terpenyl-pyrophosphate intermediates from a rat liver cell-free system were incorporated into phytoene and other carotenes by carrot and tomato cell-free systems. These results indicated that animal and plant terpene biosynthetic pathways have certain common intermediates. Varma and Chichester (1962) used tomato plastid homogenates for the incorporation of I-PP into lycopene. Also, Beeler and Porter (1962) showed the enzymatic conversion of phytoene to phytofluene with tomato plastids.

In the following year, Beeler et al. (1963) reported that carrot and tomato cell-free systems biosynthesized squalene from F-PP-4,8,12-^{14}C and MVA-2-^{14}C. Thus, this important precursor of sterols was biosynthesized using a plant cell-free system, an accomplishment later to be substantiated by Graebe (1968) using Pisum sativum (pea) fruits as the source of enzymes.

Archer et al. (1963) used a cell-free preparation from rubber and obtained results supporting some of the

earlier suggestions of Bonner (1949) and others as to the pathway of rubber biosynthesis. They showed that MVA and I-PP were incorporated into this polyterpene.

Interest in isolating and characterizing specific enzymes involved in the biosynthesis of terpenes increased in 1963-64. Using carrot root tissue, Nandi and Porter (1964) isolated and partially characterized GG-PP synthetase. Loomis and Battaile (1963) isolated MVA kinase enzymes from many plant species.

A significant series of investigations began appearing in 1965 on the biosynthesis of gibberellins and related diterpenes. West and his co-workers used cell-free extracts of Echinocystis macrocarpa Greene (wild cucumber) (Graebe et al., 1965; Upper and West, 1967; Dennis and West, 1967; West and Upper, 1969; Oster and West, 1968; Murphy and West, 1969) and Ricinus communis (castor bean) (Robinson and West, 1969a, 1969b) to provide much information concerning the biogenesis of these diterpenes as well as characterizing various properties of cell-free systems per se.

The initial cell-free data published concerning terpene biogenesis consisted mainly of identifying final products from radioactive precursors. However, during 1966, specific intermediates were reported. Using Pinus radiata (Monterey pine) Valenzuela et al. (1966a) showed that MVA-PP and I-PP were intermediates in the biosynthesis

of α-pinene. Pollard et al. (1966) reported that these same intermediates plus MVA-P and diMeallyl-PP were present and radioactive in a cell-free system using Alaska pea fruits with MVA-2-^{14}C as the substrate. Thus, some important knowledge was obtained on the step-wise metabolism of MVA in higher plants.

From 1966 to the present, many papers were published on plant cell-free systems capable of biosynthesizing terpenes. These and other cell-free systems are summarized in Table 1.

Characteristics of Plant Cell-Free Systems Which Biosynthesize Terpenes

Substrates

MVA was the most common substrate used for the biosynthesis of terpenes in cell-free systems. Most studies assumed that any products arising from MVA were terpenes (Pollard et al., 1966). MVA has been used in the form of the free acid (Archer et al., 1963; Braithwaite and Goodwin, 1960; Charlton et al., 1967; Graebe, 1967, 1969; Pollard et al., 1966; Potty and Bruemmer, 1970a; Valenzuela et al., 1966a, 1966b), lactone (Beeler et al., 1963; Graebe, 1968), and as a sodium (Anderson and Moore, 1967), potassium (Henning et al., 1961; Loomis and Battaile, 1963) or DBED salt (Beeler et al., 1963; Beytia et al., 1969; George-Nascimento et al., 1969; Graebe et al., 1965;

TABLE 1.--Summary of cell-free systems from higher plants as enzyme sources for the biosynthesis of terpenes.

Plant Species	Enzyme Source	Precursor	Form of Precursor	Products	References
Brassica oleracea	chloroplasts	(-)-17-^{14}C-kaurenoic acid	free acid	gibberellin-A_3	Stoddart (1969)
Citrus sinensis	fruit	MVA-2-^{14}C geraniol	free acid free form	MVA-P, MVA-PP geranial	Potty and Bruemmer (1970a, 1970b)
Cucurbita pepo	seedling	MVA-2-^{14}C	potassium salt	MVA-P, MVA-PP	Loomis and Battiale (1963)
C. pepo	seedling	MVA-2-^{14}C	free acid	kaurene	Graebe (1969)
Daucus Carota L. var. *sativa*	plastids and roots	a.	free form	phytoene	Anderson and Porter (1961)
D. Carota	roots	MVA-2-^{14}C Acetate-2-^{14}C	free acid free acid	β-carotene into other unidentified terpenes	Braithwaite and Goodwin (1960)
D. Carota	roots	I-PP-4-^{14}C	free form	GG-PP	Nandi and Porter (1964)
D. Carota	plastids	MVA-2-^{14}C F-PP-4,8,12-^{14}C	DBED salt free form	squalene	Beeler *et al.* (1963)
D. Carota	plastids	a.	free form	phytoene	Anderson and Porter (1962)
Echinocystis macrocarpa Greene	endosperm	MVA-2-^{14}C	DBED salt	kaurene, trans geranyl-geraniol, kaurene-19-ol, farnesol, squalene	Graebe (1965), West and Upper (1969), Oster and West (1968)
E. macrocarpa	endosperm	GG-PP-2-^{14}C	free form	kaurene	Upper and West (1967); West and Upper (1969)
E. macrocarpa	endosperm	kaurene-^{14}C	free form	kaurene-19-oic acid, kaurene-7 ol-19-oic acid	Dennis and West (1967), West and Upper (1966), West *et al.* (1967)
E. macrocarpa	embryo	MVA-2-^{14}C	DBED salt	squalene, farnesol	Graebe *et al.* (1965)
Hevea brasiliensis	latex	I-PP-1-^{14}C	potassium salt	rubber	Henning *et al.* (1961)
H. brasiliensis	latex	MVA-2-^{14}C MVA-1-^{14}C	free acid free acid	MVA-P, I-P, I-PP	Chesterton and Kekwick (1968)

TABLE 1.--continued.

Plant Species	Enzyme Source	Precursor	Form of Precursor	Products	References
H. brasiliensis	latex	MVA-2-^{14}C I-PP-^{14}C	free acid	rubber, MVA-P, I-P, I-PP	Archer et al. (1963)
Lilium longiflorum Thunb.	flower	MVA-2-^{14}C	DBED salt	unidentified	Staby and De Hertogh (1969)
Lycopsicon esculentum	fruit plastids	MVA-2-^{14}C	DBED salt	lycopene, plus unidentified terpenes (sterols?)	Shneour and Zabin (1959)
L. esculentum	fruit plastids	lycopene	free form	β-carotene	Decker and Uehleke (1961)
L. esculentum	fruit plastids	I-PP	free form	lycopene, plus unidentified terpenes (sterols?)	Varma and Chichester (1962)
L. esculentum	fruit plastids	phytoene	free form	phytofluene	Beeler and Porter (1962)
L. esculentum	fruit plastids	MVA-2-^{14}C MVA-2-^{14}C F-PP-4,8,12-^{14}C	DBED salt lactone free form	squalene	Beeler et al. (1963)
L. esculentum	fruit plastids	GG-PP-^{14}C	free form	phytoene	Shah et al. (1968)
L. esculentum	fruit plastids	a.	free form	phytoene, carotenes	Anderson and Porter (1962)
L. esculentum	fruit plastids	I-PP, F-PP	free form	phytoene	Jungalwala and Porter (1965, 1967)
Pinus radiata	needles	MVA-2-^{14}C	free acid	MVA-PP, I-PP	Valenzuela et al. (1966a, 1966b)
P. radiata	needles	MVA-2-^{14}C	DBED salt	nerol, geraniol, G-PP, isopentenol, neranyl-PP, diMeallyl-alcohol	Beytia et al. (1969); George-Nascimento (1969)
Pisum sativum	fruit	MVA-2-^{14}C	lactone	kaurene, squalene,	Graebe (1968)
P. sativum	fruit	MVA-2-^{14}C	sodium salt	kaurene	Anderson and Moore (1967)
P. sativum	plastids	MVA-2-^{14}C	free acid	farnesol, squalene, gerenylgeraniol	Graebe (1967)

TABLE 1.--continued.

Plant Species	Enzyme Source	Precursor	Form of Precursor	Products	References
P. sativum	seedlings	MVA-2-^{14}C	free acid	geraniol, farnesol, I-PP nerolidol, diMeallyl-PP, MVA-P, MVA-PP, plus higher prenols	Pollard et al. (1966)
Phaseolus vulgaris	chloroplasts	MVA-2-^{14}C	free acid	phytoene, squalene, sphtol	Charlton et al. (1967)
Pseudotsuga menziesii	seedlings	MVA-2-^{14}C	potassium salt	MVA-P, MVA-PP	Loomis and Battaile (1963)
Ricinus communis L.	seedlings	MVA-2-^{14}C GG-PP-^{14}C	DBED salt free form	beyerene, kaurene, sandar-acopomaradiene, trachyobane	Robinson and West (1969a, 1969b)
R. communis L.	seedlings	GG-PP-^{14}C	free form	kaurene plus other diterpenes	West et al. (1968)
Spinacia oleracea	seedlings	MVA-2-^{14}C	potassium salt	MVA-P, MVA-PP	Loomis and Battaile (1963)
Tulipa Gesneriana L.	flowers	MVA-2-^{14}C	DBED salt	unidentified	Staby and De Hertogh (1969)
Wedgwood Iris	shoots	MVA-2-^{14}C	DBED salt	unidentified	Staby and De Hertogh (1969)
Zea Mays	seedlings	MVA-2-^{14}C	DBED salt	possibly kaurene	West and Upper (1969)
Nicotiana Tabacum	leaves	MVA-2-^{14}C	lactone	F-PP, squalene	Benveniste et al. (1970)
Mentha piperifa	leaves	geraniol	free form	G-P, G-PP	Madyastha and Loomis (1969)

[a]Isolated ^{14}C-PP terpene intermediates from rat liver cell-free system previously fed with MVA-2-^{14}C.

Murphy and West, 1969; Oster and West, 1968; Robinson and West, 1969a, 1969b; Shneour and Zabin, 1959; Staby and De Hertogh, 1969; West and Upper, 1969). Although Popjak (1959) indicated that the lactone form of MVA was inactive in liver cell-free systems for the biosynthesis of cholesterol, data obtained with peas (Graebe, 1968), tomato and carrot (Beeler et al., 1963) cell-free systems showed this form to be an effective substrate for the biosynthesis of kaurene, squalene and phytoene. Also, Anderson and Porter (1962) showed that the lactone form served as a substrate of terpenyl-pyrophosphates in rat liver cell-free systems.

Though acetate was incorporated into terpenes using liver cell-free systems (Bucher, 1953; Rabinovitz and Greenberg, 1952; and others), it was incorporated into terpenes in only two plant systems. Acetate was converted into β-carotene (Braithwaite and Goodwin, 1960) and lycopene (Schneour and Zabin, 1959) using carrot root and tomato fruit, respectively, as sources of enzymes. Besides acetate and MVA, various terpenic intermediates, e.g. I-PP (Table 1) were incorporated into terpenes using plant extracts.

Buffers and pH

Tris and phosphate buffers were used almost exclusively as the extraction and incubation media. However, some buffers inhibited enzyme activity. For example, Tris

buffer inhibited the conversion of I-PP and F-PP to phytoene (Jungalwala and Porter, 1967) while phosphate buffer inhibited the production of lycopene (Shneour and Zabin, 1959), using extracts of tomato fruit.

In most cell-free systems, the pH which resulted in maximal product formation was approximately 7.0. However, optimal pH's have been reported to be as low as 5.0 using Citrus sinensis (orange)fruit (Potty and Bruemmer, 1970a) and as high as 8.2 with peas (Pollard et al., 1966), and bulb species (Staby and De Hertogh, 1969).

The optimum pH values for some individual enzymes involved in the biosynthesis of terpenes were: 6.5 and 5.8 for MVA kinases from orange (Potty and Bruemmer, 1970) and Cucurbita pepo (pumpkin) (Loomis and Battaile, 1963), respectively and 6.8 for GG-PP synthetase isolated from carrot root (Nandi and Porter, 1964). In extracts of pea fruits, Graebe (1968) reported an optimum pH value of 7.0 for squalene and phytoene production while the optimum pH for kaurene biosynthesis was 6.0. pH's higher than 7.5 greatly reduced the production of squalene and kaurene while little effect was noted on phytoene formation. Shneour and Zabin (1959) reported a pH value of approximately 8.0 resulted in high rates of lycopene production using extracts of tomato fruits.

Incubation Time

Incubation periods varied from a few minutes to 24 hours. Increases in products formed from MVA were approximately linear over the various times tested for kaurene (Anderson and Moore, 1967; Robinson and West, 1969b), phytoene (Anderson and Porter, 1962; Charlton et al., 1967) and for various diterpenes (Robinson and West, 1969b) as well as for the conversion of F-PP and I-PP into phytoene (Jungalwala and Porter, 1967) and GG-PP (Nandi and Porter, 1964) by extracts of various plant species.

Chesterton and Kekwick (1968), using rubber extracts, confirmed the earlier work of Pollard et al. (1966) as to the sequential production of MVA-P to MVA-PP to I-PP by time course studies. Terpenyl-PP were shown to precede phytoene production with tomato fruits (Jungalwala and Porter, 1967). Upper and West (1967), Dennis and West (1967), West and Upper (1969), Oster and West (1968), and Murphy and West (1969) showed the formation of kaurene-7βol-19-oic acid from kaurene using cucumber extracts. The sequence of formation was determined to be as follows: A protein bound C_{20} prenol was biosynthesized first followed by free kaurene. Kaurene was then quickly oxidized to kaurenol and then less rapidly to kaurenal. The reactions from kaurenal to kauren-7βol-19-oic acid via kauren-19-oic acid were shown to proceed at much slower rates.

Incubation Temperature

The most frequently used incubation temperature for the biosynthesis of terpenes from MVA was 25°C with a range from 23° (Pollard et al., 1966) to 40°C (Henning et al., 1961). Using tomato plastids as a source of enzymes, Shah et al. (1967) showed 25°C to be optimum for the conversion of GG-PP to phytoene. Temperatures of 30°C and above greatly or completely inhibited this conversion.

Aerobic vs Anaerobic Incubation

The biosynthesis of phytoene was either enhanced (Anderson and Porter, 1962; Charlton et al., 1967) or not affected (Jungalwala and Porter, 1967) by anaerobic nitrogen conditions. Squalene production under nitrogen was stimulated in tomato and carrot cell-free extracts (Beeler et al., 1963). However, no lycopene formation took place under similar conditions using the same enzyme source (Varma and Chichester, 1962). Graebe (1968) stated (no data presented) that anaerobic conditions increased yields of kaurene, squalene and phytoene with peas. The enzymatic conversion of phytoene to phytofluene in tomato plastids did not require oxygen (Beeler and Porter, 1962) and MVA kinases are not sensitive to oxygen with pumpkin extracts (Loomis and Battaile, 1963).

The participation of mixed function oxidases in kaurene metabolism necessitated that molecular oxygen be

involved (West et al., 1968, Murphy and West, 1969). Molecular oxygen was specifically required for the conversion of kaurene to kaurenol and kaurenol to kauren-19-oic acid using extracts of cucumber. The addition of $^{18}O_2$ to these cell-free extracts resulted in the label being incorporated into the oxidized derivatives of kaurene.

Energy Source

Most cell-free studies used ATP as the energy source for conversion of MVA into terpenes. The nucleotide triphosphates CTP, ITP, UTP and GTP were less effective than ATP, mole per mole (Loomis and Battaile, 1963; Potty and Bruemmer, 1970) while Pollard et al. (1966) showed that CTP could completely substitute for ATP. An ATP generating system using phosphoenol pyruvate and phosphokinase completely substituted for ATP with pumpkin cell-free systems (Loomis and Battaile, 1963).

The concentration of ATP used per assay varied depending on the particular investigation. Loomis and Battaile (1963) reported that excess ATP resulted in complexes between the added ATP and divalent cations, thus, some of the added ATP was inactivated. Beytia et al. (1969) showed that a ATP/divalent cation ratio of 1.67 resulted in maximal incorporation of MVA into various terpenes with Monterey pine extracts.

Divalent Cations

The effects of divalent cations, especially Mg^{++} and Mn^{++}, on the quality and quantity of terpenes biosynthesized in cell-free systems have been well documented (Anderson and Moore, 1967; Beytia et al., 1969; Graebe, 1968; Jungalwala and Porter, 1967; Loomis and Battaile, 1963; Nandi and Porter, 1964; Upper and West, 1967). As measured by the total amount of terpenes produced from MVA, Mn^{++} stimulated more than Mg^{++} at low concentrations while Mn^{++} was more inhibitory than Mg^{++} at high levels (Anderson and Moore, 1967; Beytia et al., 1969; Graebe, 1968; Jungalwala and Porter, 1967; Loomis and Battaile, 1963; Nandi and Porter, 1964; Shneour and Zabin, 1959). However, with cell-free extracts of cucumber the reverse was true (Upper and West, 1967).

Mn^{++} and Mg^{++} also influenced the qualitative nature of the terpenes biosynthesized. The addition of Mn^{++} selectively stimulated phytoene (Graebe, 1968; Shah et al., 1968) and GG-PP (Nandi and Porter, 1964) production in pea and tomato and carrot extracts, respectively. The presence of Mg^{++} stimulated the biosynthesis of squalene in pea (Graebe, 1968) and carrot (Nandi and Porter, 1964) cell-free systems. However, Jungalwala and Porter (1967) showed that both Mn^{++} and Mg^{++} were needed for the conversion of I-PP to phytoene while only Mg^{++} was required when GG-PP was the precursor using tomato

extracts. Using Phycomyces blakesleenus R_1 mutant, Lee and Chichester (1969) supported these findings of Jungalwala and Porter (1967).

Pyridine Nucleotides

While many investigators routinely added pyridine nucleotides to the incubation media, few reports described the effects of these compounds on product formation. Beeler et al. (1963) working with carrot and tomato extracts and Graebe (1968) using pea fruit both showed that reduced pyridine nucleotides, especially NADPH, were necessary for squalene biosynthesis. These findings were in agreement with data using mammalian liver homogenates for the biosynthesis of squalene (i.e., Krischna et al., 1966). Dennis and West (1967), Murphy and West (1969), and West and Upper (1969) reported that NADPH was required for the oxidation of kaurene to kauren-7βol-19oic acid with wild cucumber extracts.

With carrot (Anderson and Porter, 1962) and tomato plastid preparations (Anderson and Porter, 1962; Shah et al., 1968), NAD and NADP served as cofactors for the biosynthesis of phytoene. However, with Phaesolus vulgaris (bean) var. Lightning, phytoene production was not influenced by either of these two cofactors (Charlton et al., 1967). Pollard et al. (1966) reported (no data presented) that NAD and NADH were not required for the biosynthesis of various low molecular weight terpenes from

MVA using extracts of pea seedlings. The biosynthesis of lycopene from MVA (Shneour and Zabin, 1959) and the conversion of phytoene to phytofluene (Beeler and Porter, 1962) using tomato plastid cell-free extracts required NADP for maximal activity.

Inhibitors

Potassium fluoride and sodium fluoride were used to inhibit phosphatase activity normally present in many cell-free extracts. Anderson and Porter (1962) reported no increase in phytoene production in the presence of potassium fluoride using carrot plastids and Chesterton and Kekwick (1968) noted no change in the production of MVA-P, MVA-PP and I-PP with rubber extracts with the addition of this same inhibitor. However, potassium fluoride increased the presence of phosphate and pyrophosphate intermediates and decreased the amount of hexane-extractable terpenes using a tomato cell-free system (Jungalwala and Porter, 1967). Similar results were reported with the addition of sodium fluoride to Monterey pine extracts by Beytia et al. (1969) and Valenzuela et al. (1966).

Iodoacetamide specifically inhibited pyrophosphomevalonate decarboxylase and I-PP isomerase in the biosynthesis of terpenes using yeast extracts (Agranoff et al., 1960). The addition of iodoacetamide reduced GG-PP, phytoene (Jungalwala and Porter, 1967) and lycopene (Varma

and Chichester, 1962) production by 100% in tomato extracts; neutral terpene production in pea cell-free systems by 66% (Pollard et al., 1966); and MVA kinase activity by 40% in pumpkin (Loomis and Battaile, 1963). Chesterton and Kekwick (1968) and Oster and West (1968) used rubber and wild cucumber extracts, respectively, and showed an increase in MVA-P production while Potty and Bruemmer (1970a) and Jungalwala and Porter (1967) reported a decrease in MVA-PP in the presence of iodoacetamide using orange and tomato cell-free systems.

AMO 1618 (Dennis et al., 1965; Graebe, 1968; Robinson and West, 1969b) Phosfon S (Dennis et al., 1965) and Phosfon D (Dennis et al., 1965; Robinson and West, 1969b) specifically inhibited kaurene biosynthesis in the plant cell-free systems tested. These inhibitors had little or no effect on phytoene or squalene production (Graebe, 1968). From these and other results, it was generally agreed that these inhibitors were specific for copalyl and kaurene synthetase enzymes (Lang, 1970).

The results using CCC in various cell-free systems were confusing. Dennis et al. (1965) reported no effect on kaurene biogenesis in wild cucumber extracts while Anderson and Moore (1967) and Robinson and West (1969a) used pea and castor bean cell-free systems, respectively, and showed strong CCC inhibitory effects on the production of this diterpene. These differences were possibly due to:

(1) differential enzyme sensitivity to CCC by the various plant species, (2) differences in CCC concentrations used in these studies, or (3) the possibility that CCC acted at more than one site (Anderson and Moore, 1967). In regard to point three, evidence suggests that CCC also inhibits indole metabolism (Kuraishi and Muir, 1963; Moore, 1967; Norris, 1966). Taylor (1966) reviewed indole biogenesis and pointed out that some indole compounds were monoterpene derivatives. Thus, the mode of CCC action seems to be more complex than the cell-free studies indicated.

Sub-cellular Localization of Terpene Biosynthesizing Enzymes

A thorough study of the localization of enzymes capable of terpene biosynthesis on the sub-cellular level has not yet been reported. However, evidence as to these possible sites of biosynthesis are indicated in the literature.

Activities of kaurene synthetase in wild cucumber (Upper and West, 1967) and in pumpkin (Loomis and Battaile, 1963) cell-free extracts were in the 105,000 x g supernatant solution. Other reports also showed the presence of many terpenic enzymes in this soluble fraction using many plant species (i.e., Anderson and Porter, 1962; Charlton et al., 1967; Graebe, 1967). However, Graebe (1968) indicated that some of the enzymes reported to be in this soluble fraction as mentioned above may have

partly or entirely originated from organelles of the intact cell. Graebe (1968) pointed out that extraction procedures used in such studies were too harsh to maintain membrane integrity of the organelles. Therefore, enzymes normally associated with organelles may have contaminated the cytoplasm.

Enzymes necessary for the conversion of kaurene-7βol-19-oic acid to gibberellin (Stoddart, 1969), and for the incorporation of MVA into squalene (Graebe, 1968; Beeler et al., 1963), prenols (Beytia et al., 1969; Graebe, 1967), phytoene (Anderson and Porter, 1962; Graebe, 1968), lycopene (Shneour and Zabin, 1959; Varma and Chichester, 1962), kaurene (Graebe, 1968) and various carotenes (Anderson and Porter, 1962; Braithwaite and Goodwin, 1960) were all reported to be in chloroplasts from a variety of plant sources.

To date, the only active enzymes reported to be associated with microsomes and capable of terpene biosynthesis were those biosynthesizing oxidative derivatives of kaurene using wild cucumber extracts (West and Upper, 1969). In contrast, mammalian liver cell-free systems required the microsome fraction for the biosynthesis of squalene (Krischna et al., 1966; Richards and Hendrickson, 1964).

Enzyme Concentration

Most investigations with plant cell-free systems reported that increasing enzyme concentrations resulted in linear increases in terpenes biosynthesized (i.e., Anderson and Moore, 1967; Potty and Bruemmer, 1970a).

Nature of Terpenes Biosynthesized

Table 1 lists all of the major terpenes biosynthesized using plant cell-free systems. Terpene intermediates have been found to be "bound" to protein (Allen et al., 1967; Barnes et al., 1969; Beytia et al., 1969; Costes, 1966; Jungalwala and Porter, 1967; Oster and West, 1968). These protein-bound terpenes were either suggested or shown to be C_{15} prenols (Beytia et al., 1969), C_{20} prenols (Jungalwala and Porter, 1967), C_{35} - C_{40} prenyl-pyrophosphates (Costes, 1966) or GG-PP (Oster and West, 1968). Partial or complete removal of these terpenes from protein by butanol (Beytia et al., 1969) or acetone (Oster and West, 1968) indicated non-covalent bonding. Similar results were reported by Barnes et al. (1969) using cell-free extracts of Fusarium moniliforme. However, a "non-extractable" terpene fraction remained after acetone extraction with wild cucumber extracts (Oster and West, 1968). This non-extractable terpene was removed by either acid hydrolysis or alkaline phosphatase. The acid hydrolysis technique used in removing the

non-extractable terpene sometimes resulted in a qualitative alteration of this terpene, i.e., protein-bound GG-PP was released as geranyllinalool after acid hydorlysis (Goodman and Popjak, 1960; Nandi and Porter, 1964; Oster and West, 1968).

Terpenyl-pyrophosphate intermediates arising from MVA were often extracted as the free alcohol, and are referred to as prenols. For example, GG-PP can be extracted as geranylgeraniol. Prenols were thought to arise as a result of phosphatases frequently found in plant cell-free extracts (i.e., Beytia et al., 1969; Chesterton and Kekwick, 1968). Alternatively, Graebe (1968) suggested that prenols were a result of being prematurely released from enzymes because of unfavorable condition(s), a situation known to have occurred using pig liver cell-free systems (Krishna et al., 1966).

Regardless of the origin of prenols, they were normally not thought of as precursors of higher terpenes (Graebe, 1965; Lang, 1970; Upper and West, 1967). However, Costes (1966) used etiolated Zea Mays (corn) leaves and showed that geranylgeraniol, and not GG-PP, was the precursor of phytol. van Aller and Nes (1968) showed that geraniol was incorporated into squalene using pea seeds as a source of enzymes. Geraniol was also phosphorylated by enzymes of Mentha piperita (peppermint) (Madyastha and Loomis, 1969) and oxidized to geranial with orange enzymes (Potty and Bruemmer, 1970b).

Effects of Various Compounds on Terpene Biosynthesis

While various compounds were often added to cell-free systems, only a few studies reported on the effect these compounds had on the biosynthesis of terpenes. 2-mercaptoethanol stimulated terpene biosynthesis regardless of the plant species (Beytia et al., 1969; Potty and Bruemmer, 1970a; Robinson and West, 1969b; Upper and West, 1967). EDTA completely inhibited kaurene synthetase activity with wild cucumber cell-free extracts (Upper and West, 1967; West and Upper, 1969). West et al. (1967) showed that dithiothreitol was necessary for the conversion of GG-PP to phytoene using enzymes from tomato plastids, presumably by maintaining the enzyme in the reduced state.

Glutathione stimulated MVA kinase activity in orange (Potty and Bruemmer, 1970) while it inhibited the incorporation of MVA into neutral terpenes using pea seedlings as a source of enzymes (Pollard et al., 1966). With pumpkin, glutathione sometimes inhibited and sometimes stimulated MVA kinase activity (Loomis and Battaile, 1963).

Polyvinylpyrrolidone and ascorbate protected phosphorylating enzymes of orange resulting in an increase production of MVA-P and MVA-PP (Potty and Bruemmer, 1970). Phosphatidylethanolamine increased the rate of MVA incorporation into C_5 and C_{10} prenols using a soluble enzyme system from Monterey pine needles (George-Nascimento et al., 1969).

EXPERIMENTAL

Materials

Current season retarded (vegetative) Wedgwood Iris, size 10 cm, were provided by United Bulb Company, Mount Clemens, Michigan.

Except where specifically noted, reagent grade chemicals were obtained from either Mallinckrodt, St. Louis, Mo., or from J. T. Baker Chemical Co., Phillipsburg, N.J. These chemicals were not purified further.

ATP, NAD, NADH, NADP, NADPH, digitonin, alkaline phosphatase, niacinamide and chloramphenicol were supplied by Sigma Chemical Co., St. Louis, Mo. BSA and Triton X-100 were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio and LaPine Scientific Co., Irvington, N.Y., respectively.

Applied Science Laboratories Inc., State College, Pa. was the source of TMSi reagents and TMSi preparation kits. This company also supplied the GLC stationary and liquid supports mentioned in the text.

Cholesterol, stigmasterol and β-sitosterol were obtained from Analabs Inc., North Haven, Conn., and geraniol, farnesol and nerolidol standards from K and K Laboratories Inc., Hollywood, Calif. Dr. C. A. West, Department of Chemistry, University of California, Los angeles, kindly

provided us with a sample of (-)-kaurene while Dr. D. L. Davis, Department of Agronomy, University of Kentucky, Lexington, provided us with a sample of campesterol.

The sources of terpene inhibitors were: Phosfon D--Mobile Chemical Co., Richmond, Va.; CCC--American Cyanimide Co., Princeton, N.J.: SK&F 7997-A_3--Smith, Kline and French Laboratories, Philadelphia, Pa.; while AMO 1618 and iodoacetamide were gifts of Drs. H. Kende and W. W. Wells of the MSU/AEC Plant Research Laboratory and Department of Biochemistry, Michigan State University, East Lansing, respectively.

Brinkman Instruments Inc., Westbury, N.Y., was the source of silica gel G and aluminum oxide standardized, activity II-III. Prepared TLC plates coated with 0.1 mm silica gel G with fluorescent indicator was purchased from Eastman Kodak Co., Rochester, N.Y.

MVA-1-^{14}C (lactone), MVA-2-^{14}C (DBED salt) and acetate-1-^{14}C were obtained from New England Nuclear Corp., Boston, Mass. The specific activities were 1.54 and 8.6 mc/m mole for MVA-1-^{14}C and acetate-1-^{14}C, respectively. The specific activity for MVA-2-^{14}C varied between 4.7 and 6.5 mc/m mole depending on the sample lot. PPO and POPOP were also obtained from this company.

Methods

Cultural Treatment of Iris

Retarded iris were received at two week intervals. Upon arrival, the iris were placed at 9°C for six weeks prior to use. The low temperature treated iris were used as a source of enzymes for two weeks and then a new lot of similarly treated iris was employed.

Protein Determination

TCA precipitable protein in the cell-free extracts was determined by the Lowry (1951) technique. Briefly, 7.5 ml of 10% TCA was added to 2.5 ml of the cell-free extract and heated for 15 minutes at 80°C. After cooling to 5°C and centrifuging at 30,000 x g for 30 minutes, the supernatant was discarded and the pellet resuspended in 10.0 ml 0.1N NaOH. Depending on the source of the extract, aliquots ranging from 0.05 to 0.2 ml of the NaOH solution were used in the Lowry assay. BSA was used as the protein standard.

Digitonin Precipitation of Sterols

Dried neutral lipid fractions were resuspended in 2.0 ml acetone-ethanol, (v/v). To this was added 0.05 ml 10% acetate and 1.0 ml 0.5% digitonin in 50% absolute ethanol. After allowing this mixture to stand for 12 hours, it was centrifuged for 30 minutes at 30,000 x g.

The pellet was washed 3 times with 5.0 ml ethylether-acetone (2:1). The precipitate containing the sterol-digitonide complex was then resuspended in methanol. Aliquots of this fraction were either counted directly using liquid scintillation techniques or this complex was broken to release the free sterols. To free the sterols, excess pyridine was added at 70°C and allowed to stand at room temperature. Excess ethylether was then added which resulted in the precipitation of the free digitonin leaving the free sterols in solution. The mixture was then centrifuged for 30 minutes at 30,000 x g and the sterols recovered in the supernatant solution.

$^{14}CO_2$ Trapping

$^{14}CO_2$ was trapped using a radiorespirometer (Wang, 1962). Briefly, incubation flasks with sidearms to store the labelled substrates were connected via a three-way stopcock to two CO_2 traps. These traps contained fritted glass inserts through which the $^{14}CO_2$ was passed to the trapping solution. This solution consisted of 10.0 ml absolute ethanol-ethanolamine (v/v). Air or nitrogen was passed at 40 ml/min through the incubation flasks and then channeled to the trapping solution. At designated times, the trapping solution was removed and brought up to 15.0 ml with absolute ethanol. Five ml aliquots were removed and added to 10.0 ml scintilation fluor and counted using liquid scintillation techniques.

Preparation of TMSi Derivatives

Samples to be made trimethylsilyl derivatives were dried *in vacuo* at 40°C prior to the addition of TMSi reagent. The reagent consisted of hexamethyldisilazane, trimethylchlorosilane and pyridine in a ratio of 3:1:9, respectively. The amount of reagent depended on the sample source, but normally 50 µl was used. The reaction was allowed to run for one hour prior to carrying out analyzes. Storage of TMSi derivatives was at 5°C under nitrogen in glass stoppered test tubes.

Liquid Scintillation Counting

Samples soluble in organic solvents were evaporated to dryness *in vacuo* at 60°C and resuspended in 15.0 ml scintillation fluor. The fluor was made by adding 4.0 g POPOP and 100 mg PPO to one liter of toluene.

Aqueous samples were counted using a fluor containing 4.0 g POPOP, 100 mg PPO and 400 ml Triton X-100 per liter of toluene.

All samples were counted for ten minutes on a Parkard Tri-Carb Scintillation Spectrometer Model 3310. Both channels ratio and external standard methods of efficiency determination were used.

Thin-Layer Chromatography

Silica gel G and silver nitrate-impregnated silica gel G chromatograms were made on 5 x 20 cm glass plates to

a thickness of 0.25 mm using a Desaga Brinkmann applicator. The stationary phase was prepared by shaking 30 g silica gel G with 60 ml distilled water for one minute and applying immediately. With the silver nitrate plates, 0.9 g silver nitrate was added to this mixture. Also used were precoated plates of 0.1 mm silica gel G with fluorescent indicator.

All plates were dried for one hour at 100°C prior to use. The distance of the solvent run was 15 cm. The various solvents used are noted in the text.

TLC Radioactive Monitoring

All TLC were monitored for radioactivity on either a Packard Model 7201 Radiochromatogram Scanner System or a Nuclear Chicago Model 1036 Actigraph II. The scanning speed, attenuation and range selection depended on the amount of radioactivity on each plate. The radioactivity on TLC was also monitored by scraping off the R_F zones and placing them in liquid scintillation vials. To these vials, 15.0 ml of scintillation fluor was added for counting.

Column Chromatography

A 11.0 mm I.D. glass column was used in all experiments. The column was packed with 5.0 g aluminum oxide grade II to III. The dried neutral lipid residues from the cell-free studies were resuspended in a minimum volume

of the first eluting solvent. The order of eluting solvents in one series of experiments was 25.0 ml each of 2% ethylether in petroleum ether (A), 12% ethylether in petroleum ether (B), 100% ethylether (C) and 100% methanol (D). According to Stone and Hemming (1967), fractions A to D contain squalene, prenol, sterol and terpene-pyrophosphate intermediates like compounds, respectively. In a second series of experiments, a gradient solvent system ranging from 100% petroleum ether to 100% ethyl ether was used. 100 ml of petroleum and ethyl ether was used. After these two solvents passed through the column, but before the column went dry, a final rinse of 25.0 ml methanol was employed. The eluates were dried *in vacuo* at 40°C and resuspended in the proper scintillation fluor for counting.

Gas Chromatography

A Packard Gas Chromatograph Series 7300 equipped with dual hydrogen flame ionizing detectors was used. All liquid phases were coated on acid-washed and silanized solid supports. The injection and detector temperatures were maintained 10°C above the column temperature. Specific information regarding the various liquid and solid phases, temperatures, flow rates, etc., are noted in the text.

GLC Radioactive Monitoring

A Barber Coleman Radioactivity Monitor Series 5000 connected to an F & M Biomedical Model 400 Gas Chromatograph was used to monitor ^{14}C as it emerged from the gas chromatograph. The combustion-reduction chamber was held at 750°C. Propane was used as the quenching gas. A 6 ft. x 4 mm I.D. glass column packed with 3% OV-1 on 100/120 Chromosorb Q was used. The column temperature for the sample being injected are noted in the text.

Mass Spectrometry

A LKB 900 (LKB Produktor, Stockholm, Sweden) was used. This instrument consists of a gas chromatograph and a single focusing 60° magnetic sector mass spectrometer coupled directly with molecule separators of the Becker-Ryhage type. The gas chromatograph was equipped with a 4 ft. coiled glass column packed with 3% SE-30. The ion source temperature was 270°C while the column and flash heater were maintained at 240 and 250°C, respectively. The mass spectra were recorded at 60 μamp and an accelerating voltage of 3500 volts.

Studies Characterizing the Iris Cell-Free System

Standard Extraction and Assay Procedure

(1) Wedgwood Iris shoot tissue was homogenized at 0°C for two minutes at top speed in a 'Lourdes' mixer using a

0.1M Tris buffer (1:3, wt.:vol.) at pH 8.2 containing 50 µg/ml chloramphenicol; (2) The extract was filtered through cheesecloth and centrifuges for 30 minutes at 15,000 x g; (3) 2.5 ml of the supernatant was added to 1.0 ml Tris buffer, 1 x 10^{-5} moles each of $MnCl_2$, $MgCl_2$ and ATP, and 1.7 x 10^{-8} moles MVA-2-^{14}C (DBED salt) for a total volume of 4.0 ml; (4) The mixture was incubated for one hour at 25°C in a water bath shaker at 150 r.p.m.; (5) The reaction was stopped by adding 4.0 ml absolute ethanol and 0.5 ml 50% KOH and then slowly brought to a boil; (6) After cooling, neutral lipids were extracted twice with 10.0 ml hexane; and (7) The hexane was evaporated to dryness in vacuo at 60°C with the residue resuspended in scintillation fluor for counting.

The above described extraction and cell-free assay procedure is hereafter referred to as the 'standard' assay. Using this standard assay as a basis of comparison, various characteristics of this cell-free system were investigated. The following describes experiments which partially characterized this cell-free system. Each experiment was replciated at least two times over time and three times within experiments.

Experiment 1: Effect of Buffer and pH

Experiments designed to determine the optimal pH and buffer for maximal incorporation of MVA-2-^{14}C into

neutral terpenes (hereafter referred to as 'activity') were of two types. First, equal samples of tissue were homogenized in either 0.1M Tris or phosphate buffer at a pH range from 6.0 to 9.0. To reduce possible variations between samples, a second type of experiment was run. In this case, one large lot of tissue was homogenized in distilled water. After centrifugation, the supernatant was divided into equal portions and each portion adjusted to the prescribed pH with 0.1M Tris or phosphate buffer. Results were measured as the amount of radioactivity in the neutral lipid fraction.

Experiment 2: Effect of Incubation Time

Incubation periods ranging from 5 minutes to 4 hours were used to determine what effect time had on the production of ^{14}C-neutral terpenes from MVA-2-^{14}C. Time course studies were also carried out to measure the $^{14}CO_2$ released from MVA-2-^{14}C and MVA-1-^{14}C. The $^{14}CO_2$ was trapped using a radiorespirometer.

Experiment 3: Effect of Incubation Temperature

Normal extraction and assay procedures were followed. Incubation temperatures of 0, 10, 20, 30, 40, 50 and 60°C were used. Results were obtained by determining the amount of ^{14}C-neutral terpenes biosynthesized.

Experiment 4: Aerobic vs Anaerobic Incubation

Compressed air or nitrogen was passed through radiorespirometer flasks at 40 ml/min. The effects of these conditions were minitored by determining the total amount of ^{14}C-neutral terpenes biosynthesized and by measuring the amount of $^{14}CO_2$ released from MVA-2-^{14}C.

Experiment 5: Effect of Mn^{++} and Mg^{++}

Concentrations ranging from 1.25 to 5.0 x 10^{-3}M of Mn^{++}, Mg^{++} or a mixture of both were tested to determine the possible quantitative and qualitative effects on the ^{14}C-neutral terpenes biosynthesized. Quantitative results were determined by counting the radioactivity in the neutral lipid fraction. Both TLC and column chromotography were used to observe qualitative changes in the ^{14}C-products biosynthesized. The developing and eluting solvents used are noted in the text.

Experiment 6: Effect of ATP

Concentrations of ATP from 1.25 to 5.0 x 10^{-3}M were tested. Results were measured as the radioactivity incorporated from MVA-2-^{14}C into the neutral lipid fraction.

Experiment 7: Effect of Pyridine Nucleotides

Concentrations and combinations of NAD, NADH, NADP and NADPH used are noted in the text. The results were measured as the amount of ^{14}C-neutral terpenes biosynthesized.

Experiment 8: Enzyme Concentration

Enzyme concentrations, as measured by TCA precipitable protein, from 1.8 to 10.5 mg protein per assay were used. Sufficient Tris buffer was added to the incubation flasks to maintain constant volume of 6.0 ml. Results were obtained by determining the amount of ^{14}C-neutral terpenes biosynthesized.

Experiment 9: Substrates

MVA-2-^{14}C (DEBD salt), MVA-1-^{14}C (lactone) and acetate-1-^{14}C were tested to determine which was most effectively utilized by the cell-free extracts. A concentration curve for MVA-2-^{14}C was also determined. The concentration range used was from 0.6 to 9.4 x 10^{-8}M.

When acetate-1-^{14}C was used as the substrate, NADH and/or NADPH were added as sources of reductant power necessary for the conversion of acetate to MVA. The concentrations of the nucleotides differed in each experiment and are noted in the text. In all experiments when comparisons between substrates were studied, equal concentrations were utilized. Results were corrected according

to their specific radioactivities so direct comparisons could be made.

Kinetics of the $^{14}CO_2$ released from MVA-1-^{14}C and MVA-2-^{14}C were also studied. In these experiments, the $^{14}CO_2$ was trapped using a radiorespirometer. After termination, the incubation media was extracted for neutral lipids in the normal manner and the radioactivity in these fractions determined using liquid scintillation techniques.

Experiment 10: Effect of Inhibitors

Various inhibitors were tested to determine their effect on the quantity of ^{14}C-neutral terpenes biosynthesized. The inhibitors and concentrations used are noted in the text. In all experiments, a concentration range of the inhibitor was used, however, only one concentration is reported for each study.

The effects of CCC, Phosfon D, AMO 1618 and iodoacetamide on the quality of ^{14}C-neutral terpenes biosynthesized were also determined. Precoated TLC plates with silica gel G and fluorescent indicator developed in

Experiment 11: Localization of Enzymatic Activity

Relative centrifugal forces of 75, 300, 30,000 and 104,000 x g for 5, 15, 30 and 60 minutes, respectively, were used to prepare the various sub-cellular fractions. The centrifuges used were a Sorvall RC 2-B and an International Preparative Ultracentrifuge Model B-35. After separation, standard assays were run. Quantitative measurements were made by determining the total amount of ^{14}C-neutral terpenes biosynthesized and the amounts of $^{14}CO_2$ released from MVA-1-^{14}C and MVA-2-^{14}C. The products were separated qualitatively using aluminum oxide columns eluted with petroleum ether-ethyl ether-methanol in a gradient manner as previously described.

Results from the above experiments suggested that additional experiments should be run. The requirement of air on the release of $^{14}CO_2$ from MVA-2-^{14}C using 15,000 and 104,000 x g fractions spun for 30 and 60 minutes, respectively, was examined. A nitrogen atmosphere was used as the control. Four hour incubations were run and the amount of $^{14}CO_2$ released measured after 1, 2 and 4 hours using the radiorespirometer.

Experiment 12: Nature of Products

TLC, GLC, column chromatography, gas chromatography-mass spectrometer, and gas chromatograph-^{14}C analyzer techniques were used in an attempt to identify the

^{14}C-neutral terpenes biosynthesized. The specific information regarding these experiments are described in the text.

Saponification and/or acid hydrolysis and protein precipitation experiments also provided information as to the nature of the products. With regard to the saponification experiments, standard assays were run except the KOH was only added to one-half of the extracts after incubation. The ^{14}C-products were then extracted in either hexane or ethylether. Radioactivity determined in these fractions gave the quantitative results. Qualitative results were obtained by using an aluminum oxide column eluted in a gradient manner as previously described.

Concurrent experiments were also run to determine the effect of acid hydrolysis on the amount of ^{14}C-neutral terpenes biosynthesized. After incubation, (+)-saponified extracts were treated with 0.8N HCl at 37°C for one hour. The extracts were then extracted with hexane after adjusting them to the same pH. The amount of radioactivity in the hexane fraction was determined using liquid scintillation techniques.

The volatility of certain ^{14}C-neutral terpenes biosynthesized also provided information concerning their nature. ^{14}C-neutral terpenes were equally distributed on TLC plates coated with silica gel G and developed in benzene-ethyl acetate (9:1). The radioactivity for each

R_F zone was determined using liquid scintillation techniques at 0, 24 and 120 hours. In a second series of experiments, equal aliquots of a ^{14}C-neutral terpene extract were added to scintillation vials. To these vials, scintillation fluor was added directly or after the samples were dried using a stream of nitrogen or *in vacuo* at 60°C.

RESULTS

Experiment 1: Effect of Buffer and pH

Data presented in Table 2 show the effect of 0.1M Tris and phosphate buffers on the incorporation of MVA-2-^{14}C into neutral terpenes. The use of phosphate buffer resulted in an 85% inhibition compared to Tris buffer at pH 8.0. A value of 8.2 was found to be the approximate pH for maximal activity using 0.1M Tris buffer (Figure 1).

Experiment 2: Effect of Incubation Time

Results in Figure 2 show the amount of $^{14}CO_2$ released from MVA-1-^{14}C and the amount of MVA-2-^{14}C incorporated into neutral terpenes over a two hour period. The release of $^{14}CO_2$ was immediate while there was a lag period of approximately seven minutes before ^{14}C-neutral terpenes were detected. The total amount of radioactivity detected in CO_2 was higher than the radioactivity found in the neutral lipid fraction. The production of $^{14}CO_2$ and ^{14}C-neutral terpenes was linear for one hour under the conditions of the experiment.

Experiment 3: Effect of Incubation Temperature

Results presented in Figure 3 indicated that 33°C was the approximate temperature for maximal incorporation

TABLE 2.--Effect of Tris and phosphate buffers, pH 8.0, on the incorporation of MVA-2-^{14}C into neutral terpenes.

Buffer (0.1M)	Incorporation (cpm)
Tris	7037
phosphate	1128

Standard extraction and incubation used as described in experimental except buffers as noted and homogenizing ratio of 1:8.

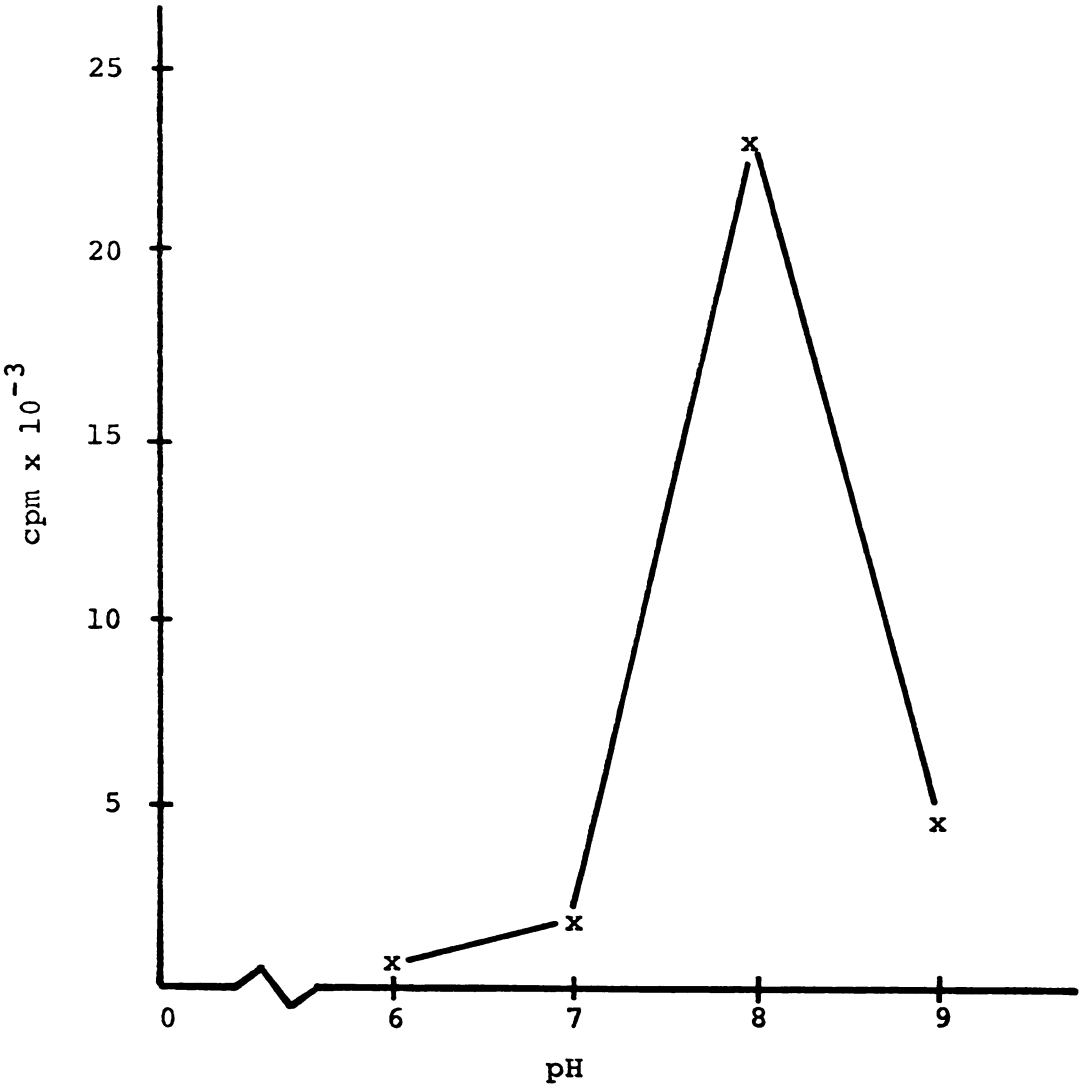

Figure 1.--Effect of pH on the incorporation of MVA-2-^{14}C into neutral terpenes using 0.1M Tris buffer.

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

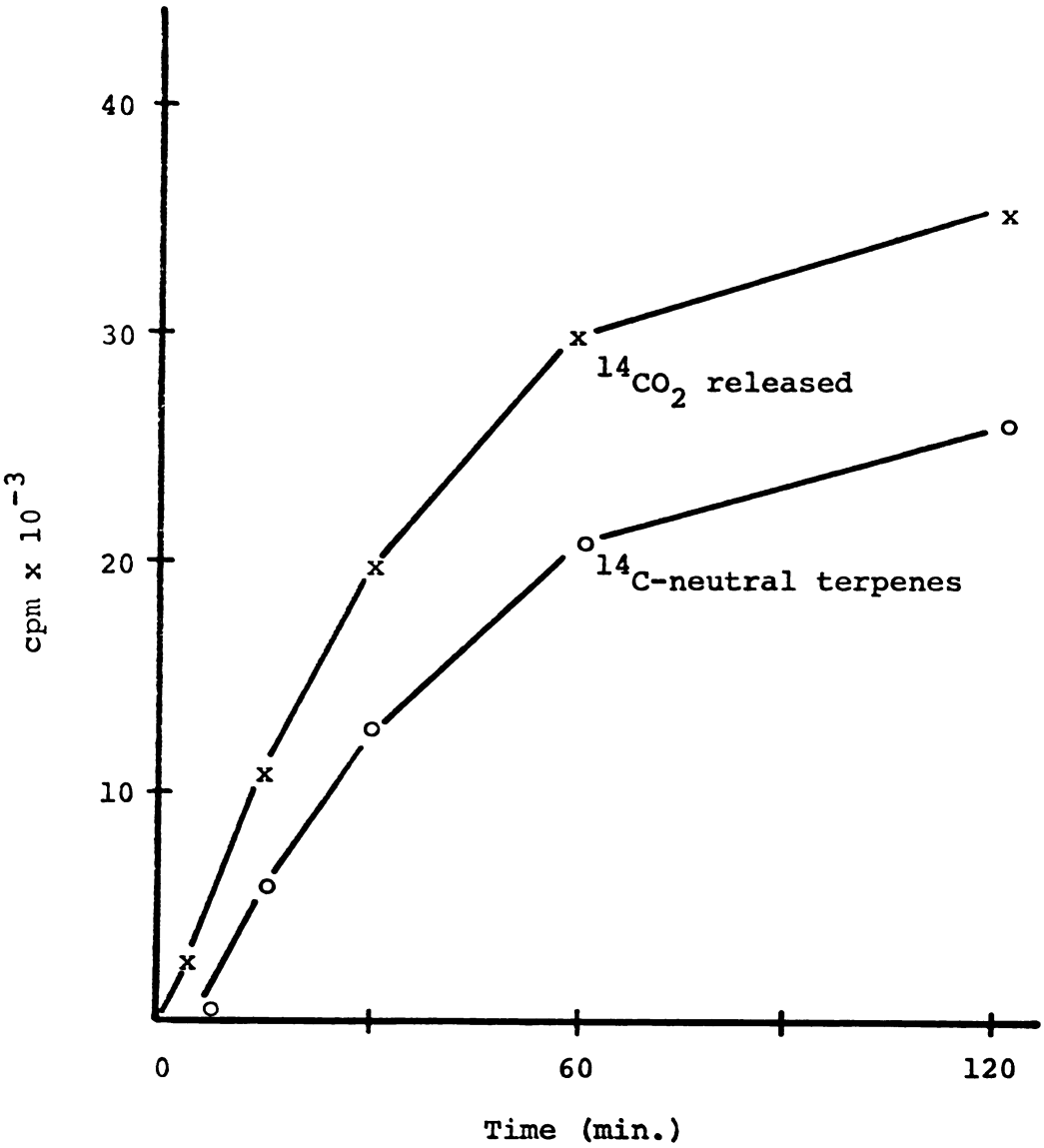

Figure 2.--Effect of time on the incorporation of MVA-2-^{14}C into neutral terpenes and on the release of $^{14}CO_2$ from MVA-1-^{14}C.

Standard extraction and incubation used as described in Experimental except for incubation time and substrate indicated.

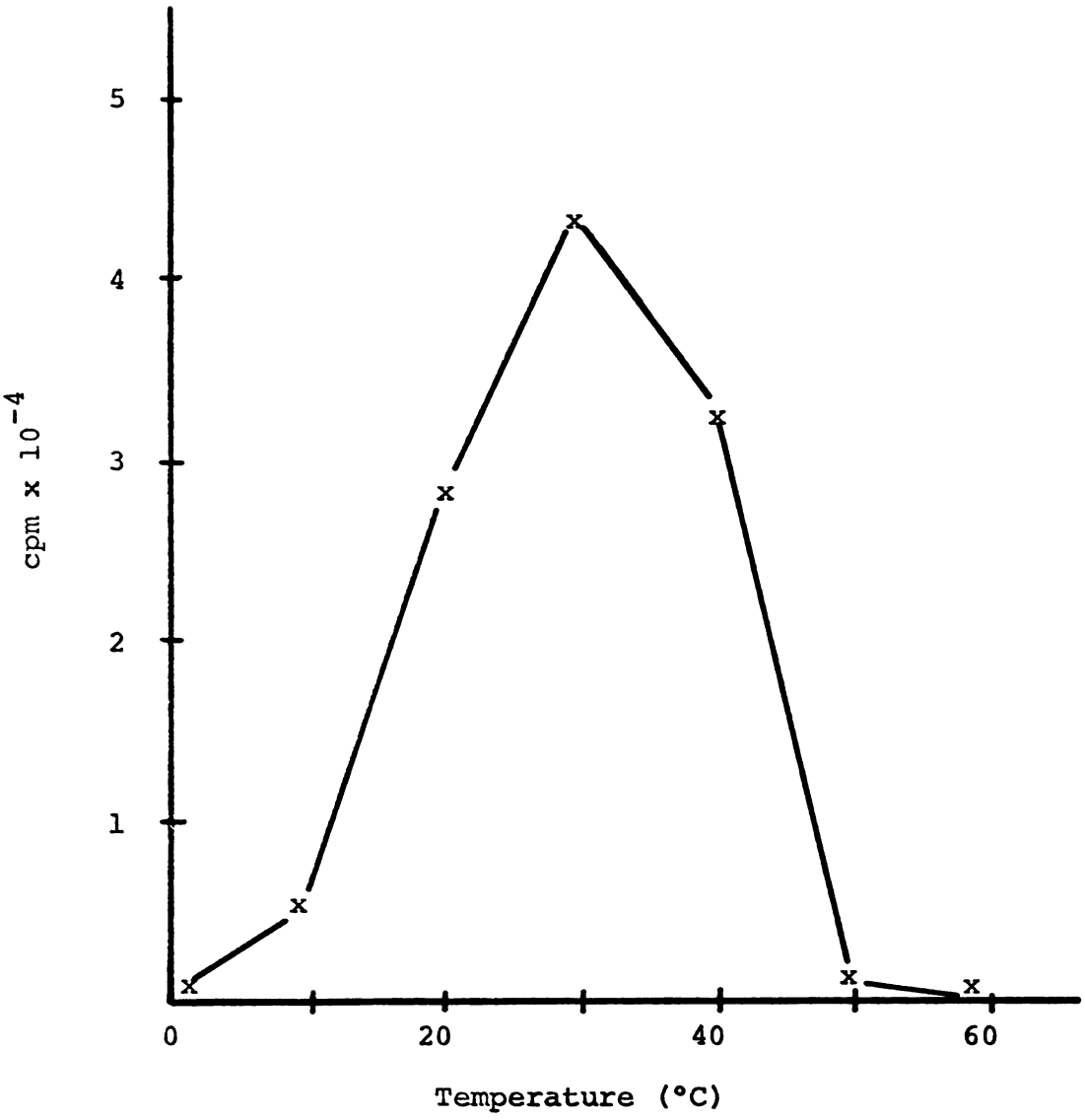

Figure 3.--Effect of temperature on the incorporation of MVA-2-^{14}C into neutral terpenes.

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

of MVA-2-^{14}C into neutral terpenes. Temperatures above 50° or below 10°C either completely or severely inhibited the amount of radioactivity incorporated.

Experiment 4: Aerobic vs Anaerobic Incubation

The data in Table 3 show that anaerobic (nitrogen) incubation conditions increased the incorporation of MVA-2-^{14}C into neutral terpenes by 5%. These same anaerobic conditions also increased the amount of $^{14}CO_2$ released from MVA-2-^{14}C (Figure 4).

Experiment 5: Effect of Mn^{++} and Mg^{++}

Results presented in Table 4 and Figure 5 show the quantitative effects of these cations. Mn^{++} stimulated more incorporation of MVA-2-^{14}C into neutral terpenes at lower concentrations but inhibited when concentrations above 2.5×10^{-3}M were used (Figure 5). On the other hand, Mg^{++} stimulated an approximate linear increase in incorporation up to the highest concentration tested of 5.0×10^{-3}M. A Mn^{++} concentration of 5.0×10^{-3}M resulted in more activity than 2.5×10^{-3}M each of Mn^{++} and Mg^{++} (Table 4).

Qualitative effects of Mn^{++} and Mg^{++} on the types of ^{14}C-neutral terpenes biosynthesized are presented in Table 5 and Figures 6 to 8. Experiments in which aluminum oxide columns were used showed that Mn^{++} stimulated the biosynthesis of less polar neutral terpenes with a

TABLE 3.--Effect of aerobic and anaerobic (nitrogen) incubation conditions on the incorporation of MVA-2-^{14}C into neutral terpenes.

Treatment	Incorporation (cpm)
aerobic	42,756
anaerobic	44,971

Standard extraction and incubation used as described in experimental except where nitrogen replaced air.

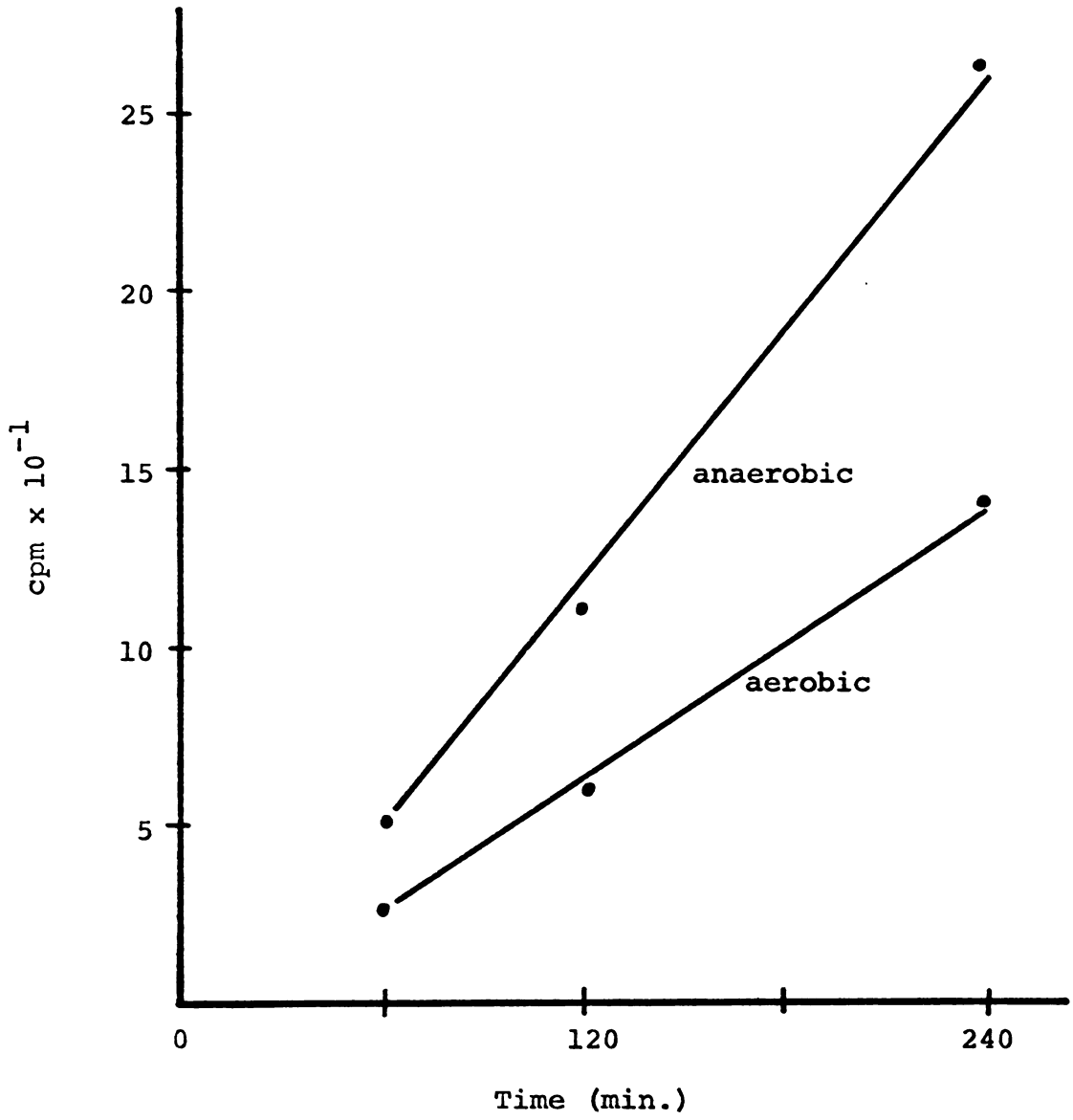

Figure 4.--Effect of anaerobic (nitrogen) and aerobic conditions on the release of $^{14}CO_2$ from MVA-2-^{14}C over time.

Standard extraction and incubation used as described in Experimental except for nitrogen and incubation time indicated.

TABLE 4.--Effect of $MnCl_2$ and $MgCl_2$ on the incorporation of MVA-2-^{14}C into neutral terpenes.

Cation Concentration (x 10^{-3}M)		Incorporation	
$MgCl_2$	$MnCl_2$	(cpm)	(increase over control)
0	0 (control)	6,227	0
0	5	43,819	7.0
5	0	34,176	5.5
2.5	2.5	37,267	6.0

Standard extraction and incubation used as described in Experimental except: no KOH; terpenes extracted with ethyl ether; and cations indicated.

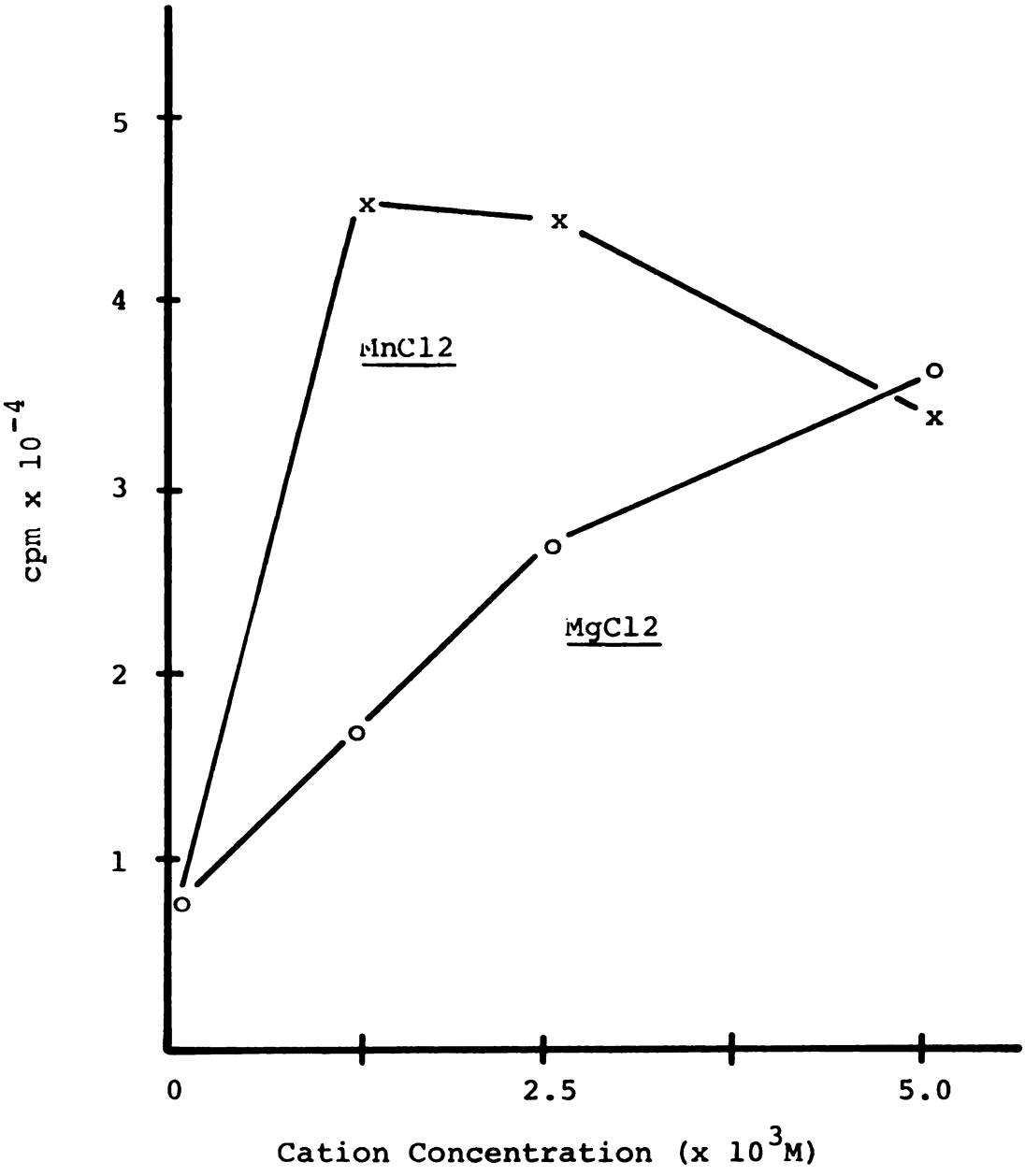

Figure 5.--Effect of $MnCl_2$ and $MgCl_2$ on the incorporation of MVA-2-^{14}C into neutral terpenes.

Standard extraction and incubation used as described in Experimental except for cation concentrations indicated.

TABLE 5.--Effect of $MnCl_2$ and $MgCl_2$ on the quality of neutral terpenes biosynthesized from MVA-2-^{14}C.

Treatment	Incorporation/fraction (%)*		
	A	B	C
$MnCl_2$ (20 µmoles)	10	76	14
$MgCl_2$ (20 µmoles)	2	63	35
$MnCl_2$ + $MgCl_2$ (10 µmoles each)	5	81	14

Standard extraction and incubation used as described in Experimental except for cations as indicated.

*Aluminum oxide column eluted with 2% ether in pet ether (A); 12% ethyl ether in petroleum ether (B) and 100% ethyl ether (C) as described in Experimental.

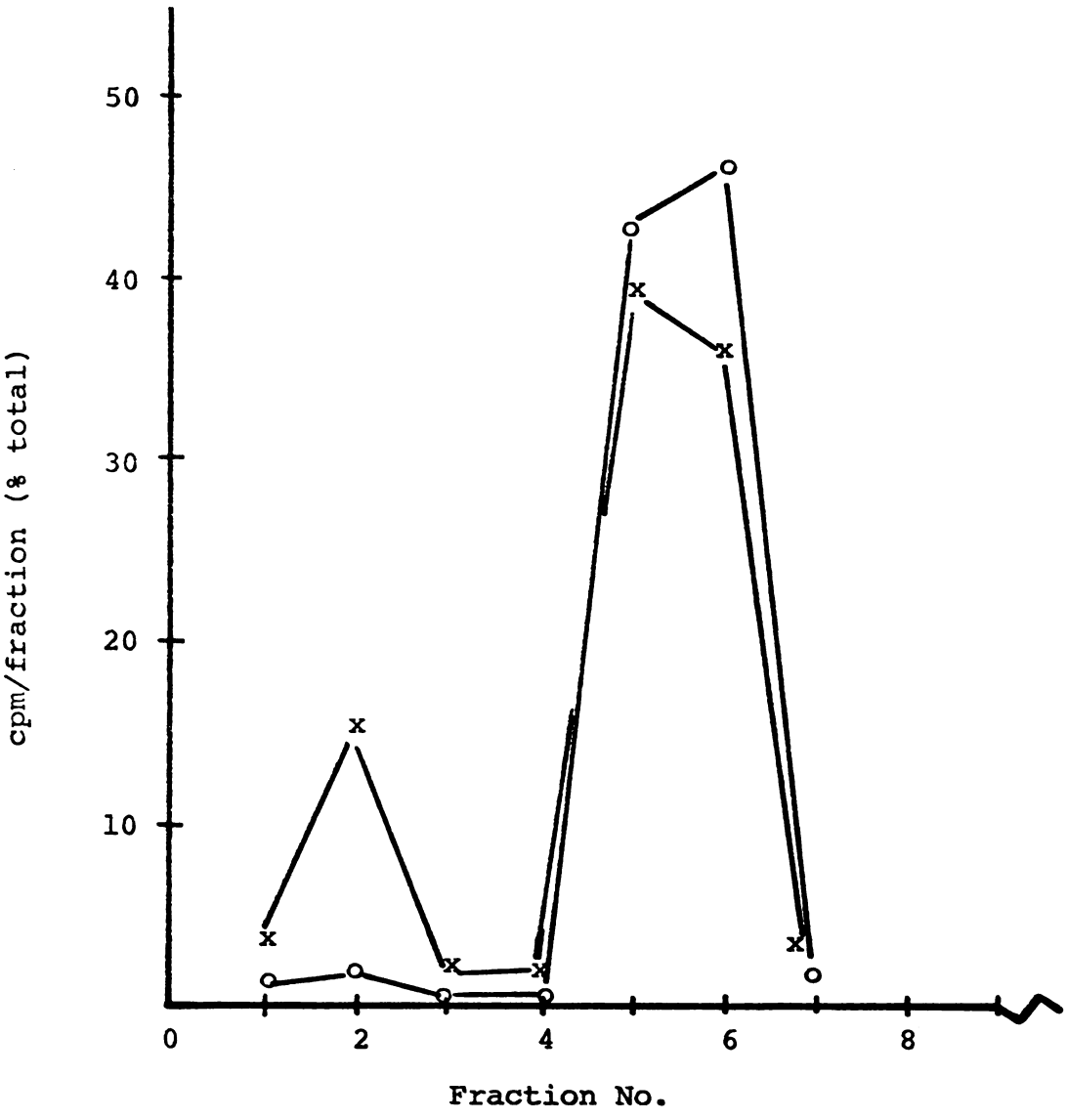

Figure 6.--Qualitative effect of $MnCl_2$ (x—x) and $MgCl_2$ (o—o) on the incorporation of MVA-2-^{14}C into neutral terpenes.

Standard extraction and incubation used as described in Experimental except: no KOH, terpenes extracted with ethyl ether. Column eluted in gradient manner.

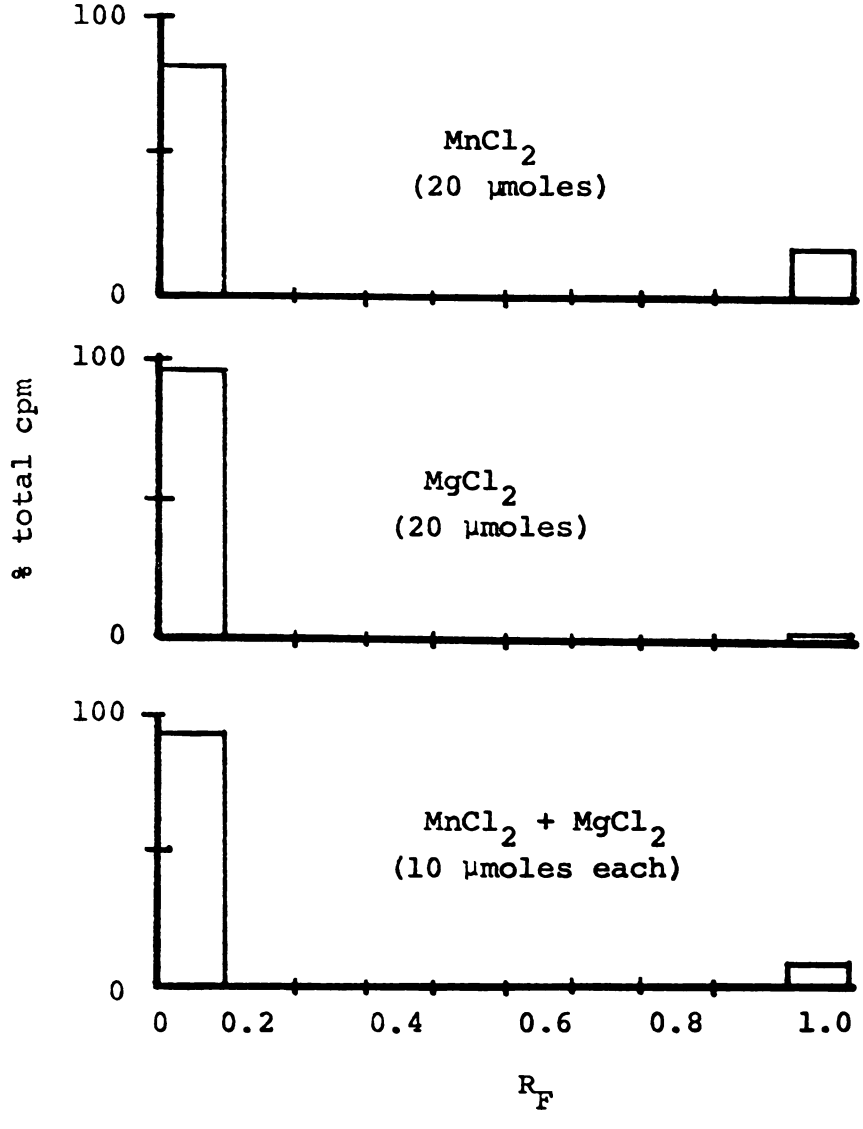

Figure 7.--Qualitative effect of $MnCl_2$ and/or $MgCl_2$ on the incorporation of MVA-2-^{14}C into neutral terpenes.

Standard extraction and incubation used as described in Experimental except for cation indicated. TLC developed in hexane-ethyl ether-acetic acid (90:10:1).

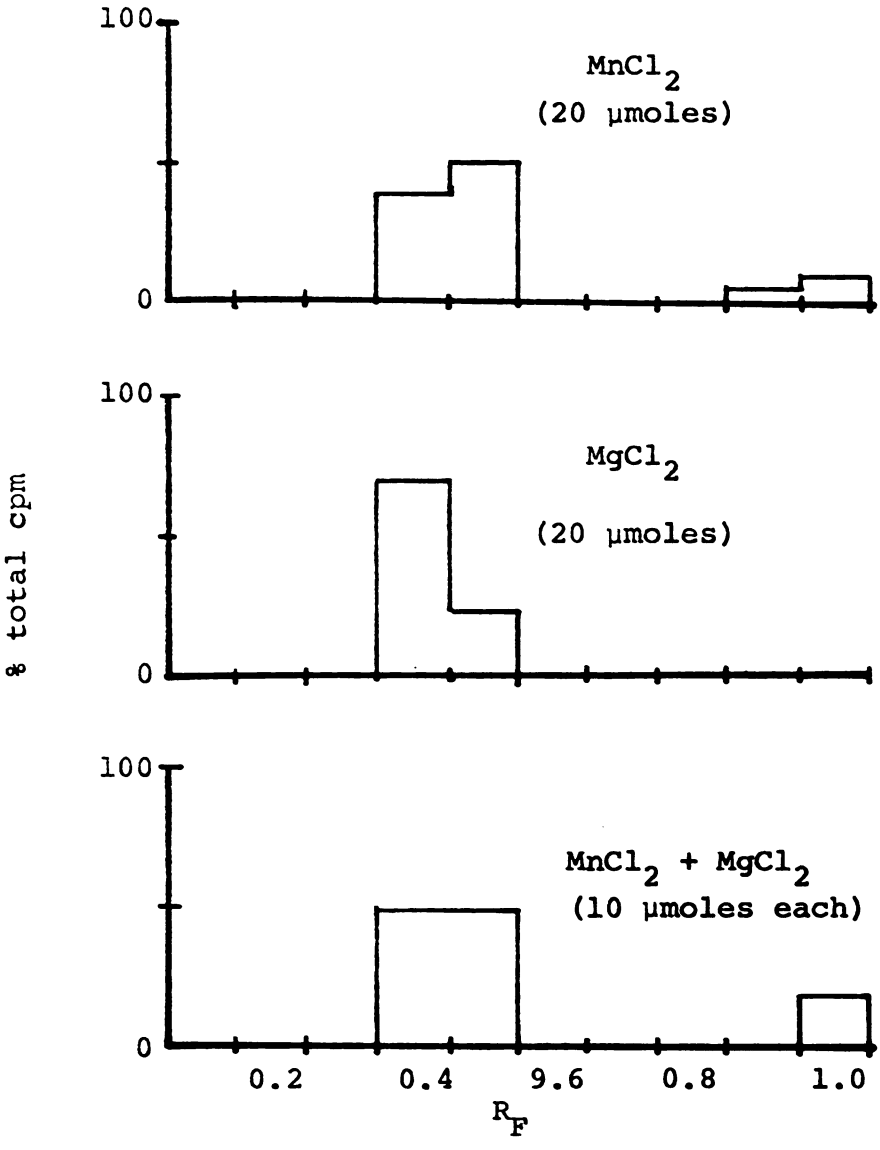

Figure 8.--Qualitative effect of $MnCl_2$ and/or $MgCl_2$ on the incorporation of MVA-2-^{14}C into neutral terpenes.

Standard extraction and incubation used as described in Experimental except for cation indicated. TLC developed in benzene-ethyl acetate (9:1).

concurrent reduction in the more polar products (Table 5 and Figure 6). These results were confirmed using TLC with two solvent systems (Figures 7 and 8).

Experiment 6: Effect of ATP

Results presented in Table 6 indicated that the cell-free system had an absolute requirement for ATP. Data in Figure 9 show that the ATP concentration was lower for maximal incorporation for non-cold treated iris than for iris which had received low temperature treatment prior to enzyme extraction.

Experiment 7: Effect of Pyridine Nucleotides

NAD, NADP and NADPH had little effect on the incorporation of MVA-2-^{14}C into neutral terpenes (Table 7). The data (Table 8) show that NADH and NADPH did not substantially increase the incorporation of acetate-1-^{14}C into neutral terpenes compared to the radioactivity incorporated using MVA-2-^{14}C as the substrate.

Experiment 8: Enzyme Concentration

Results presented in Figure 10 indicated that enzyme concentrations, as determined by TCA precipitable protein, of up to 10.5 mg protein per assay resulted in linear increases in the incorporation of MVA-2-^{14}C into neutral terpenes.

TABLE 6.--Effect of ATP on the incorporation of MVA-2-^{14}C into neutral terpenes.

Treatment	Incorporation (cpm)
plus ATP	10,897
minus ATP	221
plus ATP (boiled enzyme)	236

Standard extraction and incubation used as described in Experimental except no $MnCl_2$ added.

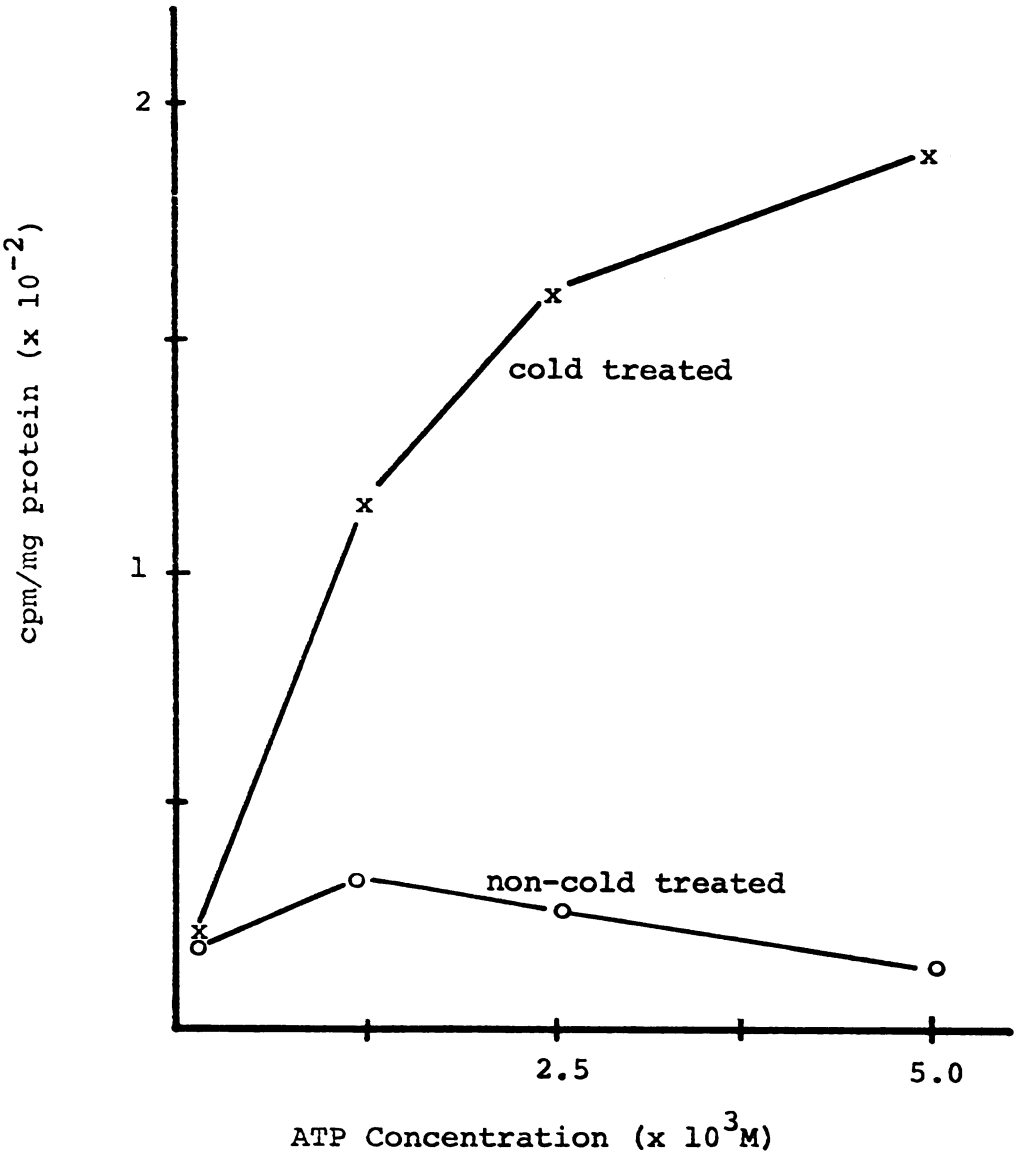

Figure 9.--Effect of ATP concentration on the incorporation of MVA-2-^{14}C into neutral terpenes using cell-free extracts of non-cold and cold treated Wedgwood Iris.

Standard extraction and incubation used as described in Experimental except for ATP concentrations and enzyme sources indicated.

TABLE 7.--Effect of NAD, NADP and NADPH on the incorporation of MVA-2-^{14}C into neutral terpenes.

Treatment	Experiment	Incorporation (cpm)
No pyridine nucleotides	A	20,114
plus NAD (2 µmoles)		19,433
plus NADP (2 µmoles)		19,022
No pyridine nucleotides	B	35,331
plus NADPH (0.36 µmoles)		34,408
No pyridine nucleotides	C	39,203
plus NADPH and NAD (1.5 µmoles each)		36,692

Standard extraction and incubation used as described in Experimental except for pyridine nucleotides indicated.

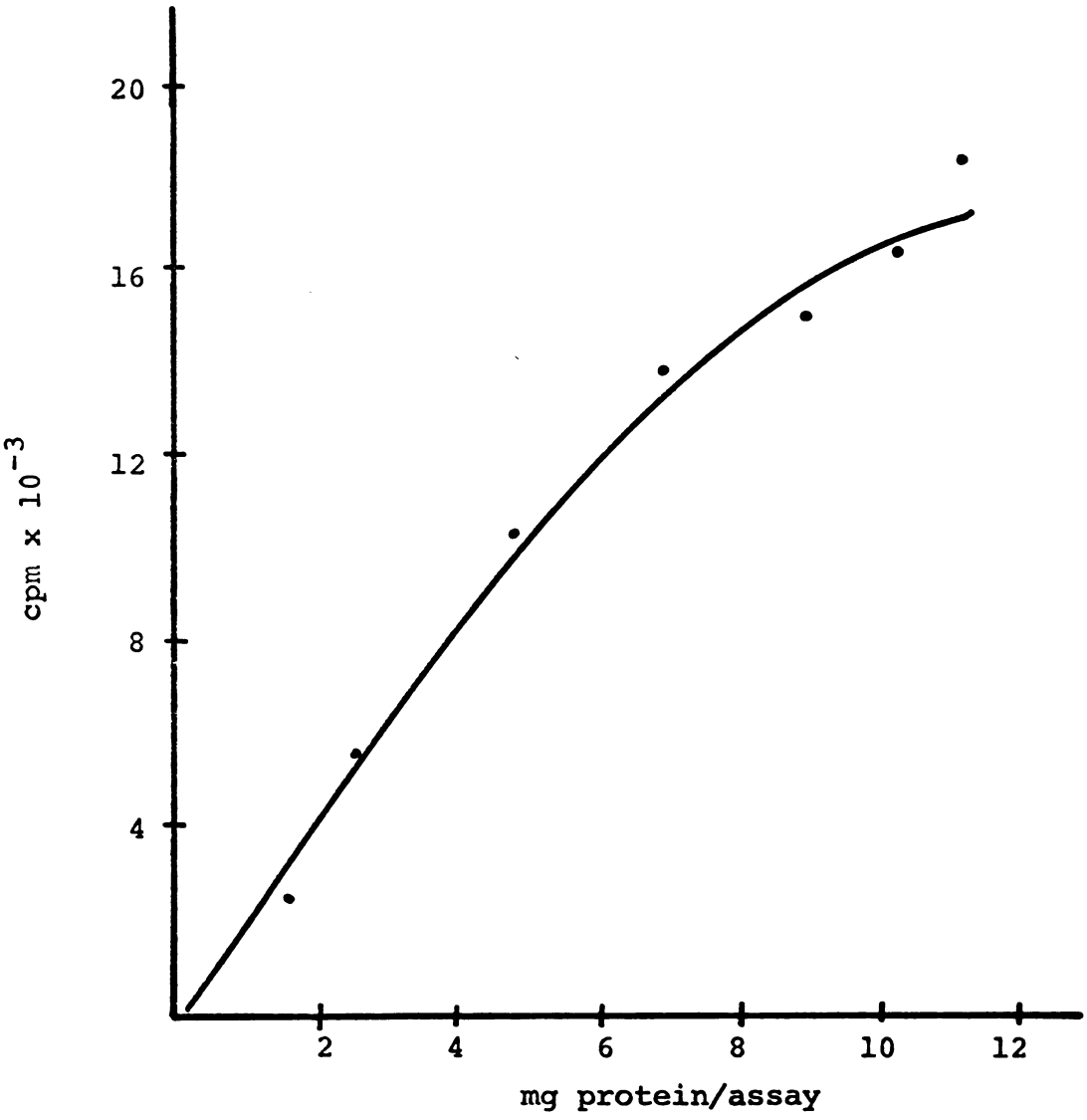

Figure 10.--Effect of protein concentration on the incorporation of MVA-2-^{14}C into neutral terpenes.

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

Experiment 9: Substrates

Acetate-1-^{14}C in the presence of NADPH and/or NADH was not substantially incorporated into neutral terpenes (Table 8). However, both MVA-1-^{14}C and MVA-2-^{14}C were actively used as substrates by enzymes in the cell-free system (Table 9). Also, data presented in Table 9 show that no radioactivity was present in the neutral terpene fraction when MVA-1-^{14}C was used as the substrate.

Results presented in Table 9 and Figure 11 revealed that a small but measurable amount of $^{14}CO_2$ was released when MVA-2-^{14}C was used as the substrate. This release of $^{14}CO_2$ from MVA-2-^{14}C had a lag period of approximately 45 minutes before detectable amounts of $^{14}CO_2$ was found. The total amount released was approximately 6% of that released when MVA-1-^{14}C was the substrate.

Linear increases in the concentrations of MVA-2-^{14}C resulted in linear increases in ^{14}C-neutral terpenes biosynthesized (Figure 12). The highest concentration tested of 9.4×10^{-6}M resulted in an incorporation rate of 3.0×10^{-9} moles d-MVA-2-^{14}C/mg protein/hour. This incorporation rate represented 60% of the added d-MVA-2-^{14}C being converted to ^{14}C-neutral terpenes.

Experiment 10: Effect of Inhibitors

The effects of various inhibitors on the incorporation of MVA-2-^{14}C into neutral terpenes is presented in Table 10. SK&F 7997-A_3, CCC and Amo 1618 had little or

TABLE 8.--Effect of ATP, NADH and NADPH on the incorporation of acetate-1-^{14}C into neutral terpenes.

Cofactor			Incorporation (cpm)
ATP	NADPH	NADH	
-	-	-	49
+	-	+	110
+	+	+	167
-	+	+	172
+	-	-	175
+	+	-	226
+	-	-	34,055*

Standard extraction and incubation used as described in Experimental except: ATP (25mM); NADPH (0.165mM); NADH (0.165mM) and acetate-1-^{14}C instead of MVA-2-^{14}C.

*Used MVA-2-^{14}C as substrate.

TABLE 9.--Time course release of $^{14}CO_2$ from MVA-2-^{14}C and MVA-1-^{14}C and the incorporation of ^{14}C into neutral terpenes.

Incubation Time (min)	$^{14}CO_2$ Released (cpm)		^{14}C Recovered in Neutral Terpenes (cpm)	
	MVA-1-^{14}C	MVA-2-^{14}C	MVA-1-^{14}C	MVA-2-^{14}C
20	1,834	0	-	-
40	4,366	0	-	-
60	7,293	69	-	-
120	10,936	255	-	-
240	13,818	1,065	0	9,656

Standard extraction and incubation used as described in Experimental except for incubation time and substrate indicated.

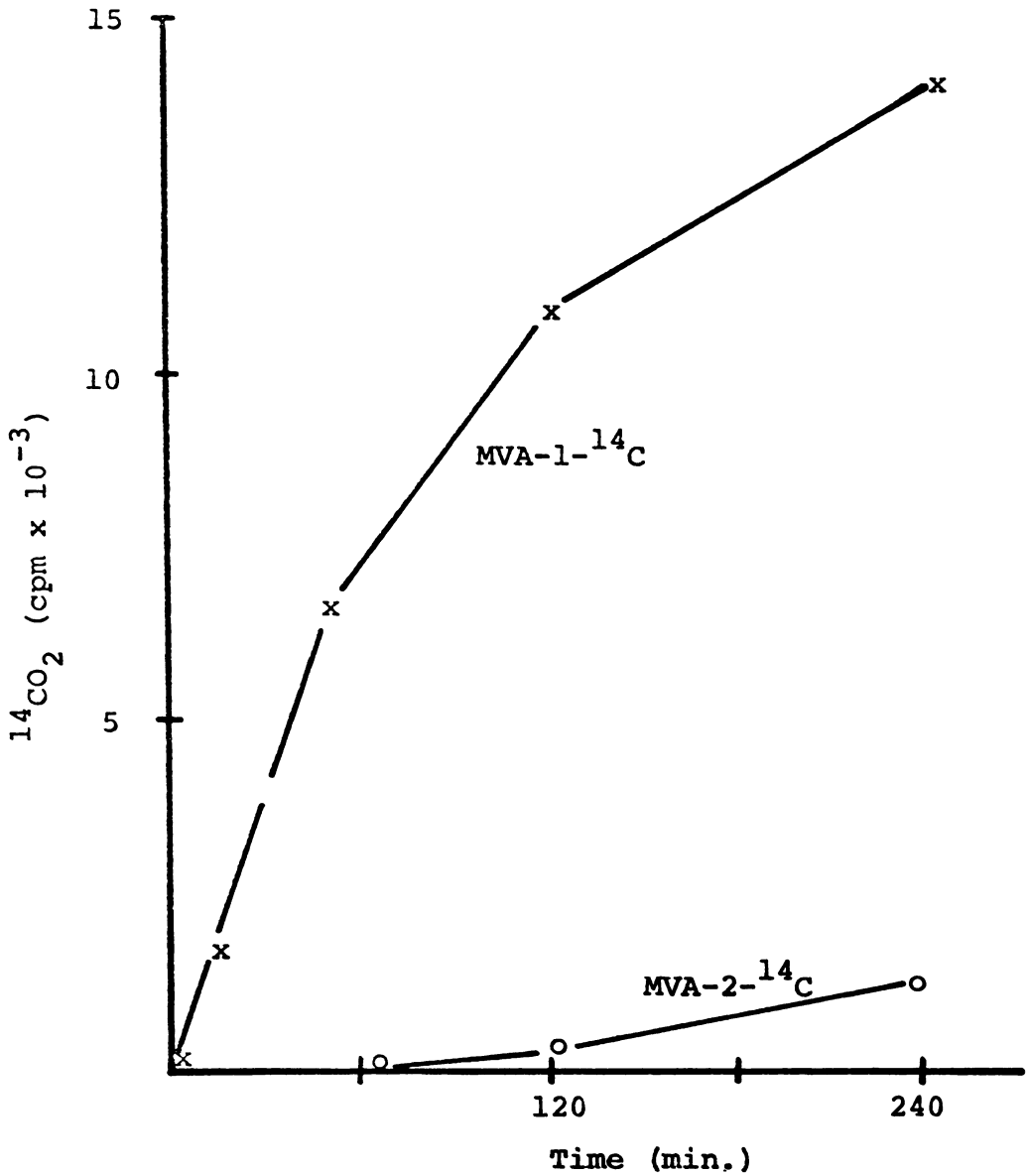

Figure 11.--Time course release of $^{14}CO_2$ from MVA-1-^{14}C and MVA-2-^{14}C.

Standard extraction and incubation used as described in Experimental except for incubation time and substrates indicated.

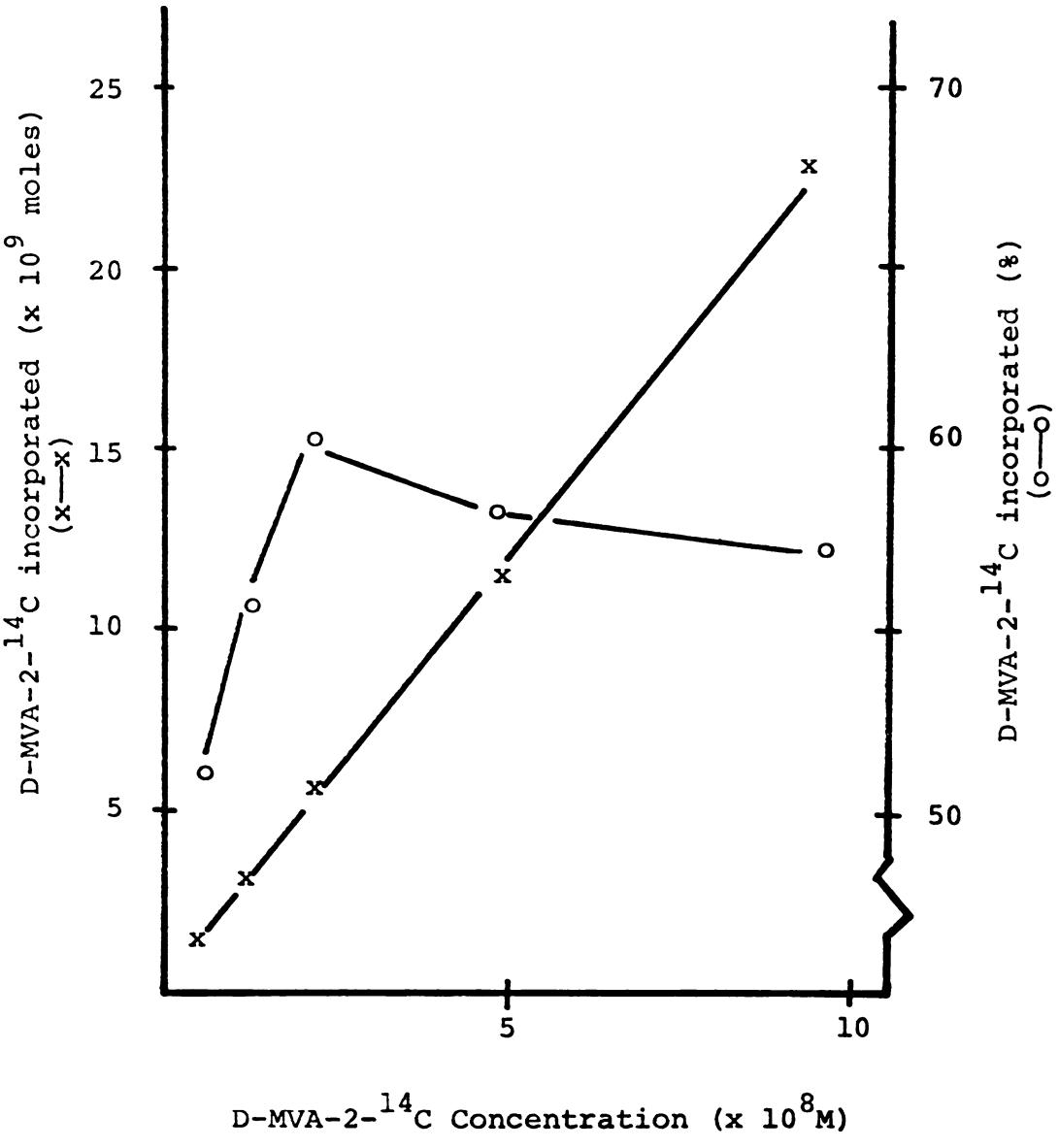

Figure 12.--Effect of D-MVA-2-^{14}C concentration on the incorporation of D-MVA-2-^{14}C into neutral terpenes.

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

TABLE 10.--Effect of various inhibitors on the incorporation of MVA-2-^{14}C into neutral terpenes.

Inhibitor	Concentration (mg/assay)	Concentration (μM)	Incorporation (% of control)
None (control)	--	--	100
SK&F 7997-A_3	2.00	1,025	100
CCC	3.14	5,000	98
AMO 1618	3.60	2,500	98
Sodium fluoride	5.04	30,000	93
Streptomycin	2.90	500	92
Chloramphenicol	0.20	155	85
Niacinamide	8.54	2,000	83
Phosfon D	8.00	5,000	16
Iodoacetamide	3.70	5,000	9

Standard extraction and incubation used as described in Experimental except for inhibitors indicated.

no effect. The addition of sodium fluoride, streptomycin and chloramphenicol resulted in inhibitions of 17, 8 and 15%, respectively. The two most potent inhibitors at the concentrations tested were Phosfon D and iodoacetamide. Phosfon D inhibited by 84% and iodoacetamide by 91%.

The effects of Phosfon D, CCC, AMO 1618 and iodoacetamide on the quality of ^{14}C-neutral terpenes biosynthesized are presented in Figure 13. It was found that CCC and iodoacetamide did not effect the nature of the products. However, the addition of AMO 1618 and Phosfon D did alter the ratio of these products. With AMO 1618 treated assays, there was a complete inhibition of the products associated with R_F zones 0.7 and 1.0. The only R_F zone exhibiting radioactivity was at R_F 0.8. Phosfon D, on the other hand, caused a large decrease in ^{14}C-neutral terpenes associated with R_F zones 0.7 and 0.8. As a result of this decrease, a large percentage increase in activity in R_F 1.0 was shown: although there was no substantial increase in absolute radioactivity in R_F 1.0 (data not presented).

Experiment 11: Localization of Enzymatic Activity

Results presented in Table 11 indicated that the active enzymes were present in the soluble fraction of each centrifugation. Further quantitative results presented in Figure 14 show the influence that a crude and

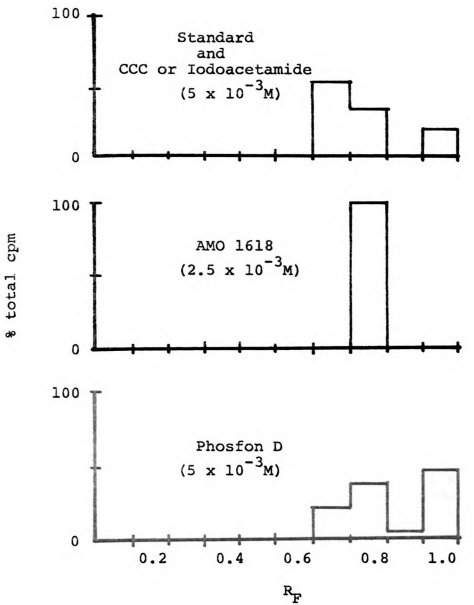

Figure 13.--Qualitative effects of inhibitors on the incorporation of MVA-2-^{14}C into neutral terpenes.

Standard extraction and incubation used as described in Experimental except for inhibitors indicated. TLC developed in benzene-ethyl acetate (9:1).

TABLE 11.--Distribution of enzymatic activity in supernatant solutions after various centrifugation treatments.

Centrifugation Treatment		Incorporation (cpm)
Rel. cent force (x g)	Time of spin (min)	
none (crude)	0	39,680
75	5	38,407
300	15	40,531
30,000	30	43,590
104,000	60	41,335

Standard extraction and incubation used as described in Experimental except for centrifugation indicated.

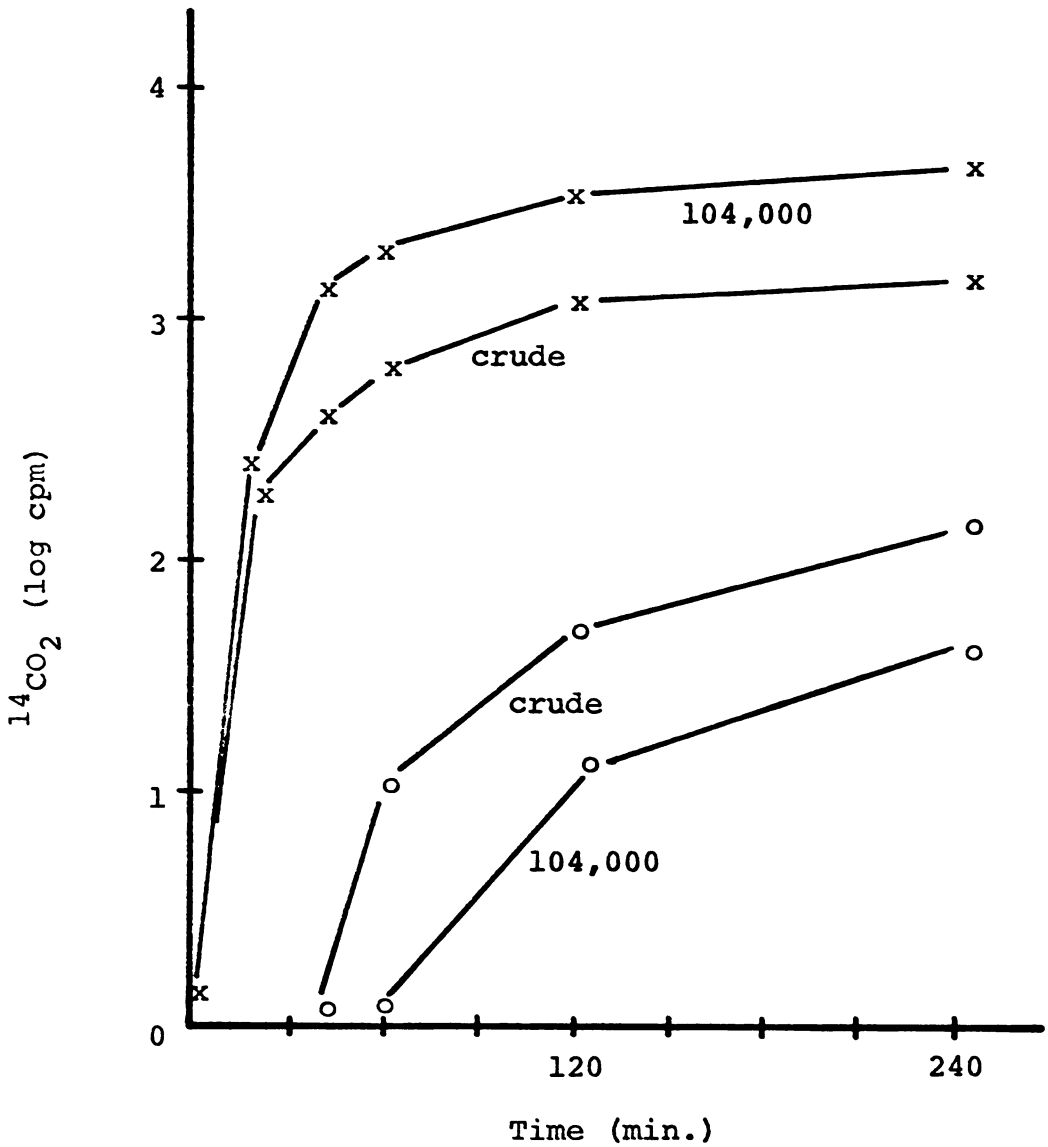

Figure 14.--Time course release of $^{14}CO_2$ from MVA-1-^{14}C (x—x) and MVA-2-^{14}C (o—o) using crude and 104,000 x g cell fractions.

Standard extraction and incubation used as described in Experimental except: incubation time, centrifugation and substrate as indicated.

104,000 x g supernatant solution had on the release of $^{14}CO_2$ from MVA-1-^{14}C and MVA-2-^{14}C. With MVA-1-^{14}C as the substrate, more $^{14}CO_2$ was released from the high spin fraction. On the other hand, when MVA-2-^{14}C was used, the results were reversed. The release of $^{14}CO_2$ from MVA-2-^{14}C was greater with the crude fraction and also, the lag period before $^{14}CO_2$ was detected was shorter.

Effects of crude and 104,000 x g fractions on the types of ^{14}C-neutral terpenes biosynthesized are presented in Figure 15. With the 104,000 x g fraction there was a large decrease in the more non-polar neutral terpene represented under fraction I. Also, fractions II and V remained approximately the same while fraction III increased. A new radioactive product (peak IV) was noted with the 104,000 x g fraction.

Experiment 12: Nature of Products

Figure 16 presents a TLC radioactive scan of a ^{14}C-neutral terpene fraction developed in benzene-ethyl acetate (9:1). Three main radioactive peaks were detected. Similar results in regard to the number of peaks were obtained using four other solvent systems (data not reported).

The main radioactive peak with each solvent system used (i.e., R_F zones 0.4 and 0.5 in Figure 16) was shown to contain cholesterol, campesterol, stigmasterol and β-sitosterol using GLC co-chromatography (Table 12).

no effect. The addition of sodium fluoride, streptomycin and chloramphenicol resulted in inhibitions of 17, 8 and 15%, respectively. The two most potent inhibitors at the concentrations tested were Phosfon D and iodoacetamide. Phosfon D inhibited by 84% and iodoacetamide by 91%.

The effects of Phosfon D, CCC, AMO 1618 and iodoacetamide on the quality of ^{14}C-neutral terpenes biosynthesized are presented in Figure 13. It was found that CCC and iodoacetamide did not effect the nature of the products. However, the addition of AMO 1618 and Phosfon D did alter the ratio of these products. With AMO 1618 treated assays, there was a complete inhibition of the products associated with R_F zones 0.7 and 1.0. The only R_F zone exhibiting radioactivity was at R_F 0.8. Phosfon D, on the other hand, caused a large decrease in ^{14}C-neutral terpenes associated with R_F zones 0.7 and 0.8. As a result of this decrease, a large percentage increase in activity in R_F 1.0 was shown: although there was no substantial increase in absolute radioactivity in R_F 1.0 (data not presented).

Experiment 11: Localization of Enzymatic Activity

Results presented in Table 11 indicated that the active enzymes were present in the soluble fraction of each centrifugation. Further quantitative results presented in Figure 14 show the influence that a crude and

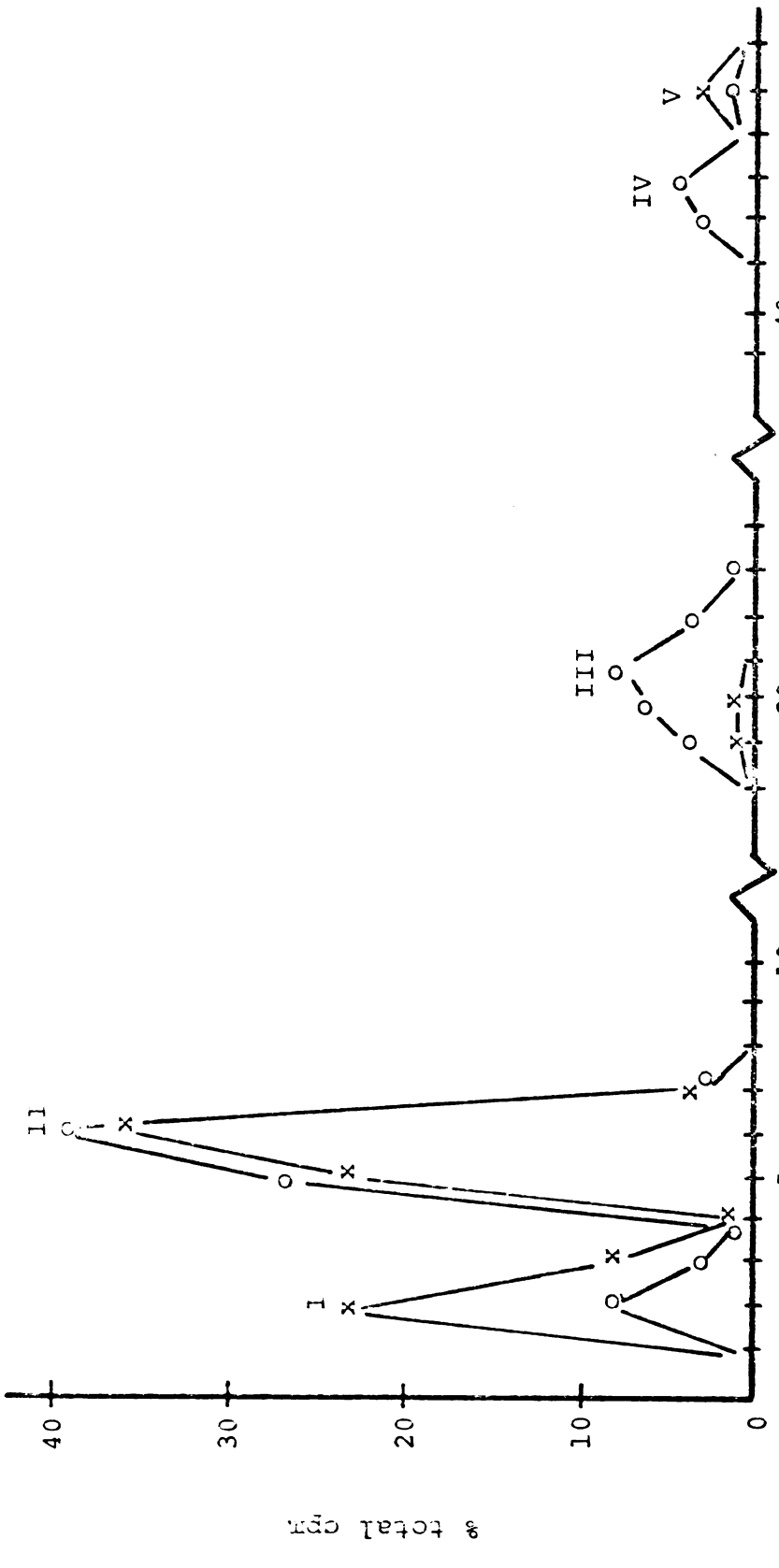

Figure 15.--Qualitative comparison of neutral terpenes biosynthesized from MVA-2-^{14}C with a crude (x—x) and 104,000 (o—o) x g cell fraction.

Standard extraction and incubation used as described in Experimental except for centrifugation indicated. Al_2O_3 column eluted in gradient manner.

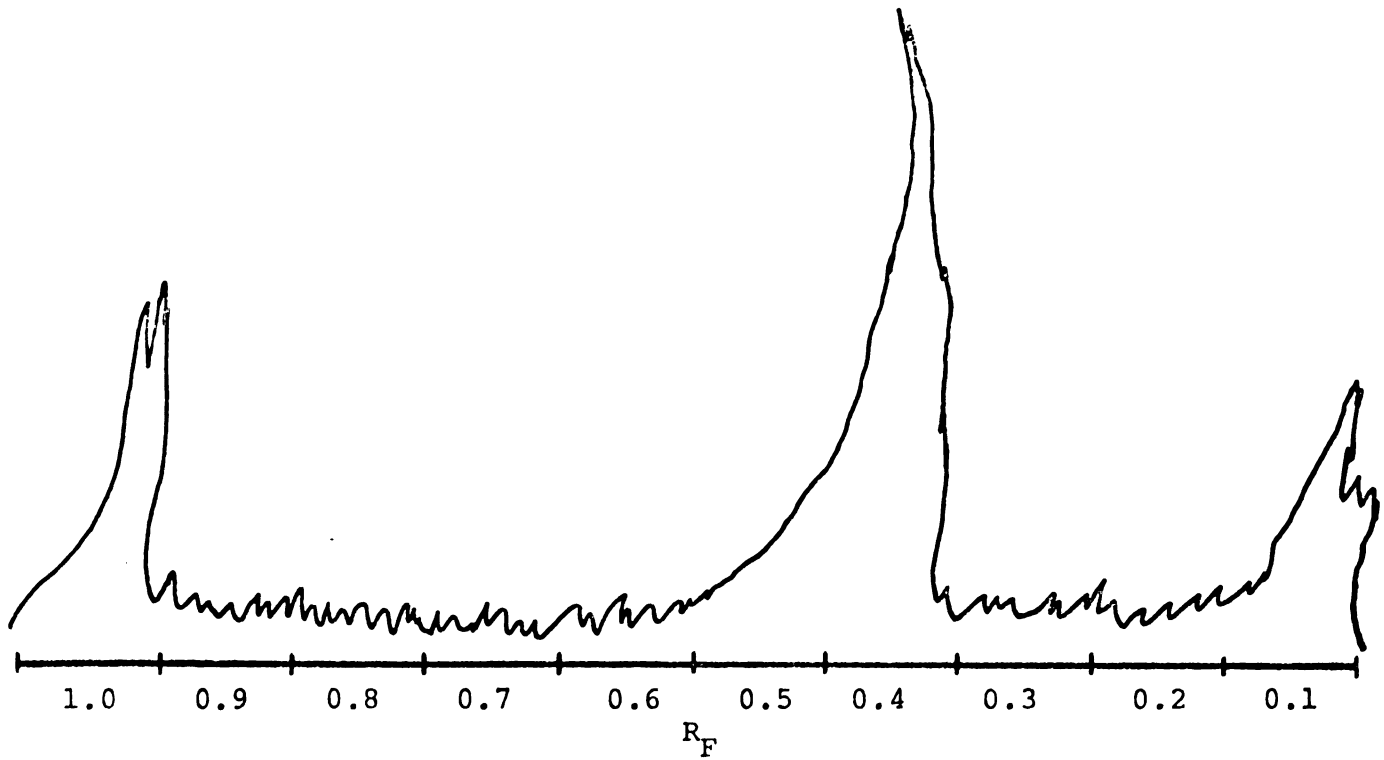

Figure 16.--TLC scan for radioactive neutral terpenes biosynthesized from MVA-2-^{14}C.

Standard extraction and incubation used as described in Experimental. TLC developed in benzene-ethyl acetate (9:1).

TABLE 12.--Retention times of certain TMSi-neutral terpenes extracted from Wedgwood Iris and TMSi-sterol standards using GLC.

Standard	Experiment*	Retention Time (min)	
		Standard	Unknown
Cholesterol	A	15.7	16.2
	B	17.2	17.5
Campesterol	A	20.5	20.7
	B	22.2	22.5
Stigmasterol	A	22.3	22.5
	B	24.4	24.4
beta-Sitosterol	A	27.7	27.8
	B	28.3	28.4

*Experiment A = 6 ft. column packed with 2% Ov-1 on 100/120 Chromosorb W held at 240°C. Flow rate = 45 ml/min.

Experiment B = 6 ft. column packed with 3% SE-30 on 100/120 Chromosorb W held at 250°C. Flow rate - 40 ml/min.

All samples TMSi as described in Experimental.

Mass spectra data (Figures 17 to 19) confirmed the presence of campesterol, stigmasterol and β-sitosterol but showed that cholesterol was not present.

Collection of the effluent off of the gas chromatograph to determine the specific activities of the sterols associated with the radioactive peak using TLC met with failure (data not reported). Digitonin precipitation of these sterols resulted in an average of less than 1% of the sterols being radioactive (Table 13).

Data presented in Table 14 reconfirmed the presence of three ^{14}C-neutral terpenes being biosynthesized. By comparison of the retention times at 182 and 230°C for ±TMSied samples, the retention values were influenced within temperatures for peaks I and II. However, the influence of TMSi on peak III was negligible. Subsequent studies showed that peaks I and II were associated with the radioactivity in R_F zones 0.4 and 0.5 in Figure 16 and peak III associated with R_F 1.0 (data not presented).

Results from TLC experiments presented in Tables 15 and 16 indicated that farnesol may be one of the ^{14}C-neutral terpenes found under R_F zones 0.4 and 0.5 in Figure 16. These results also showed that geraniol, nerolidol and (-)-kaurene did not co-chromatograph with radioactive products of the cell-free system. The small radioactive peak at R_F 0.1 noted in Figure 16 was found to co-chromatograph with MVA-2-^{14}C (data not reported).

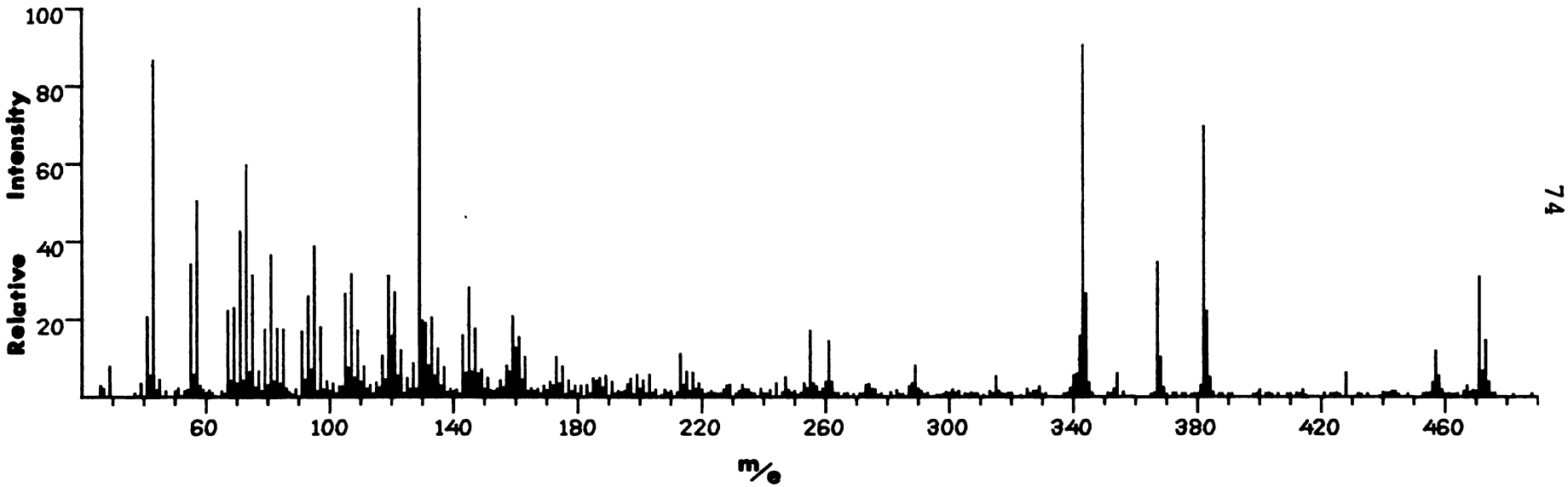

Figure 17.--Mass spectrum of TMSi-derivative of campesterol extracted from Wedgwood Iris.

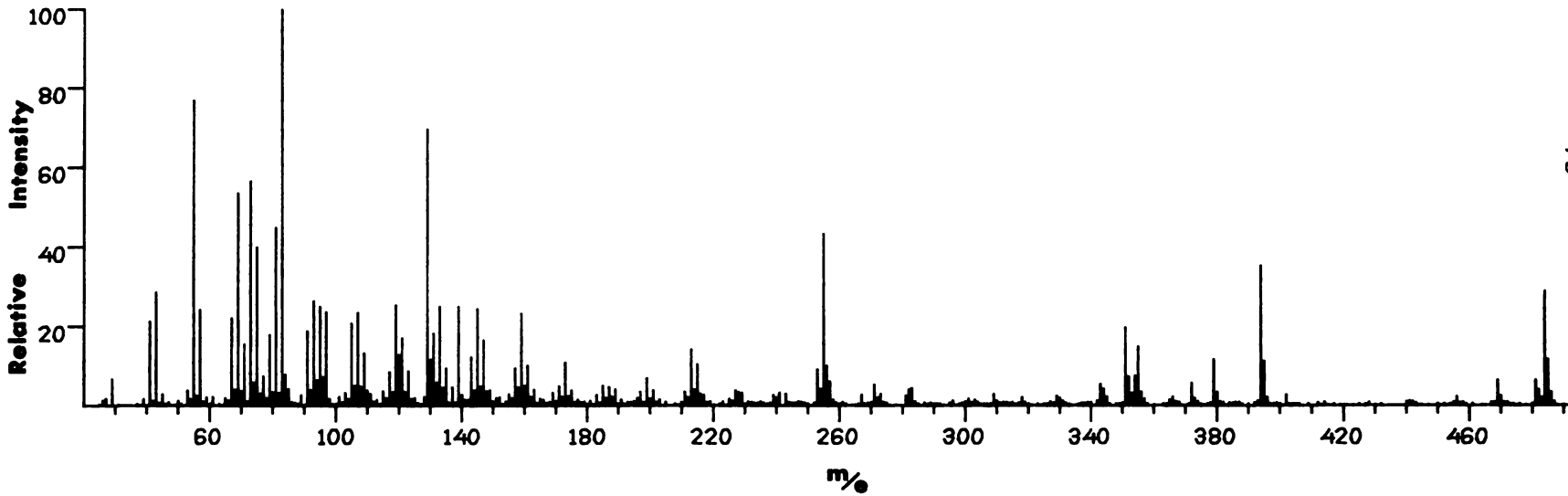

Figure 18.--Mass spectrum of TMSi-derivative of stigmasterol extracted from Wedgwood Iris.

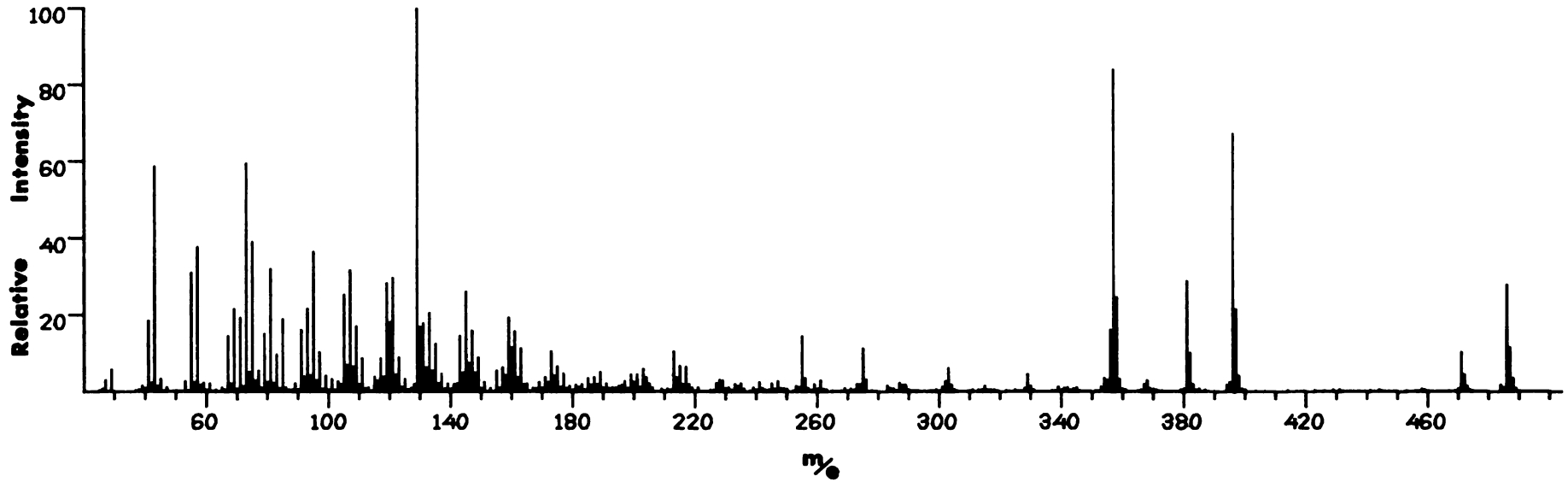

Figure 19.--Mass spectrum of TMSi-derivative of β-sitosterol extracted from Wedgwood Iris.

TABLE 13.--Amount of radioactivity (cpm) in neutral lipid extracts associated with digitonin precipitable sterols following incubation with MVA-2-^{14}C.

Fraction	Experiment	cpm	% ^{14}C-sterols
Neutral lipid	A	39,203	
Sterol		154	0.39
Neutral lipid	B	40,115	
Sterol		655	1.63
Neutral lipid	C	36,692	
Sterol		138	0.37
Neutral lipid	D	42,234	
Sterol		127	0.30
Sterol (minus digitonin)		113	--

Standard extraction and incubation used as described in Experimental except for experiment C where 6×10^{-3}M NAD and NADPH were added.

TABLE 14.--GLC retention times of radioactive neutral terpenes biosynthesized from MVA-2-^{14}C.

Column Temperature (°C)*		Retention times of ^{14}C-products (min)		
		I	II	III
170	(+TMSi)	4.5	27.0	54.0
182	(+TMSi)	2.6	11.0	17.0
182	(-TMSi)	2.1	12.4	16.5
230	(+TMSi)	1.8	3.5	14.5
230	(-TMSi)	(solvent front)	2.8	14.5

*6 ft. x 2 mm I.D. glass column packed with 3% OV-1 on Chromosorb Q. Flow rate - 40 ml/min.

TABLE 15.--Thin-layer co-chromatography of ^{14}C-neutral terpenes biosynthesized from MVA-2-^{14}C with geraniol (G), farnesol (F), nerolidol (N) and (-)-kaurene (K) using three solvent systems.

R_F	Solvent System*					
	A		B		C	
	cmp (%)	standard	cpm (%)	standard	cpm (%)	standard
0.1	40	G, F	0		1	
0.2	2	N	0		1	
0.3	1		0		0	
0.4	0		0		1	G
0.5	2		0		26	F
0.6	0		0		2	
0.7	5		2	G	0	N
0.8	34		21	F	0	
0.9	5		29	N	1	
1.0	10	K	48	K	68	K

Standard extraction and assay used as described in Experimental.

*Solvent A = hexane-ethyl ether-acetic acid (90:10:1)
Solvent B = chloroform-acetone (0:1)
Solvent C = benzene/ethyl acetate (9:1)

TABLE 16.--Thin-layer co-chromatography of the non-polar ^{14}C-neutral terpene biosynthesized from MVA-2-^{14}C with (-)-kaurene under various experimental conditions.*

Treatments**		Solvent***	Radioactivity Associated with (-)-kaurene (cpm)
$AgNO_3$	TMSi		
-	+	1	1,359
-	+	2	1,154
-	+	3	3,190
-	-	1	5,687
-	-	2	1,772
-	-	3	2,089
+	+	1	617
+	+	2	0
+	+	3	0
+	-	1	1,442
+	-	2	0
+	-	3	0

Standard extraction and incubation used as described in Experimental.

*The non-polar ^{14}C-neutral terpene used was that from R_F 1.0 (see Figure 16).

**Silica gel G TLC plates $\pm$ $AgNO_3$ and preparation of TMSi derivatives as described in Experimental.

***Solvent 1 = benzene/ethyl acetate (9:1);
Solvent 2 = hexane/benzene (1:3);
Solvent 3 = hexane-ethyl ether-acetic acid (90:10:1).

Acid hydrolysis and/or saponification of the cell-free media after incubation increased the amount of radioactivity recovered in the hexane fraction (Table 17). Subsequent experiments on the effects of saponification on the nature of the products are presented in Table 18 and Figure 20. It was found that some of the ^{14}C-neutral terpenes biosynthesized were associated with protein extracted with TCA (Table 18). Furthermore, saponification caused a partial release of these ^{14}C-products associated with protein to the fraction extractable with hexane. Extraction of a protein-^{14}C-neutral terpene complex with butanol resulted in all of the radioactivity being transferred to the butanol.

The effect of saponification on the quality of ^{14}C-neutral terpenes biosynthesized is seen in Figure 20. These results showed that saponification chemically altered at least one of the ^{14}C-products. The true ^{14}C-neutral terpene product in the cell-free system is less polar than the artifact created by saponification. Only one of the ^{14}C-products were found to be affected.

The nature of the products biosynthesized with regard to certain evaporation characteristics were determined and results are presented in Tables 19 and 20. While only a small amount of radioactivity was lost using different scintillation counting techniques (Table 19), approximately 50% of the radioactivity was lost from TLC plates stored for 5 days at room temperatures.

TABLE 17.--The effect of saponification and acid hydrolysis on the amount of radioactivity recovered with hexane from cell-free systems incubated with MVA-2-^{14}C.

Treatment		Hexane Fraction (cpm)
Saponification	Acid Hydrolysis	
+	-	20,803
+	+	22,095
-	-	5,813
-	+	6,447

Standard extraction and incubation used as described in Experimental except for saponification and acid hydrolysis as indicated.

TABLE 18.--Association of ^{14}C-terpenes with protein as influenced by various treatments.

Treatment	^{14}C-terpene (cpm)
Saponified*	46,689
Non-saponified*	15,366
Protein (saponified)	33,090
Protein (non-saponified)	59,265
Butanol wash of protein	67,595
Protein after butanol wash	0

Standard extraction and incubation used as described in Experimental except ether was used instead of hexane.

*Represents radioactivity not associated with protein.

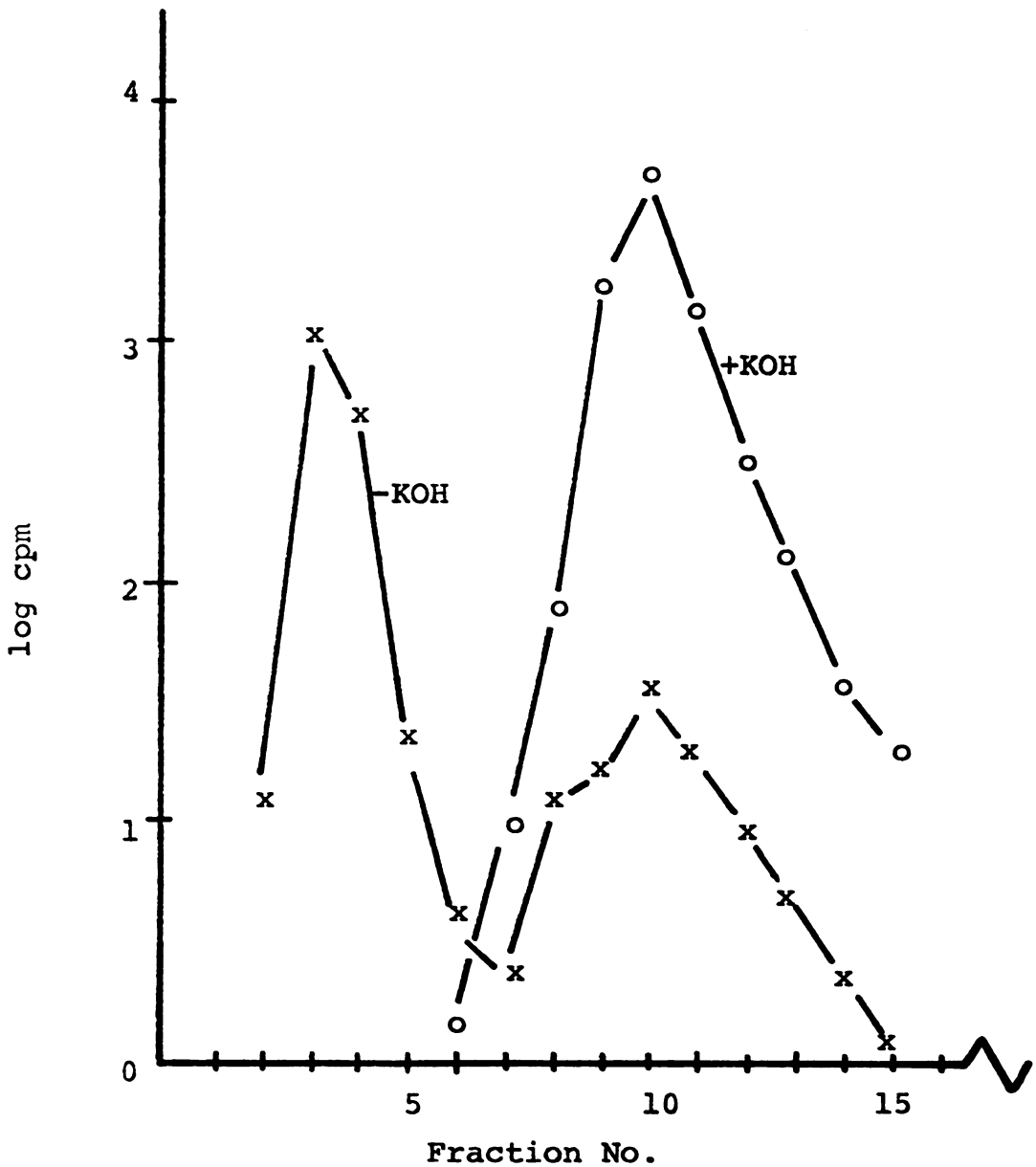

Figure 20.--Effect of saponification on the quality of ^{14}C-neutral terpenes extracted from cell-free assays with MVA-2-^{14}C as the substrate.

Standard extraction and incubation used as described in Experimental except terpenes extracted with ethyl ether prior to the addition of KOH for the +KOH treatment.

TABLE 19.--Effect of scintillation counting techniques on the loss of ^{14}C-neutral terpenes from evaporation.

Technique*	^{14}C-neutral Terpenes	
	(dpm)	% loss
A	4,446	0
B	4,216	5
C	4,344	2

Standard extraction and incubation used as described in Experimental except for scintillation techniques noted.

*Scintillation fluor added:
A, directly to hexane aliquot;
B, to hexane aliquot dried in vacuo at 60°C; and
C, to hexane aliquot dried under a nitrogen stream at room temperature.

TABLE 20.--Effect of time on the loss of ± TMSi ^{14}C-neutral terpenes from thin-layer plates by evaporation.*

Time (days)	TMSi	^{14}C-neutral Terpenes	
		cpm/TLC plate	% loss
0	+	47,798	(control)
	-	52,750	(control)
1	+	46,788	2
	-	49,122	7
5	+	24,516	49
	-	27,311	48

Standard extraction and incubation used as described in Experimental.

*Silica gel G TLC plates developed in benzene-ethyl acetate (9:1).

DISCUSSION

Richards and Hendrickson (1964) stated: ". . . attainment of mevalonate constitutes, in a sense, passage through the gateway to isoprenoid biogenesis." Thus, based on this statement alone, the radioactive products arising from MVA-2-^{14}C with the Wedgwood Iris cell-free system were terpenes. The fact that these radioactive terpenes were non-saponifiable showed that they were neutral terpenes.

The iris cell-free system incorporated MVA-2-^{14}C into at least three neutral terpenes (Table 14). While unequivocal identification of these biosynthesized neutral terpenes requires further experimentation, the data obtained in the present study indicated this cell-free system is unique for the study of terpene biogenesis. The system shows a high degree of experimental flexibility and above all, the amount of MVA-2-^{14}C incorporation is exceptionally high (3.0×10^{-9} moles d-MVA-2-^{14}C/mg protein/hour).

Results presented by Archer et al. (1963); Nicholas, (1967); Bloch (1965); Heftmann (1970; Chesterton and Kekwick (1968); and George-Nascimento et al. (1969) have conclusively shown that terpene biosynthesis from MVA

proceeds initially to I-PP by the step-wise addition of 3 ATP to MVA and the loss of the number one carbon of MVA as CO_2 (hereafter referred to as the 'normal' terpene biogenetic pathway). While the intermediates from MVA to I-PP were not isolated nor identified in the present study, data obtained indicated that the biosynthesis of terpenes in iris proceeded in this normal manner, namely: (1) there was an absolute requirement for ATP (Table 6); (2) Mn^{++} and/or Mg^{++} was stimulating (Table 4); (4) the kinetics of the $^{14}CO_2$ released from MVA-1-^{14}C (Figures 2 and 11); (4) the inhibitory effect of iodoacetamide (Table 10 and Figure 13); (5) the localization of enzymes necessary for the initial steps from MVA to I-PP (Table 11); (6) no requirement for air (Table 3); and (7) the effect of acid hydrolysis (Table 17).

ATP was the only nucleoside triphosphate tested with the iris cell-free system. This should not be interpreted to mean that ATP was the only possible donor of high energy phosphates. Loomis and Battaile (1963), Pollard et al. (1966) and Potty and Bruemmer (1970a) showed with pumpkin, pea and orange cell-free systems, respectively, that other nucleosides could act as phosphate donors. Regardless of the possible donor, high energy phosphates were required in the iris cell-free system for the biosynthesis of neutral terpenes (Table 6), a requirement for the normal pathway.

Stoichiometrically, much more ATP was required for maximal incorporation of MVA-2-^{14}C into neutral terpenes in relation to the amount of MVA-2-^{14}C added. Considering the 3:1 ratio of ATP:MVA needed for the normal conversion of MVA to I-PP, up to 400 times this theoretical concentration ratio did not result in maximal activity in the iris cell-free system. Similar findings were reported by Graebe (1968), Loomis and Battaile (1963), and Beytia et al. (1969). These results might be explained in several ways. Some of the added ATP was complexed with divalent cations as shown by Beytia et al. (1969) and Loomis and Battaile (1963). Also, the added ATP was acted upon by phosphatases in the cell-free extract thus lowering the actual concentration of available ATP.

As with all kinases (White et al., 1959), the MVA kinases thought to be present in the iris cell-free system required Mn^{++} and/or Mg^{++} for maximal activity (Table 4). This divalent cation requirement has been shown with many other plant cell-free systems for similar reactions (i.e. George-Nascimento et al., 1969; Loomis and Battaile, 1963). The activity present in the iris system without the addition of either divalent cation was possibly due to the presence of these cations in the extract.

The release of $^{14}CO_2$ from assays which contained MVA-1-^{14}C was immediate and preceeded the recovery of radioactivity in the neutral terpene fraction from

MVA-2-^{14}C by approximately seven minutes (Figures 2 and 11). The rapid release of $^{14}CO_2$ from MVA-1-^{14}C, coupled with the fact that no radioactivity was recovered in the neutral terpene fraction when MVA-1-^{14}C was used, is expected if the biosynthesis of terpenes in iris was proceeding normally (i.e., Block, 1965; Chesterton, 1968). The delay before radioactivity from MVA-2-^{14}C was detected in neutral terpenes may have represented a rate limiting enzyme(s) after the decarboxylation step. It was also noted with the iris cell-free system that more $^{14}CO_2$ was decarboxylated from MVA-1-^{14}C than ^{14}C incorporated into neutral terpenes from MVA-2-^{14}C. This might be explained by the fact that not all of the ^{14}C-products from MVA-2-^{14}C were neutral terpenes. For example, phosphate or pyrophosphate intermediates formed in the biosynthesis of terpenes and terpenes like gibberellins would not be extracted with the neutral terpene fraction.

While iodoacetamide can inhibit many enzymatic reactions in which sulfhydryl groups are important (White, 1959), this compound is especially effective as an inhibitor of pyrophosphomevalonate decarboxylase and I-PP isomerase (Agranoff et al., 1960). Thus the nearly complete inhibition in the iris cell-free system of neutral terpene biosynthesis by iodoacetamide (Table 10) added further evidence that terpene biosynthesis in iris was proceeding via the normal pathway.

It has been shown that the initial reactions from MVA to I-PP require soluble enzymes (Graebe, 1968, 1967; Charlton et al., 1967; Anderson and Porter, 1967) and also that these reactions do not require oxygen (Beeler et al., 1963; Graebe, 1968; Murphy and West, 1969). The data obtained in the present study agreed with these findings (Tables 3 and 11).

The increased amount of extractable radioactivity from the neutral lipid fraction after acid hydrolysis (Table 17) suggested that ^{14}C-phosphate and/or ^{14}C-pyrophosphate intermediates were present. Some of these phosphorylated intermediates (i.e., I-PP, G-PP, GG-PP, F-PP) should be present if the terpene biosynthetic pathway was proceeding normally. Similar conclusions have been made with data from other cell-free systems (Beytia et al., 1969; Chesterton and Kekwick, 1968; Oster and West, 1968; Valenzuela et al., 1966a). However, with the iris system, the addition of acid increased the amount of extractable radioactivity only slightly compared to the increases after similar treatment reported in the references cited above. This result suggested that phosphatase activity was very high in the iris cell-free extracts. It was also shown with the iris system that potassium fluoride did not reduce this suspected phosphatase activity (Table 10). Potassium fluoride was also ineffective as a phosphatase inhibitor with other cell-free

systems (Anderson and Porter, 1962; Chesterton and Kekwick, 1968).

Thus, all of the data obtained supported the suggestion that the iris cell-free system was biosynthesizing terpenes from MVA to I-PP via the normal biosynthetic pathway.

The ^{14}C-neutral terpenes biosynthesized with the iris cell-free system were not positively identified. However, early investigations suggested that sterols were among the radioactive products. This conclusion was reached because of the following data: (1) the use of five solvent systems with TLC showed that the majority of the radioactivity co-chromatographed with sterols (data not reported); (2) the positive identification of sterols in the neutral lipid extract (Figures 17 to 19); (3) the delayed release of $^{14}CO_2$ from MVA-2-^{14}C spiked assays (Figure 2) which is known to occur when certain triterepene intermediates in the biosynthesis of sterols are decarboxylated (Richards and Hendrickson, 1964); and (4) the increased release of $^{14}CO_2$ from MVA-2-^{14}C when microsomes were present in the incubation medium (Figure 14), since microsomes are necessary for this release (Bloch, 1965; Richards and Hendrickson, 1964). Although these data were correct, it proved to be misleading. Subsequent studies showed that: (1) digitonin precipitation of sterols resulted in little or no readioactivity associated with the digitonin (Table

13); (2) GLC-^{14}C analysis experiments indicated that no detectable radioactivity co-chromatographed with sterols (Tables 12 and 14); (3) the addition of SK&F 7997-A_3, which should block the biosynthesis of lanosterol (Bonner et al., 1963) and therefore the release of $^{14}CO_2$ from MVA-2-^{14}C, did not do so (Table 10 and data not reported); (4) pyridine nucleotides, which are required for sterol biosynthesis (Richards and Hendrickson, 1964), did not effect the nature of terpenes produced (Table 7); and (5) the increased release of $^{14}CO_2$ from MVA-2-^{14}C under anaerobic conditions (Figure 4), a condition which is inhibitory for this release (White et al., 1959).

The release of $^{14}CO_2$ from MVA-2-^{14}C incubations might have been a catabolic disposal of certain prenol intermediates and not an indication of sterol biosynthesis. Popjak (1959) reported that prenols were converted into carboxylic acids in the absence of cofactors in the biosynthesis of squalene and sterols under prolonged incubations. These carboxylic acids were then the source of ^{14}C lost to the atmosphere (presumably as $^{14}CO_2$) by a mechanism as yet not determined. He also found that microsomes in the incubation medium increased the formation of the carboxylic acids from the prenols and thus, increased the loss of ^{14}C. Results in the present study agreed with his findings. The release of $^{14}CO_2$ from MVA-2-^{14}C incubations occurred only after a lag period

(Figure 11) and microsomes increased this release (Figure 14).

The main radioactivity associated with sterols was subsequently shown to be a prenol-like substance using TLC, GLC and aluminum oxide chromatography. The ^{14}C-prenol-like substance had similar retention times using TLC with three solvent systems as farnesol (Table 15). This product was conclusively shown not to be geraniol nor nerolidol (Table 15). However, two lines of evidence suggested that the product was geranylgeraniol and not farnesol. First, the radioactive peak of this prenol-like substance had a slightly longer retention time than farnesol using GLC (data not reported). Secondly, evaporation studies showed that this product was less volatile than farnesol (Table 20). Baumann (1969) showed that within 24 hours at room temperature, approximately 50% of the ^{14}C-farnesol applied to TLC plates evaporated. Also, she reported that drying a sample of ^{14}C-farnesol under a stream of nitrogen or *in vacuo* resulted in substantial losses of radioactivity. In the present study, it took five days for 50% of the ^{14}C-prenol-like substance to evaporate from similar TLC plates and only slight radioactivity was lost drying the sample (Table 20). Thus, the longer retention time and lower volatility of the ^{14}C-prenol-like product from the iris cell-free system suggested that it had a higher molecular weight than

farnesol. Geranylgeraniol has a higher molecular weight than farnesol and thus, may be this product. This prenol-like product may also have been a larger prenol since such prenols have been reported in Micrococcus lysodeikticus by Allen et al. (1967).

The more non-polar ^{14}C-neutral terpene biosynthesized in the iris cell-free system was initially thought to be kaurene using three solvent systems with TLC (Table 15). Also, AMO 1618, which specifically inhibits kaurene biosynthesis (Graebe, 1967; Lang, 1970), inhibited the biosynthesis of this non-polar neutral terpene (Figure 13), again suggesting that kaurene was being biosynthesized. It was demonstrated, however, that this terpene was probably not kaurene. First, radioactivity thought to be kaurene did not co-chromatograph with a standard using GLC (data not reported) and silver nitrate impregnated TLC developed in three solvent systems (Table 16). Second, CCC and Phosfon D did not inhibit the biosynthesis of this non-polar product (Figure 13). Third, microsomes were necessary for its biosynthesis (Figure 15). It was possible that the kaurene standard deteriorated in storage because it did not respond properly to various TLC techniques reported in the literature for the identification of kaurene (data not reported). In reference to the second point, the reasons why the inhibitors did not affect the biosynthesis of this non-polar product could

be explained by insufficient concentrations of the added inhibitor or the possibility that CCC and Phosfon D do not inhibit kaurene biosynthesis in this system. Lang (1970) has reviewed these possibilities and reported that CCC and Phosfon D were often ineffective and less specific, respectively, as kaurene inhibitors. Finally, Murphy and West (1969) and others have shown that kaurene biosynthesis proceeds without microsomes and that microsomes are only necessary for the oxidation of kaurene.

Regardless of the exact identification of the prenol-like product, it is significant to note that saponification of the incubation medium resulted in chemical changes in the products. The saponification procedure increased extractable radioactivity with hexane (Table 18) and caused a qualitative change in the ^{14}C-prenol-like product (Figure 20). Oster and West (1968) and Barnes et al. (1969) reported similar results with regard to quantitative affects of saponification. In both studies as well as results of the present study, the ^{14}C-prenol-like products were shown to be non-covalently bound to protein. Whether this binding was discriminate or not, remains to be determined. It is known that enzyme-bound terpene intermediates do occur (Krischna et al., 1966) indicating that the binding between protein and prenol-like substances in the iris system may be real. The addition of sodium hydroxide (Oster and West, 1968) and potassium hydroxide

(Barnes et al., 1969 and results presented in Table 17) in some way caused the release of the radioactivity from the protein complex. What has not been reported in the literature is the effect of saponification on the quality of the terpenes being biosynthesized. It was found in the iris cell-free system that saponification of the incubation media chemically altered the ^{14}C-prenol-like product (Figure 20). The real radioactive product exhibited greater non-polar characteristics than the product after saponification. Thus, it may be possible that there was an ester linkage between the prenol-like product and some other substance and that saponification hydrolyzed this ester bond to give the free ^{14}C-prenol-like product and a salt of the other substance. It would be interesting to know if saponification affected the terpenes biosynthesized in other cell-free systems in a similar manner. If so, then certain products identified in these other systems may have been artifacts.

The quantitative and qualitative nature of the ^{14}C-neutral terpenes biosynthesized were controlled by the conditions of the assay. The addition of Mn^{++} stimulated the production of the more non-polar ^{14}C-neutral terpene (Figures 6 to 8). While many other reports have shown similar changes by altering the divalent cations (i.e., Beytia et al., 1969; Graebe, 1968; Loomis and Battaile, 1963; Upper and West, 1967), the reasons behind these

changes are not known. With the present system, it can be hypothesized that either Mn^{++} or Mg^{++} functioned as the cofactor for the initial series of MVA kinase reactions but that only Mn^{++} was a cofactor for the biosynthesis of the more non-polar terpene product. Further qualitative changes were induced by removing the microsomes from the incubation medium (Figure 15). Also, the addition of AMO 1618 and Phosfon D altered the types of ^{14}C-neutral terpenes biosynthesized (Figure 13). Thus, by altering the divalent cation, cell fraction or by the addition of certain inhibitors, it was possible to control which ^{14}C-neutral terpenes were to be biosynthesized.

The inhibitory effect of phosphate buffer may have been a result of insufficient Mn^{++} and Mg^{++} ions available as cofactors for the cell-free biosynthesis of terpenes. At high pH's using phosphate buffer, Mn^{++} and Mg^{++} are precipitated out of solution. It is interesting to note that the inhibitory effect of the minus Mn^{++} and Mg^{++} treatment (Table 4) were the same, indicating that the phosphate buffer at pH 8.0 did result in precipitation of the cations.

A significant advantage of the iris cell-free system over most of the reported systems for the study of terpene biogenesis was the highly efficient rate of MVA-2-^{14}C incorporated into neutral terpenes (3.0×10^{-9} moles D-MVA-2-^{14}C/mg protein/hour. Up to 80% of the active MVA

added was incorporated within one hour. In comparison, only one plant cell-free system reported, using extracts of wild cucumber, had an efficiency of greater than 50% (Oster and West, 1968) while the remaining had lower efficiencies of MVA incorporation.

SUMMARY

Part I of this dissertation reports a series of experiments which partially characterized a cell-free system for the biosynthesis of neutral terpenes from MVA using extracts of Wedgwood Iris. The results of these experiments are summarized as follows:

1. Tris buffer (0.1M) at pH 8.2 produced the maximal rates of MVA incorporation into neutral terpenes. Phosphate buffer (0.1M) at pH 8.0 was inhibitory.

2. There was an absolute requirement for ATP as a source of high energy phosphates.

3. Mn^{++} and Mg^{++} stimulated MVA incorporation. Mn^{++} was more effective at low (1.25×10^{-3}M) concentrations but inhibitory at high (5.0×10^{-3}M) concentrations when compared with Mg^{++}.

4. The optimal temperature for maximal MVA incorporation was approximately 33°C. Temperatures below 10°C severely inhibited while temperatures above 50°C completely inhibited MVA incorporation.

5. Acetate-1-^{14}C was not incorporated into neutral terpenes even when reduced pyridine nucleotides were added.

6. Using MVA-1-^{14}C as the substrate, an immediate release of $^{14}CO_2$ was detected. Small but detectable levels

of $^{14}CO_2$ were also obtained when MVA-2-^{14}C was used as the substrate, but only after a lag period of approximately 45 minutes.

7. Enzymatic activity was always located in the supernatant solutions after increasing centrifugations up to 104,000 x g for one hour.

8. Anaerobic (nitrogen) incubation conditions slightly stimulated MVA incorporation and the release of $^{14}CO_2$ from MVA-2-^{14}C.

9. NAD, NADP and NADPH slightly inhibited MVA incorporation.

10. Sodium fluoride, niacinamide, streptomycin, chloramphenicol, CCC, SK&F 7997-A_3 and AMO 1618 had little or no effect on the amount of MVA incorporated. Phosfon D and iodoacetamide were highly inhibitory.

11. Campesterol, stigmasterol and β-sitosterol were identified in the iris extract. Little or no radioactivity was associated with these sterols when precipitated with digitonin.

12. At least three radioactive neutral terpenes were biosynthesized from MVA-2-^{14}C. Unequivocal identification of these ^{14}C-products was not made. Data suggested that one product was prenol-like, possibly farnesol or geranyl-geraniol.

13. Mn^{++} and microsomes were necessary for the biosynthesis of a non-polar ^{14}C-neutral terpene. AMO 1618 inhibited the biosynthesis of this non-polar product.

14. Either Mn^{++} or Mg^{++} were effected cofactors for the biosynthesis of the prenol-like product(s).

15. Some of the ^{14}C-prenol-like product(s) was non-covalently associated with TCA precipitable protein. Saponification released some of the radioactivity from the protein. Saponification also chemically altered at least one of the prenol-like products. This resulted in a shift to a more polar product.

16. Acid hydrolysis of the incubation media increased the hexane extractable radioactivity.

17. Under optimized incubation conditions, MVA incorporation rates as high as 3.0×10^{-9} moles d-MVA-2-^{14}C/mg protein/hour were observed.

PART II

INTRODUCTION

The study presented in this part of the dissertation was undertaken to determine: (1) Are changes in the quantity of GAs in tulip and iris during normal forcing treatments a response to changes in their biosynthetic rates in situ? (2) What tissues biosynthesize gibberellins in tulip and iris? A cell-free system was used in an attempt to answer these two questions. A discussion of the isolation and partial characterization of this cell-free system is presented in Part I.

It must be pointed out that only part of the life cycles of tulips and iris was investigated. Rees (1966) referred to three stages in the horticultural life cycles of bulbs: (1) the storage of dry bulbs after harvest, (2) growth to flowering and (3) growth from flowering to death of the above ground parts and harvesting. In the present study, only stage 2 and parts of stages 1 and 3 were investigated. Studies of the entire life cycle were not possible but they should be carried out in the future.

Temperature ranges referred to in the following pages were "optimum" in that rapid flowering was achieved. Temperatures other than optimum, but within the minimum and maximum limits would, in most cases, produce a flowering bulb but at a slower rate.

LITERATURE REVIEW

A literature review on how enrironmental stimuli, especially temperature, influence the growth and development of bulbs can take many approaches. In this dissertation, special emphasis was placed on environmentally induced physiological and biochemical changes in bulbs. Attention was also given to certain morphological developments of Tulipa Gesneriana L. and Wedgwood Iris, the experimental materials used in this study. More complete discussions of developmental bulb morphology can be found in the following references (Algera, 1936, 1947; Blaauw, 1941; Hartsema and Luyten, 1955; Hartsema, 1961; Hekstra, 1968; Kamerbeek, 1962; Luyten et al., 1926; Sass, 1944).

Morphological Development of Tulipa Gesneriana L. and Wedgwood Iris

Aspects of the morphological development of tulip and of iris are reviewed separately.

Tulip bulbs grown for forcing are harvested in midsummer after flowering is complete and the above ground parts have senesced. At harvest time the bulbs are vegetative and consist of fleshy scales, tunic, immature lateral buds, basal plate with preformed root initials and an apical meristem on a rosette stem with 1-2 foliage

leaves. Subsequent flower and bulb development depends on various conditions i.e. cultivar, size of bulb, the growing season of the previous year, temperature and other environmental conditions.

Under warm temperature conditions after harvest, flower development will proceed only after 2-3 more leaves are formed (Hartsema, 1961). During this warm temperature period, various stages of flower development become discernible. Mulder and Luyten (1928) and Beijer (1942) have described these stages. The most advanced stage of organogenesis is when the six tepals and anthers and the tri-lobed stigma are visible. This stage was called stage "G" by Beijer (1942) or VII by Mulder and Luyten (1928). At this stage of development the main growing axis consists of the flower, flower stalk and leaves.

The attainment of stage "G" or VII was thought to be a signal for commencement of low temperature treatments (De Hertogh et al., 1967; Hartsema, 1961). Application of low temperatures prior to reaching this stage often resulted in malformed plants. These low temperatures can be imposed on the bulbs when the bulbs are dry and not planted (precooling) or after the bulbs have been planted under moist conditions. During either type of low temperature treatment, some elongation growth of the floral organs, floral stalk and leaves takes place. This

low temperature period is necessary for proper stalk elongation under subsequent forcing conditions. Le Nard and Cohat (1968) have also shown that the low temperature treatment is needed for bulbing.

After the low temperature requirement has been met, which can only be determined by empirical knowledge of the cultivar, forcing period, etc., the bulbs are placed under warm, moist, lighted conditions for forcing. During this period, the floral stalk elongates rapidly, anthesis occurs, and finally the flower, floral stalk and leaves senesce.

Bulb development of the tulip during these same periods is undoubtedly related to flower development, however, bulb development was described separately to avoid confusion.

At harvest time, the new main bud usually cannot be detected. This bud, which is located in the axil of the innermost scale of the mother bulb, can only be identified after flower formation has begun (Hartsema, 1961; Rees, 1966). During subsequent cultural treatments, this bud will develop into the new main bulb and will consist of 5-6 scales and 1-2 foliage leaves. Hekstra (1968) referred to this bud as the apical or A bud.

A number of other lateral buds can develop depending on the size of the bulb. The scale next to the innermost scale produces the B bud, as denoted by Hekstra

(1968). C, D and E buds can also develop from subsequently more outer scales. The H bud develops from the axil of the tunic on the outside of the mother bulb. At harvest time it is possible to observe the C and D bud while the B bud is less often detected.

During moist, low temperature treatments, the lateral buds begin to develop into bulblets. At the same time, adventitious roots emerge from the basal plate of the mother bulb and the scales of the mother bulb begin to senesce. It is also possible for some of the developing bulblets to root and flower if they had been attached to the mother bulb for a sufficient period of time. This is particularly true for the H bulb.

When placed under forcing conditions, the scales of the mother bulb continue to senesce while the bulblets continue to enlarge. The termination of bulblet enlargement occurs when the above ground parts and scales of the mother bulb have completely senesced. These bulblets are now mature bulbs and the process is again renewed.

In contrast to the warm temperature requirement for flower formation in tulip, Wedgwood Iris require low temperatures for flower initiation and development. After the iris are harvested in mid-summer, a six week period of 9-13°C will initiate flowering of the main axis (Blaauw, 1941). A second flower is also initiated in the axil of the second spathe-leaf. If this low temperature

treatment is preceded by a 31°C treatment for 1-2 weeks, flower formation under subsequent low temperature is greatly accelerated (Hartsema and Luyten, 1940; Stuart and Gould, 1949).

To retard flower initiation and development, iris bulbs can be stored at constant high temperatures (28-30°C) immediately after harvesting (Hartsema and Blaauw, 1935). When iris are held in this manner, it is possible to have a year-round source of vegetative bulbs for forcing or experimental use. These are known as retarded iris.

After a low temperature treatment, the iris are planted and placed under forcing conditions. At the onset of this forcing period, the flowers are nearly or completely formed. Extension growth only comes after flower formation is complete (Hartsema, 1961). As with the tulip, the various floral stages of the iris have been described (Blaauw, 1935).

In iris, leaf development is related to flower initiation and development. At harvest, an average of 4 leaves are present (Hartsema and Luyten, 1955). If the low temperature treatment is applied starting directly after harvest, 2-3 more leaves are formed, however, if retarding temperatures are used then more leaves are formed before the bulb becomes reproductive. For example, Hartsema and Luyten (1955) showed that storage of iris

after lifting at 23°C resulted in an average of 8.8 leaves. It is important to note that regardless of the number of leaves, the last two formed will become spathe-leaves.

New bulbs are formed from bulblets in the axils of sheathing and foliage leaves of the mother bulb. A two year period is normally needed before these bulblets reach flowering size (Hartsema, 1961). It is important for the bulb producers to prevent flowering during this period to allow maximum bulb growth. A temperature sequence of 7 weeks at 5°C followed by 5 weeks at 20°C after harvesting will prevent flowering and stimulate growth of the new bulbs (Hartsema, 1961).

Physiological and Biochemical Development of Bulbs

The physiology and biochemistry of bulbs has received little attention until recent years. The general research approach has been to single out certain compounds or processes (e.g. respiration) and to monitor them under various environmental conditions. In this review, the following process and compounds are discussed in relation to growth and development of bulbs: carbohydrates, proteins and amino acids, enzymes, growth regulators and respiration.

Carbohydrates

Pinkof (1929) studied the changes of carbohydrates in various cultivars of tulips. He concluded that temperature influenced the chemical composition of these bulbs and specifically that low temperature caused a decrease in starch with a concurrent increase in non-reducing sugars.

Algera (1936, 1947) expanded Pinkof's work of 1929 using tulips and hyacinths as experimental material. Using a May harvest of the tulip cultivar Murillo, he found reducing sugars remained at a constant low level through December regardless of the temperature treatment. From December to February, the levels increased with planted bulbs at all temperatures. Non-planted bulbs held at 17°C during this same period showed no change from the previous low level. The earliest rise in reducing sugars occurred when a low temperature treatment preceded planting. He showed further that non-reducing sugars and starch had an inverse relationship. The non-reducing sugars quickly decreased after lifting as the starch content increased. Following this initial change, the non-reducing sugars increased slowly as starch decreased. These changes were found to be dependent on temperature. As with reducing sugars, non-planted bulbs stored at 17°C showed no or little change in starch or non-reducing sugars. Of special interest was the

"after-effect" of applying a low temperature period to tulips in the summer. The effects of this low temperature period were not observed until fall when the breakdown of starch was enhanced.

Similar carbohydrate studies were reported with Wedgwood Iris. Rodrigues Pereira (1962) found a decrease in starch, sucrose, reducing sugars and polyfructosides in iris scales during flower formation at 13°C with a concurrent increase of these substances in the buds. Halevy et al. (1963) and Kamerbeek (1962) reported similar findings.

In an attempt to determine how low temperatures accelerated flowering with lily, Stuart (1952) studied changes in carbohydrates in relation to temperature. His results showed that at low temperatures there was a more rapid hydrolysis of carbohydrates with an increase of reducing sugars and sucrose when compared to bulbs held at high temperatures.

Proteins-Amino Acids

Fowden and Steward (1957a, 1957b) and Zacharius et al. (1957) reported on nitrogenous compounds in bulbs of the Liliaceae family, with special emphasis on Tulipa Gesneriana L. Their findings with T. Gesneriana L. are summarized as follows: (1) two unusual amino acids, λ-methyleneglutamic acid and λ-methyleneglutamine, were

present in the tulip; (2) these two amino acids appeared to have an inactive metabolism; (3) the amino acid content, especially arginine, aspartate and glutamine, changed quantitatively depending on temperature, light, tissue and growth period; (4) amino acid turnover in excised, mature leaves was slow; and (5) total soluble nitrogen was lowest during certain stages of corolla and androecium development.

Higuchi and Sisa (1967) showed that protein content in tulip scales decreased during low temperature treatment. They attempted to determine the stage when sufficient low temperature was 'perceived' by the bulb, to ensure proper growth and development, by comparing levels and types of proteins. To date, this stage has not been delineated by this or any other method.

Halevy and Shoub (1964b) studied nitrogen changes in iris bulbs from flowering to senescence. They showed that nitrogen levels decreased steadily until the scape began to senesce. They suggested that this was a result of proteins (amino acids) being translocated from the desiccating scape to the developing bulbs.

Barber and Steward (1968) studied proteins of tulip in relation to morphogenesis. Using the tulip cultivars Greenland and Golden Harvest, they reported that there were organ specific proteins and that these proteins showed marked changes soon after floral initiation. In

particular, vegetative and floral organs exhibited large differences in the enzyme proteins studied, i.e. isozymes of esterase and malic acid dehydrogenase.

Enzymes

Peroxidase, catalase, invertase and malic acid dehydrogenase were some of the enzymes that have been studied in bulbs. The changing activity of these enzymes was often correlated to developmental stages of bulbs which were influenced by the environment.

Peroxidase and catalase have been studied in iris during curing (Halevy et al., 1963; Van Laan, 1955), precooling (Halevy et al., 1963) and from flowering to senescence (Halevy and Shoub, 1964). Halevy et al. (1963) reported small changes in peroxidase and catalase activity in iris when stored at either 25 or 2°C from the time of lifting. During this period, flower initiation did not occur. A normal forcing temperature sequence of 30-25-10°C increased enzyme activity after two weeks into the 10°C period, but only after flower initiation had begun. Similar results were obtained by Van Laan (1955) with catalase. He noted, however, a decrease in catalase activity during curing temperatures. Both studies used whole bulbs as sources of enzymes.

In the period from anthesis to senescence, catalase activity in iris buds and scales increased while peroxidase

activity decreased in both tissue (Halevy and Shoub, 1964). Respiration, believed to be correlated with the activity of these enzymes (Burris, 1960), increased in the bud and decreased in the scales.

Invertase was reported to increase during low temperature treatments in tulips (Hartsema, 1961), however, no data was presented to support this statement.

Malic acid dehydrogenase activity was one of the enzymes studied by Barber and Steward (1968) in relation to morphogenesis of tulip. From 4 to 7 isozymes of this enzyme were reported to be present in each of the various organs tested of the tulip for a total of 9 different isozymes. Certain isozymes were related to reproductive growth because they were detected only in reproductive tissue. Furthermore, after tulip bulbs were placed under warm temperatures necessary for floral initiation, these 'reproductive' isozymes were detected in the apex prior to any visible reproductive tissue.

Growth Regulators

Extractable gibberellin-like substances (GAs) (Aung and De Hertogh, 1967, 1968; Aung et al., 1969a, 1969b, 1970; Barendse, et al., 1970; Einert et al., 1970a, 1970b; Halevy, 1970; Rodrigues Pereira, 1965) and ethylene (Staby and De Hertogh, 1970b) have been isolated from many bulb species while auxin has only been detected in

lilies (Stewart and Stuart, 1942; Tsukamoto, 1970). Considering the importance of growth regulators, surprisingly little is known about their presence or roles in bulbs.

As identified by the Avena coleoptile curvature bioassay, auxin was present in lily (Stewart and Stuart, 1942; Tsukamoto, 1970). During the 'dormant' period after lifting, the stem tip contained about 1000 fold greater auxin concentration than the scales or basal plate and 5 fold greater concentration than the entire stem tissue (Stewart and Stuart, 1942). Storage of the bulbs for 30 days in moist peat at 25°C increased the levels in the stem tips and basal plates while decreasing levels were measured in scales and stems.

Ethylene has been isolated from the internal atmosphere of five bulb species (Staby and De Hertogh, 1970b). The source of this gas may have been the bulb tissue itself, microorganisms normally associated with bulbs or a combination of the two. A protective fungicide, 'Benlate' (Methyl 1-(butylcarbamoyl)-2-bensimidazole carbamate), reduced the amount of extractable ethylene suggesting that fungi were at least partially responsible for the gas.

Many roles of ethylene in plants have been suggested or determined (Pratt and Goeschl, 1969), however, roles of ethylene in bulbs are unknown. Certain disorders in

bulbs have been suggested to be ethylene induced (Kamerbeek, 1970; de Munk, 1970). Exogenously applied ethylene hastened the flowering of iris (Stuart et al., 1966) while similar concentrations induced injuries with many other bulb species (Doubt, 1917; Hitchcock et al., 1932). The extent of the responses to ethylene, whether positive or negative, depended on the concentration of the gas, duration of exposure, temperature, bulb species and developmental stage of the bulbs when applied.

GAs were detected in many bulb species (Aung and De Hertogh, 1967, 1968; Aung et al., 1969a, 1969b, 1970; Barendse, et al., 1970; Einert et al., 1970a, 1970b; Halevy, 1970; Rodrigues Pereira, 1965). They changed both quantitatively and qualitatively in tulips depending on the cooling (Aung and De Hertogh, 1968) and forcing (Aung et al., 1969b) treatments and on moisture (Aung et al., 1970). Flowers and developing bulblets were high sources of GAs in tulip while roots and scales had low amounts (Einert et al., 1970a). Data also indicated that GAs were biosynthesized in the anthers of tulips (Marré, 1946; Staby and De Hertogh, 1970a), and three species of lily (Barendse et al., 1970). The biosynthesis and/or release of GAs in anthers has been thought to be a general phenomenon with other plant species (Greyson and Tepfer, 1967; Plack, 1957, 1958). Emasculation or spontaneous atrophy of tulip anthers resulted in subsequent

growth inhibition (Marré, 1946). This type of inhibition can be reversed by gibberellins in some plant species (Greyson and Tepfer, 1967).

The experiments of Rodrigues Pereira (1961, 1962, 1964, 1965, 1966) and Halevy and Shoub (1964) with iris suggested that GAs were involved in flower initiation and development and scape elongation. These findings were supported by experiments with exogenous and endogenous gibberellins (Halevy and Shoub, 1964; Rodrigues Pereira, 1962, 1964, 1965). Sites of gibberellin biosynthesis in iris were thought to be foliar and sheath leaves and scales (Rodrigues Pereira, 1964).

Respiration

Working with tulip and hyacinth, Algera (1936, 1947) reported that respiration decreased after lifting followed by an increase depending on the cultural practices. This increase in respiration was further enhanced following planting. The cultural practices imposed on the bulbs varied mainly with temperature. Differences in light and moisture as well as differences in bulb development confounded respiration results when attempting to interpret them solely on the basis of temperature. It can be concluded from Algera's work, however, that low temperature treatments prior to planting with tulip enhanced CO_2 and O_2 exchange and stimulated greater rates of respiration after planting.

Algera (1936) concluded that low temperature did not regulate tulip respiratory enzymes based on mathematical manipulation of his data. Instead, he stated that one of two explanations may be correct in relating low temperature to respiration: (1) the hydrolysis of of starch into sugars resulted in a release of heat. Making use of the principle of moving equilibrium, any process which evolves heat will increase its products (sugars) upon lowering the temperature, (2) low temperatures increased the amount of 'free' water in the bulbs. This increase in 'free' water enabled more sugars to be dissolved thus shifting the equilibrium of starch breakdown in favor of sugars.

More respiratory information is known in the case of iris. Studying the respiration rates from lifting to flowering, Kamerbeek (1962), Halevy et al. (1963) and Rodrigues Pereira (1962) basically agreed on the temperature influenced respiratory changes. Their findings are summarized as follows.

After the iris bulbs were lifted from the field in mid-summer, respiration decreased rapidly at high temperatures until a steady stage was reached 2-3 weeks later. Heat curing further decreased respiration. Following this curing period, respiration increased at low temperatures. This increase reached a peak when the floral organs began to differentiate. This peak was followed

by another decrease. During sprouting after planting, respiration increased to a second peak and then decreased again prior to anthesis.

Using 2-thiouracil (an inhibitor of nucleic acid biosynthesis) and gibberellic acid (GA_3), Rodrigues Pereira (1966) concluded that there was only a very indirect relationship between transition to the reproductive stage and the increase in respiration rate. Halevy et al. (1963) concluded that low temperature itself did not increase respiration rates in iris and will only do so when inserted at the proper stage in the thermoperiodic sequence of this bulb. In this regard, low temperatures imposed on the iris right after lifting decreased respiration to a rate lower than that observed at curing or retarding temperatures.

Halevy and Shoub (1964) investigated respiration rates from flowering to senescence. During this period, the rate of respiration in the scales decreased. In contrast, the rate in the buds initially increased and then decreased at senescence. These findings emphasized that the choice of tissue for measuring respiration was critical.

EXPERIMENTAL

Preliminary experiments were carried out during the 1968-69 season using T. Gesneriana L. cvs. Ralph and Elmus as enzyme sources for studying the biosynthesis of terpenes. More detailed experiments were performed in 1969-70 using the same tulip cultivars and the Wedgwood Iris. The materials and methods for the 1968-69 and 1969-70 experiments are described separately.

1968-69 Experiments

Materials

The tulip cultivars Ralph and Elmus, size 12 cm and up, were received from the Laboratory for Flower-Bulb Research in Lisse, The Netherlands. The sources of all chemicals were the same as noted in Part I of this dissertation.

Methods

Cultural treatments.--On arrival, all bulbs were stored dry at 20°C until the treatments were initiated. When treatments necessitated, the bulbs were planted in 8 x 8 x 4 inch wooden flats with 16 bulbs per flat. A soil mixture of equal volumes sandy loam, sand and peat was used. Watering was done when necessary to keep the

plants moist at all times. No fertilizers were added. Table 21 summarizes the treatments for the 1968-69 experimental season.

Extraction and assay procedures.--Periodic extractions were made throughout the treatment period to determine the relative incorporation rates of MVA-2-^{14}C into neutral terpenes. Depending on the particular treatment and development of the bulb; roots, fleshy scales, leaves and/or floral tissue were used as the enzyme sources. A total of 48 bulbs were used per extraction.

Bulb tissue was extracted and assayed in the following manner: (1) tissue was homogenized at 0°C for two minutes at top speed in a 'Lourdes' mixer using a 0.1M Tris buffer (1:3, wt.:vol.) at pH 8.2 containing 0.02M niacinamide, (2) the extracts were filtered through cheesecloth and centrifuged at 15,000 x g for 30 minutes, (3) 2.5 ml of the supernatant was added to 1.0 ml buffer, 5 x 10^{-6} moles each of $MgCl_2$ and ATP, and 1.7 x 10^{-8} moles MVA-2-^{14}C (DBED salt) for a total volume of 4.0 ml, (4) the mixture was incubated for 2 hours at 25°C in a water bath shaker at 150 r.p.m., (5) the reaction was stopped by adding 4.0 ml ethanol and 0.5 ml 50% KOH and the mixture was slowly brought to a boil, (6) after cooling, neutral lipids were extracted twice with 10.0 ml hexane each and (7) the hexane was evaporated in vacuo at 40°C

TABLE 21.--Summary of treatments imposed during 1968-69 season.

Tulip cv.	Treatment Code	Arrival Date	Planting Date	Temperature Treatments (°C)		Date into Greenhouse
				Before Planting	After Planting	
Ralph	R-1	8/31/68	10/11/68	6W-9*	3W-9; 5W-5	12/6/68
Ralph	R-2	8/31/68	9/19/68	3W-20	5W-9; 10W-5	1/3/69
Ralph	R-3	8/31/68	11/9 /68	10W-20	3W-9; 4W-5; 6W-2	2/21/69
Ralph	R-4	8/31/68	not planted	14W-20	--	--
Elmus	E-1	8/31/68	11/9/68	10W-20	3W-9; 4W-5; 6W-2	2/21/69
Elmus	E-2	8/31/68	not planted	12W-20	--	--

*6W-9 signifies, for example, 6 weeks at 9°C.

with the residue resuspended in scintillation fluor for counting.

Liquid scintillation counting.--Liquid scintillation counting techniques were the same as described in Part I of this dissertation.

Thin-layer chromatography.--TCL techniques were the same as described in Part I of this dissertation. The various developing solvents are noted in the text.

1969-70 Experiments

Materials

The sources of the tulip cultivars Ralph and Elmus, size 12 cm and up; Wedgwood Iris, size 10 cm; and of all chemicals were the same as previously noted.

Methods

Cultural treatments.--Table 22 summarizes all treatments for the 1969-70 experimental season. Wooden flats, measuring 20 x 14 x 4 inches, were used with 50 bulbs per flat. Otherwise, similar cultural treatments were employed as described for the 1968-69 experiments.

Extraction and assay procedures.--The extraction and assay procedures were similar to those described for the 1968-69 experiments. Differences from the previous procedures were: (1) the concentration of ATP and $MgCl_2$

TABLE 22.--Summary of the treatments imposed during 1969-70 season.

Bulb Species	Arrival Date	Planting Date	Temperature Treatments (°C)		Date Into Greenhouse
			Before Planting	After Planting	
T. Gesneriana cvs.					
Ralph	9/7 /69	9/28/69	3W-20*	6W-9; 7W-5	1/5 /70
Elmus	10/12/69	10/26/69	2W-20	3W-9; 5W-5; 8W-2	2/15/70
Wedgwood Iris	10/5 /69	11/30/69	2W-20; 6W-9	greenhouse temps.	11/30/69

*3W-20 signifies, for example, 3 weeks at 20°C.

was increased to 1 x 10^{-5} moles per assay, (2) 1 x 10^{-5} moles of $MnCl_2$ was added per assay, (3) the incubation time was reduced to one hour, (4) when stamens were used as the source of enzymes, the homogenizing ratio was altered to 1:10, wt.:vol., (5) no niacinamide was added to the buffer, (6) 50 µg/ml chloramphenicol was added to the buffer and (7) 100 bulbs were used per determination.

Liquid scintillation counting.--Liquid scintillation counting techniques were the same as described in Part I of this dissertation.

Protein determinations.--The determination of protein was the same as described in Part I of this dissertation.

Pollen development.--Various stages of pollen development were determined by smearing the anthers in the presence of acetocarmine. This stain was prepared by adding 1.0 g carmine to 100 ml of boiling 45% acetic acid. The iron source was the dissecting needles. The smears were flamed and then examined under a microscope.

RESULTS

Results of the 1968-69 and 1969-70 experiments are presented separately. Results on the incorporation of MVA-2-^{14}C into neutral terpenes are expressed as the complete reaction mixture less the minus ATP cell-free assay. Data, not reported, showed that the minus ATP assay was equivalent to the boiled enzyme control plus ATP.

'Enzyme activity' refers to the activity of terpene biosynthesizing enzymes in the cell-free system which transform MVA-2-^{14}C into neutral terpenes. This 'enzyme activity' is presented as the radioactivity (cpm) in the neutral lipid fraction after either two (1968-1969) or one (1969-70) hour incubations.

1968-69 Experiments

Figures 21 to 26 summarize the results of the treatments listed in Table 21. Both elongation growth data and relative enzyme activity of scape tissue are shown.

With both tulip cultivars, from 97 to 100% of the enzyme activity was found to be associated with scape tissue (data not reported). For this reason, no enzyme activity data is presented from extracts of root or fleshy scale tissue.

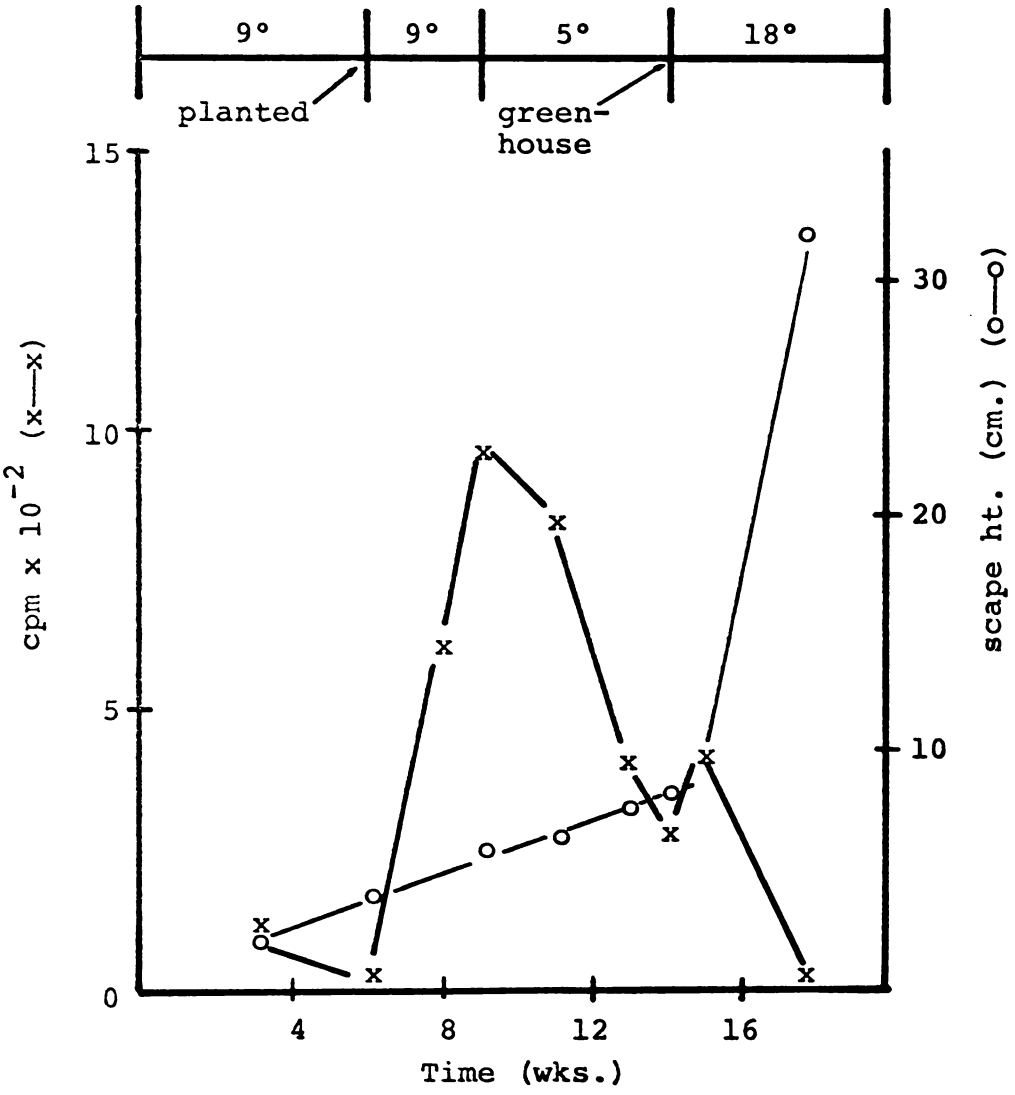

Figure 21.--The incorporation of MVA-2-^{14}C into neutral terpenes using scape extracts of T. Gesneriana L. cv. Ralph and scape elongation growth during forcing treatments (treatment R-1).

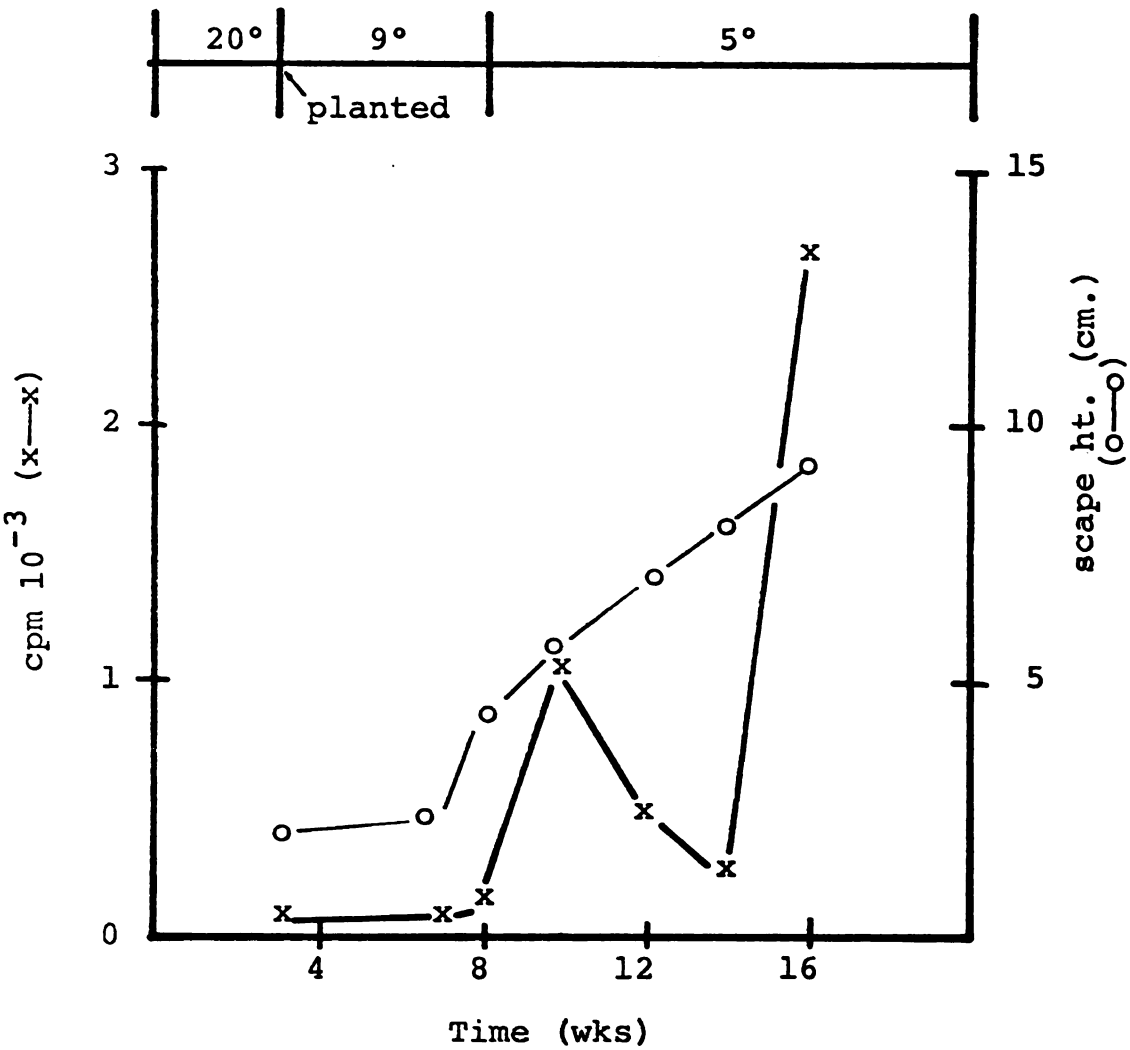

Figure 22.--The incorporation of MVA-2-^{14}C into neutral terpenes using scape extracts of T. Gesneriana L. cv. Ralph and scape elongation growth during forcing treatments (treatment R-2).

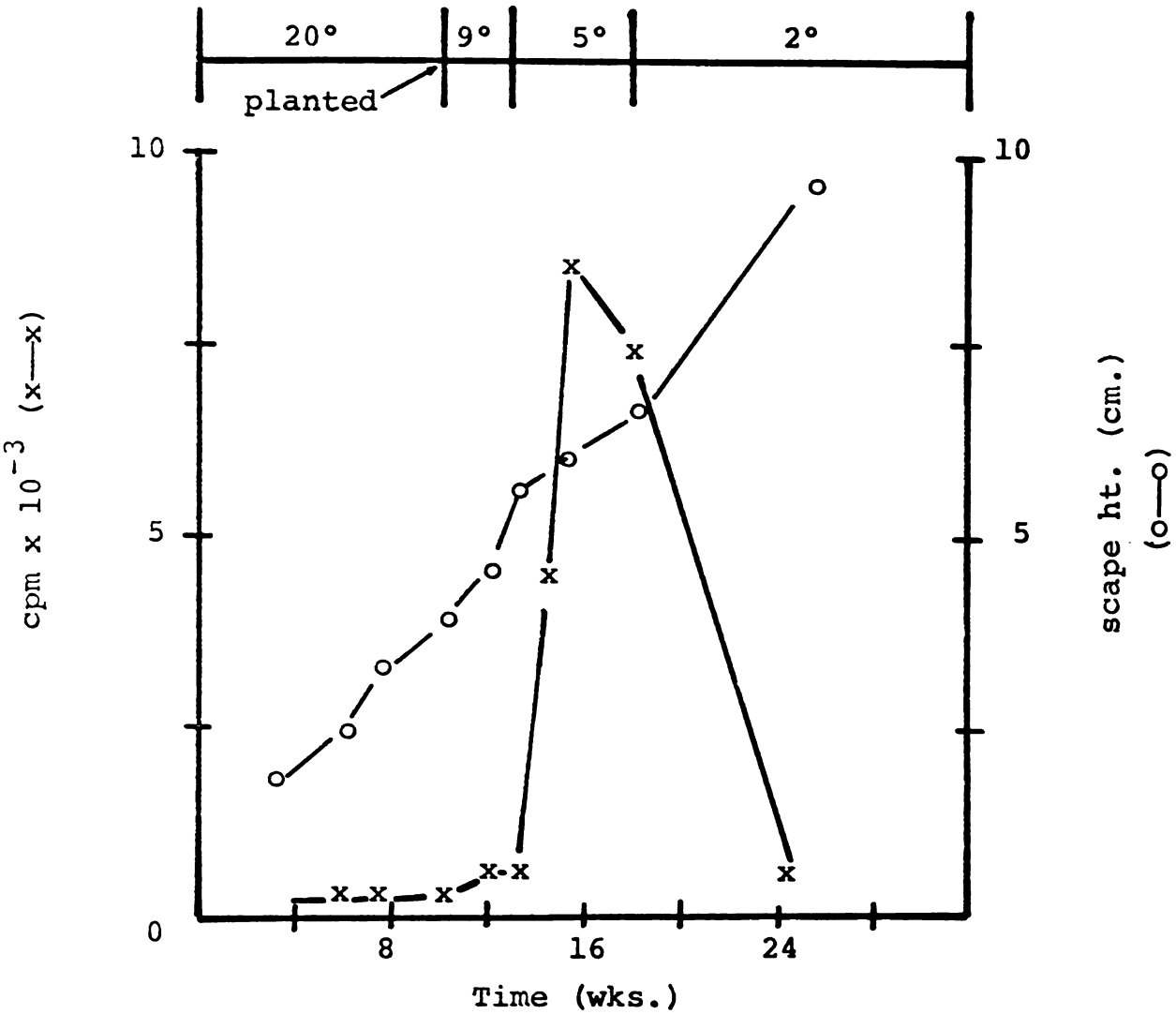

Figure 23.--The incorporation of MVA-2-^{14}C into neutral terpenes using scape extracts of T. Gesneriana L. cv. Ralph and scape elongation growth during forcing treatments (treatment R-3).

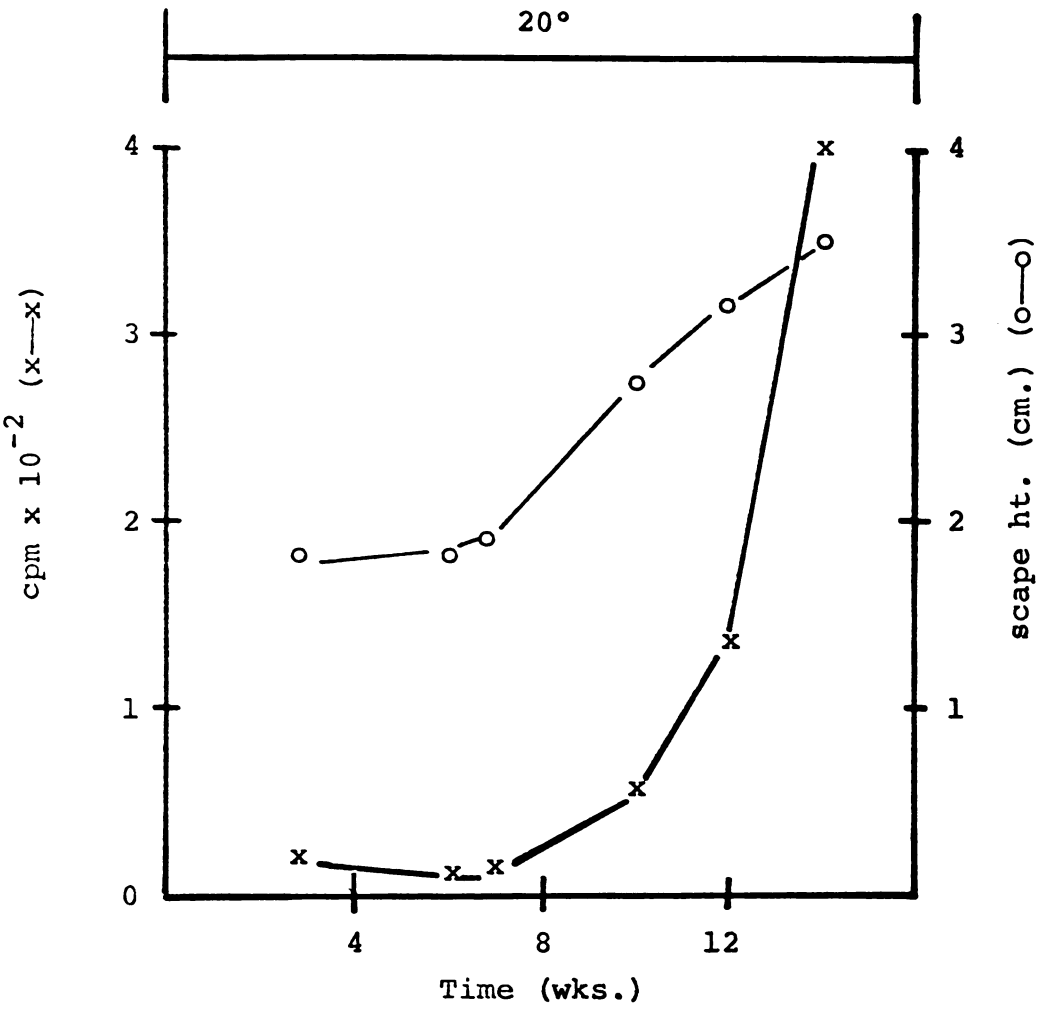

Figure 24.--The incorporation of MVA-2-^{14}C into neutral terpenes using scape extracts of T. Gesneriana L. cv. Ralph and scape elongation growth during forcing treatments (treatment R-4).

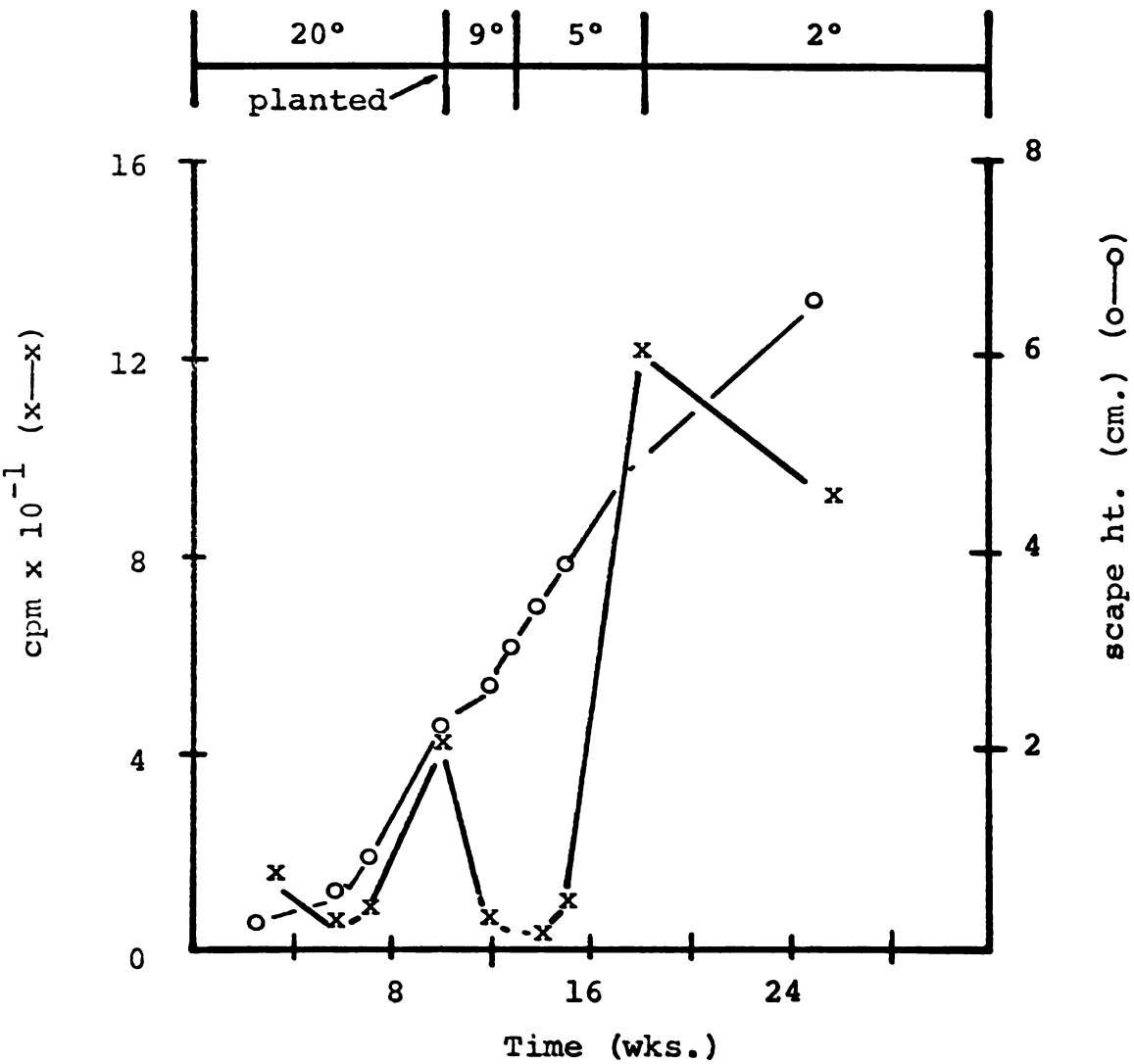

Figure 25.--The incorporation of MVA-2-^{14}C into neutral terpenes using scape extracts of T. Gesneriana L. cv. Elmus and scape elongation growth during forcing treatments (treatment E-1).

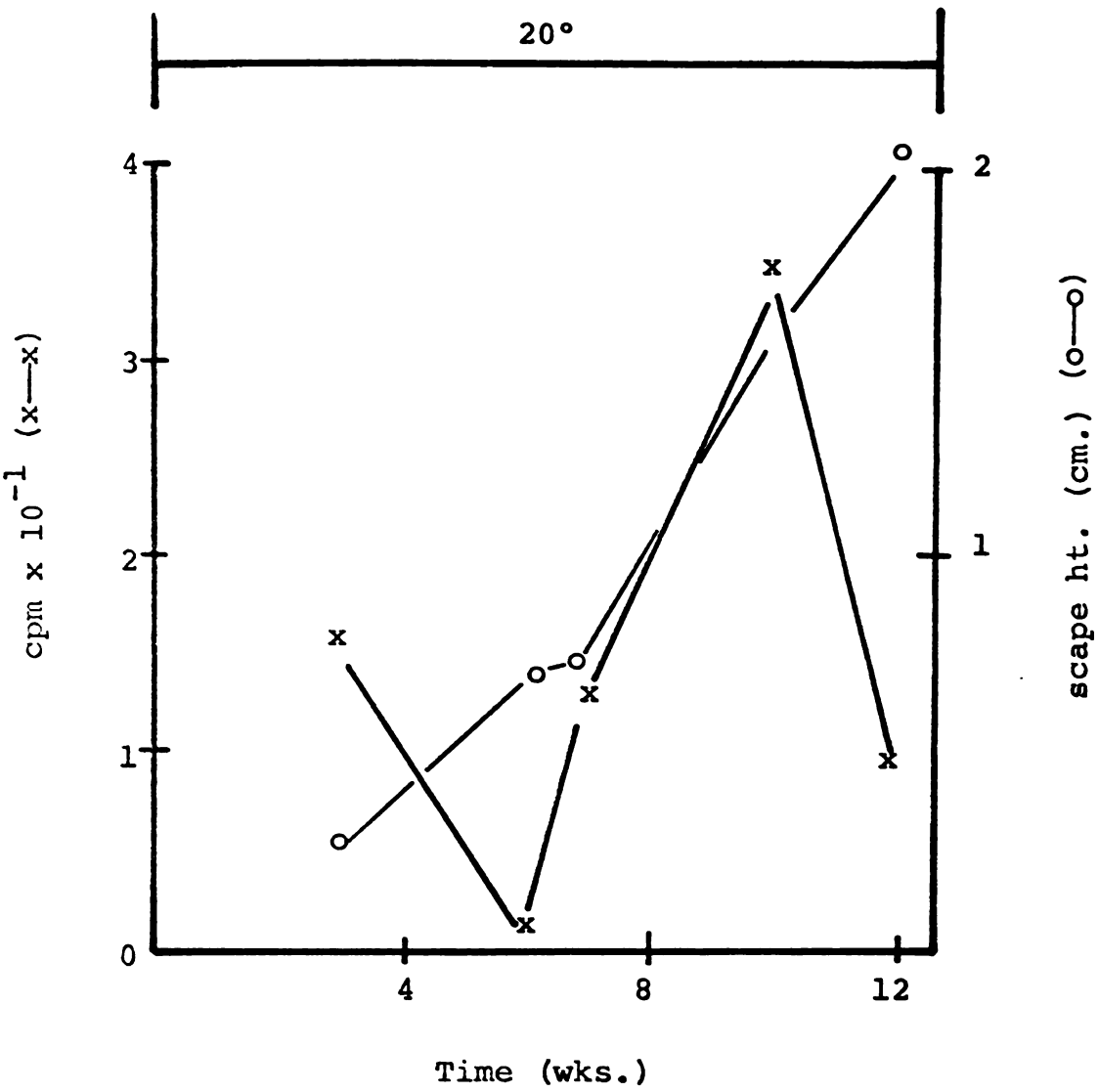

Figure 26.--The incorporation of MVA-2-^{14}C into neutral terpenes using scape extracts of T. Gesneriana L. cv. Elmus and scape elongation growth during forcing treatments (treatment E-2).

Only in treatment R-1 (Figure 21) was enzyme activity data determined through flowering. With treatments R-2, R-3 and E-1 (Figures 22, 23, 24), monitoring of enzyme activity was discontinued prior to placing the bulbs into the greenhouse. The experimental tissue from these discontinued treatments was used, instead, as enzyme sources to investigate other problems. However, sample lots of treatments R-2, R-3 and E-1 were grown through flowering to obtain growth and flowering data (Table 23). Termination of treatments R-4 and E-2 (Figures 24, 26) was due to decaying of these non-planted bulbs.

Scape Elongation Growth

With all treatments (Figures 21-26), scape length increased with time. The fastest growth rates occurred after placing the bulbs into the greenhouse. Treatment R-1 exemplified this response (Figure 21). Planted bulbs exposed to moisture and low temperatures exhibited faster growth rates (Figures 21, 22, 23, 25) when compared to non-planted bulbs stored at 20°C (Figures 24,26).

Enzyme Activity

Enzyme activity rates were generally high with the cell-free extracts of cultivar Ralph (Figures 21-24). Low temperatures alone and in combination with moisture increased enzyme activity. Precooling (R-1) resulted in

TABLE 23.--Summary of the mean elongation growth and flowering data of T. Gesneriana L. cvs. Ralph and Elmus for the 1968-69 experiments.

Treatment Code*	Final Plant ht. (cm)	Flower Size (cm)	Flowering Date**	Days to Flower***
R-1	32.9	4.8	1/4/ 69	29
R-2	33.0	3.7	1/27/69	25
R-3	25.0	4.5	3/7 /69	15
R-4	Were not planted and forced.			
E-1	40.0	5.0	3/16/69	24
E-2	Were not planted and forced.			

*See Table 21 for explanation.

**Date at which 50% or more of the plants are in flower.

***Determined from date into greenhouse.

an earlier increase in activity after planting (Figure 21) compared to non-precooled bulbs of treatments R-2 and R-3 (Figures 22, 23). This earlier rise in activity with treatment R-1 occurred even though the non-precooled bulbs of treatment R-2 were planted prior to the precooled bulbs of treatment R-1. Activity increase was further delayed by storing the bulbs at 20°C dry for longer periods prior to planting (R-3, Figure 23). Continuous storage or Ralph at 20°C dry resulted in an increase in activity up until the bulbs decayed (Figure 24), however, this increase was small compared to planted and cooled bulbs of this same cultivar (Figures 21-23).

Changes in enzyme activity with the cultivar Elmus were slight (Figures 25, 26). The increase in activity with treatment E-1 during the 5°C treatment and the decrease at 2°C (Figure 25) was consistent with activity changes noted with treatment R-3 (Figure 23).

Other experiments with the cultivar Elmus revealed a drastic loss of enzyme activity when the bulbs were placed into the greenhouse (Table 24). This decrease in activity was also reflected in the results of treatment R-1 (Figure 21).

High enzyme activity was observed with the cultivar Elmus at the time the bulbs were moved from low temperature treatments into the greenhouse (Table 24). This activity level was higher than any other level previously

TABLE 24.--Effect of greenhouse forcing conditions on the incorporation of MVA-2-^{14}C into neutral terpenes by enzymes extracted from stamens of T. Gesneriana L. cv. Elmus.

Days in Greenhouse	Incorporation (cpm)	Scape ht. (cm)
0	8440	9.0
7	70	23.3
14	0	38.9

detected with this cultivar (Figures 25, 26). However, these bulbs exhibiting high activity were not a part of the original shipment of Elmus used in the treatments listed in Table 21. These 'higher activity' Elmus were of better quality than the main lot of Elmus and were forced for a later flowering period.

Localization of Enzyme Activity

With both tulip cultivars, from 97 to 100% of the enzyme activity was located in the cell-free extracts of scape tissue (data not reported). Of the various scape tissue tested, only the stamens produced an active extract (Table 25).

Identification of ^{14}C-Products

Absolute identification of the ^{14}C-products with Ralph and Elmus extracts were not determined. Results presented in Table 26 tentatively identified farnesol as one of the ^{14}C-products with both tulip cultivars. Kaurene, geraniol and nerolidol were definitely not present as radioactive products.

1969-70 Experiments

Table 27 and Figures 27 to 30 summarize the protein, growth and enzyme activity results of the 1969-70 experiments. It must be re-emphasized that the levels of

TABLE 25.--Incorporation of MVA-2-^{14}C into neutral terpenes by enzymes from various sources of T. Gesneriana L. cv. Ralph.

Treatment Code*	Enzyme Source	Incorporation (cpm)
R-3 (week 18)	Flowers	18,044
	Scape	8,389
R-2 (week 15)	Stamens	12,276
	Tepals + Gynoecium	231

*See Table 21 for explanation.

TABLE 26.--Thin-layer co-chromatography of the ^{14}C-products biosynthesized in a cell-free system using extracts of T. Gesneriana L. cvs. Ralph and Elmus with geraniol (G), farnesol (F), nerolidol (N) and (-)-kaurene (K) standards (% total cpm).

R_F	Solvent*								
	A			B			C		
	Ralph	Elmus	Standards	Ralph	Elmus	Standards	Ralph	Elmus	Standards
0.1	92	92	G, F	0	1		0	0	
0.2	0	2	N	0	0		2	0	
0.3	0	0		0	0		0	0	
0.4	8	6		0	0		38	33	G
0.5	0	0		0	0		44	62	F
0.6	0	0		0	0		0	2	
0.7	0	0		1	1	G	15	0	N
0.8	0	0		71	75	F	1	2	
0.9	0	0		15	9	N	0	0	
1.0	0	0	K	13	14	K	0	0	K

*A = hexane-ethlyether-acetic acid (90:10:1)
B = chloroform-acetone (9:1)
C = benzene-ethyl acetate (9:1)

TABLE 27.--Localization of enzyme activity for the incorporation of MVA-2-^{14}C into neutral terpenes with extracts from T. Gesneriana L. cvs. Ralph and Elmus and Wedgwood Iris (% total cpm incorporated).

Bulb Species	Tissue			
	Root	Fleshy Scale	Shoot	Stamens
Wedgwood Iris	9	18	73	*
T. Gesneriana L.				
Ralph	0	5	0	95
Elmus	0	6	0	94

*Was not determined.

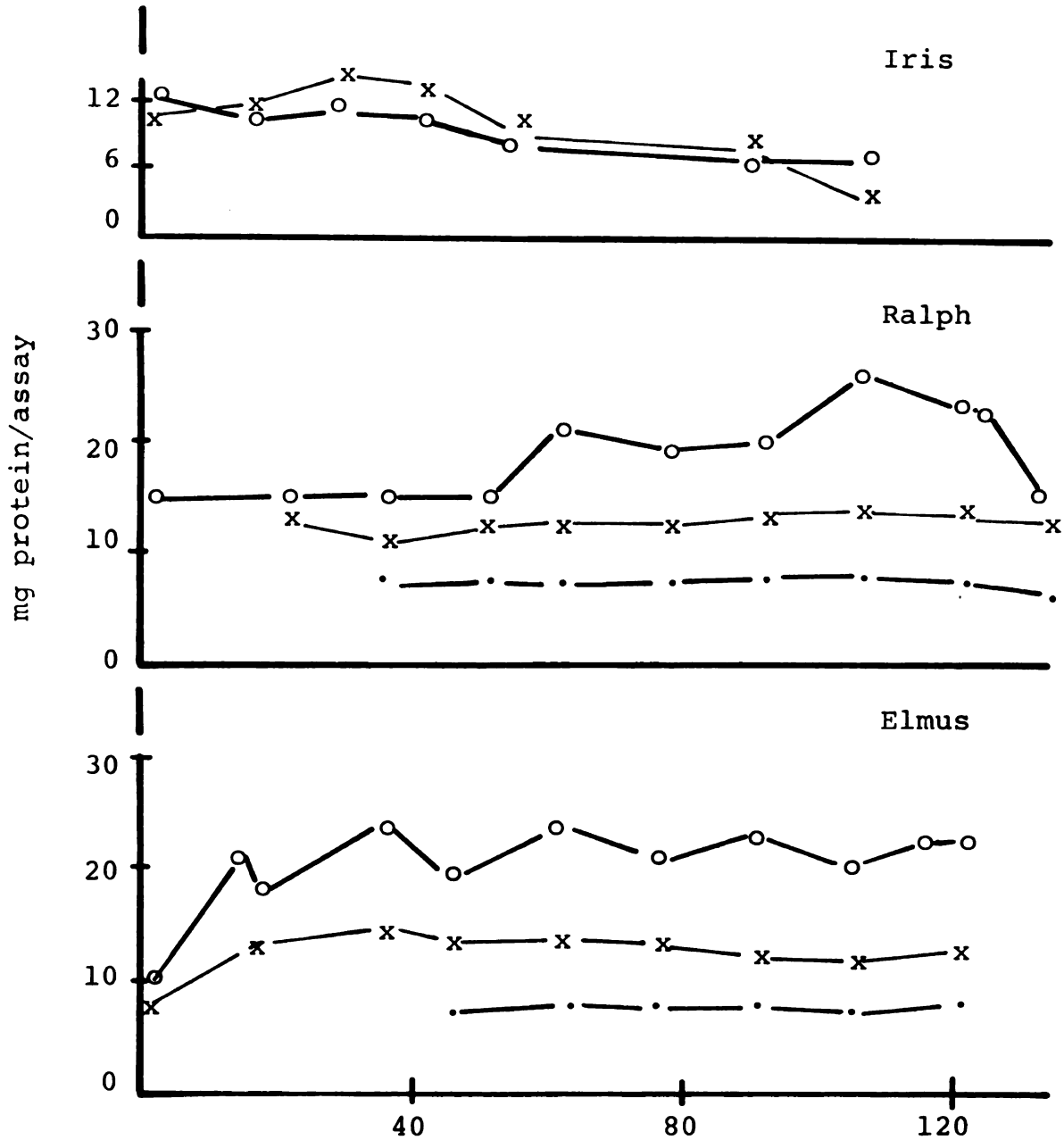

Figure 27.--Changes in TCA precipitable protein levels during forcing treatments using shoot (●--●) and scale (x—x) extracts of Wedgwood Iris and scape (o—o) scale (■—■) and stamen (•—•) extracts of T. Gesneriana L. cvs. Ralph and Elmus.

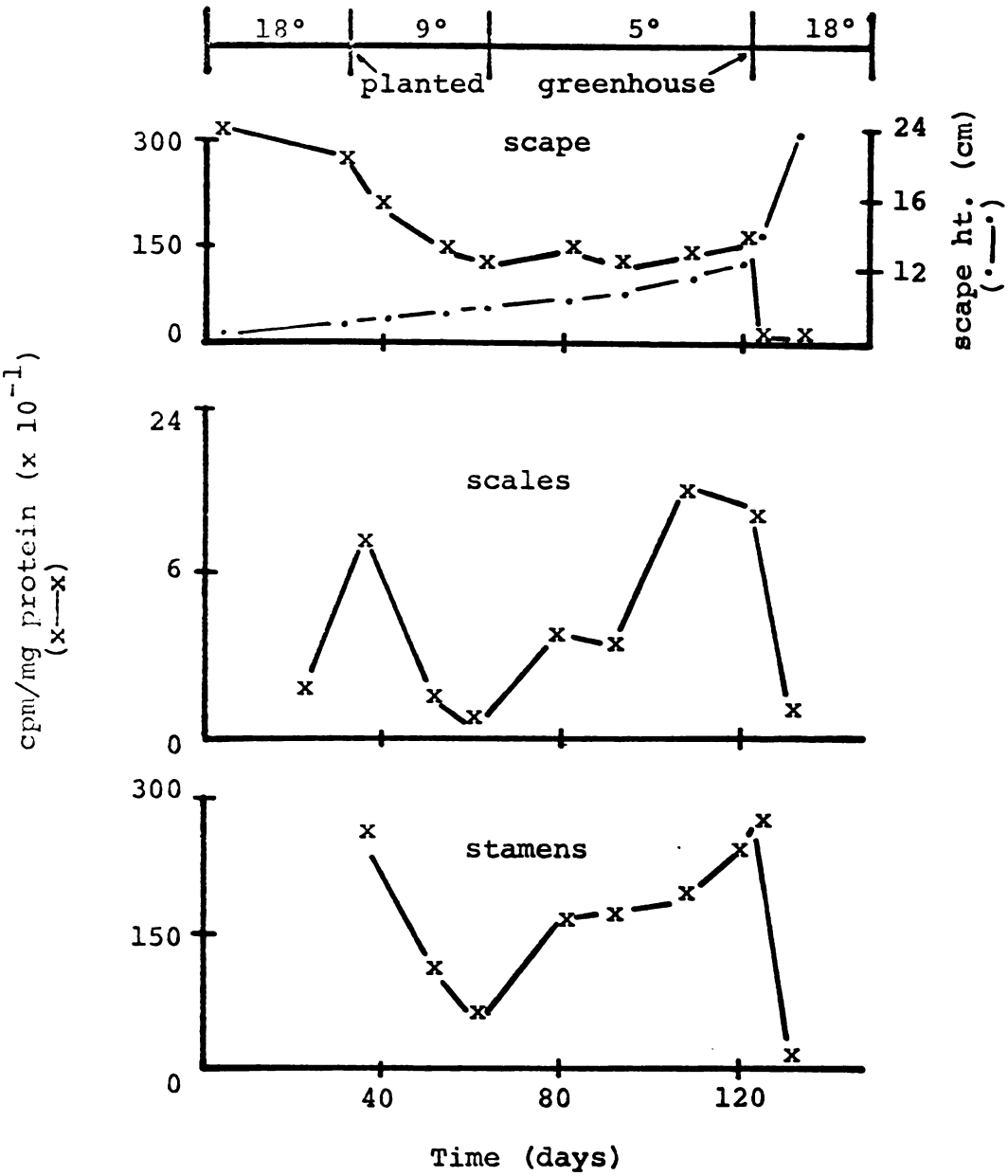

Figure 28.--The incorporation of MVA-2-^{14}C into neutral terpenes using scape, scale and stamen extracts of T. Gesneriana L. cv. Ralph and scape elongation growth during forcing treatments.

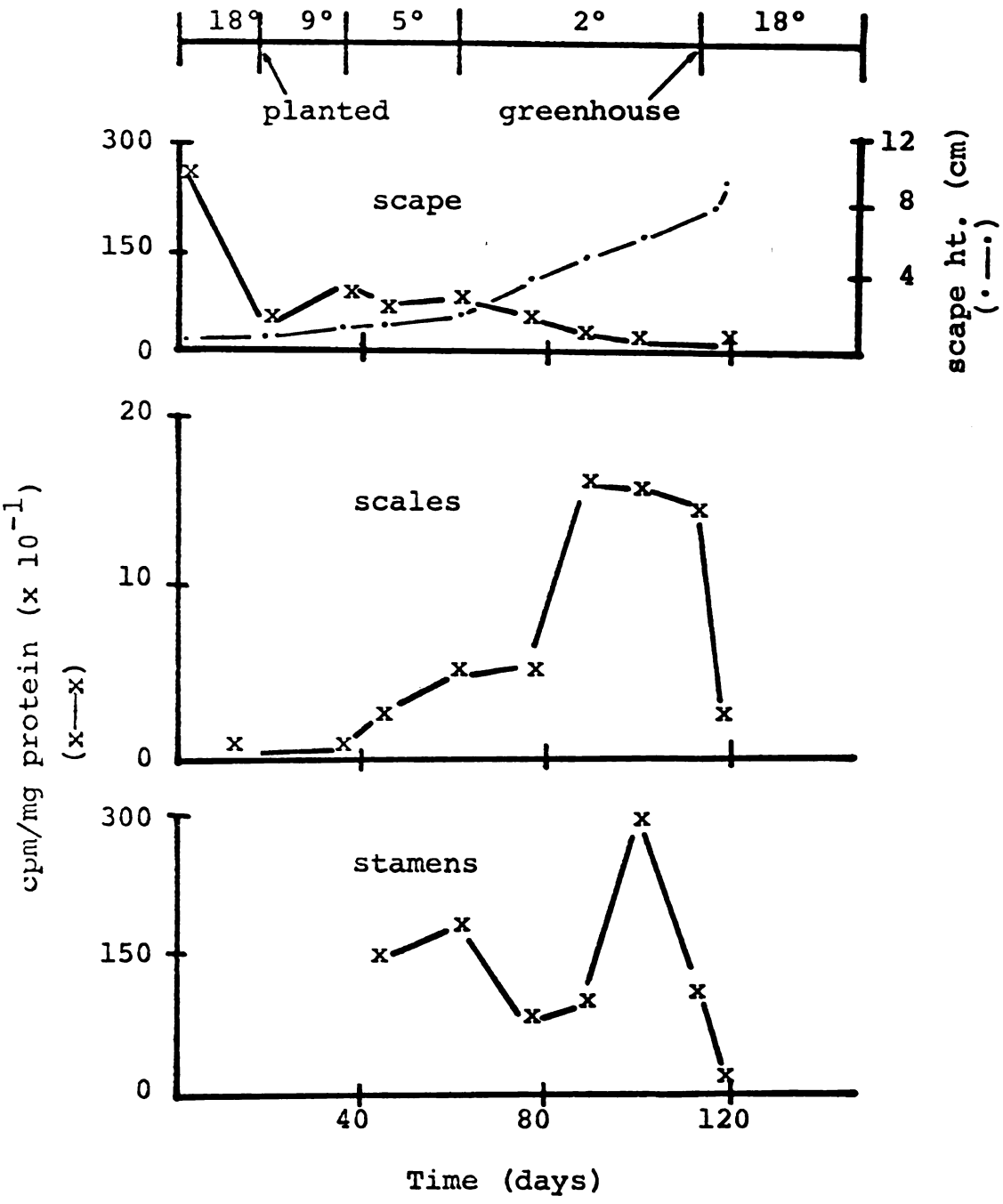

Figure 29.--The incorporation of MVA-2-^{14}C into neutral terpenes using scape, scale and stamen extracts of T. Gesneriana L. cv. Elmus and scape elongation growth during forcing treatments.

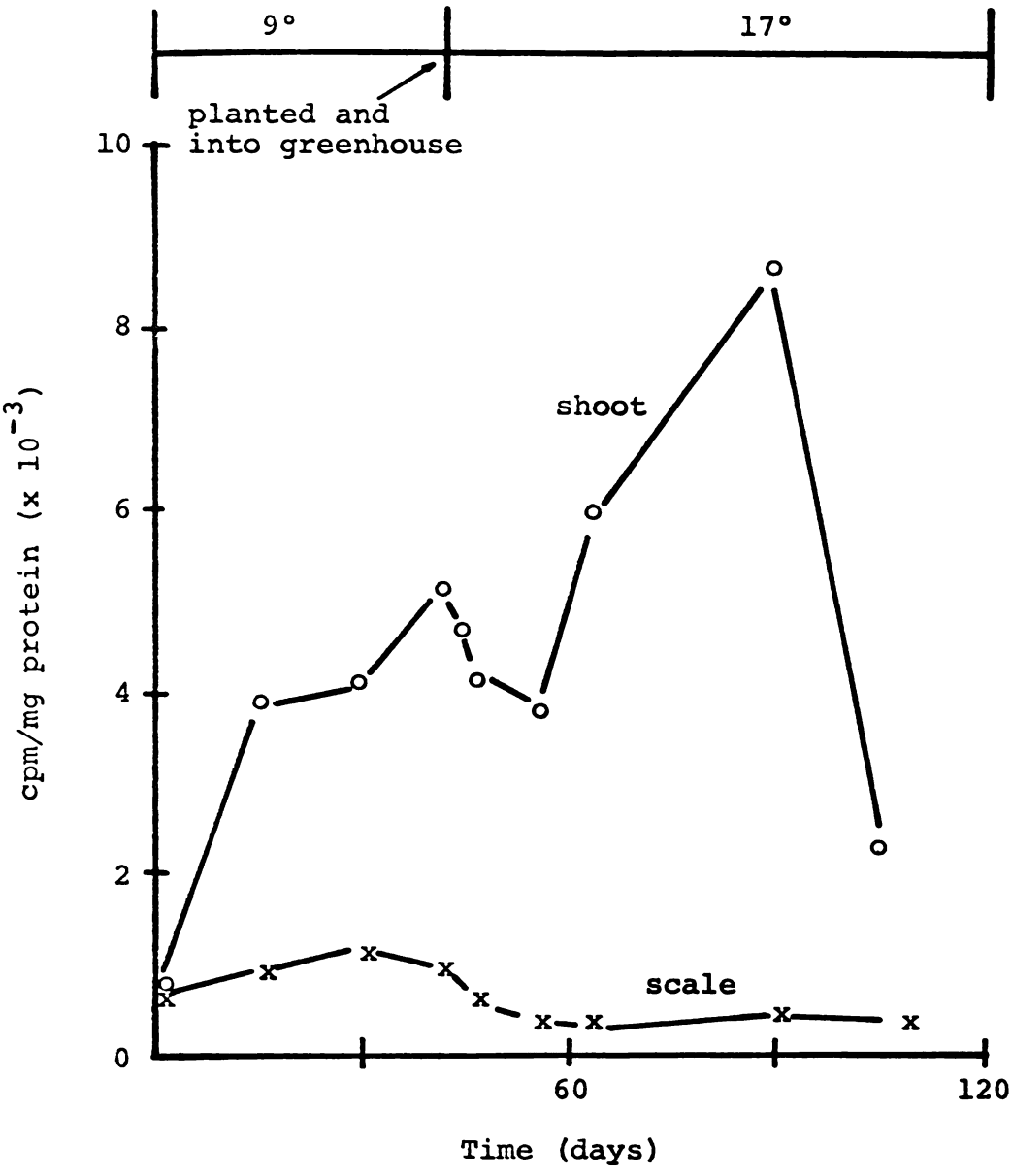

Figure 30.--The incorporation of MVA-2-^{14}C into neutral terpenes using shoot and scale extracts of Wedgwood Iris during forcing treatments.

ATP and $MgCl_2$ were increased, $MnCl_2$ added to and niacinamide omitted from the assay medium compared to the 1968-69 experiments.

Protein

Protein levels in iris generally decreased with time under the conditions of the experiment (Figure 27). The levels in fleshy scales were generally lower to various degrees in comparison to shoot tissue, except for the first and last determinations. With tulip tissue, only small changes in protein levels were noted except for the increase in the scapes of Ralph during the 5°C period. Protein levels of stamens should be increased approximately by a factor of 3 before a comparison with other tissue is made because of the differences in homogenizing ratios as noted in Experimental.

Scape Elongation Growth

A steady growth rate of scape tissue with both tulip cultivars was noted until the bulbs were placed into the greenhouse (Figures 28, 29). At this time, growth rates increased sharply. This increase was similar to the increase observed with treatment R-1 of the 1968-69 experiments (Figure 21). No growth data was obtained for the Wedgwood Iris experiments.

Localization of Enzyme Activity

Results presented in Table 27 confirmed the results presented in Table 25 as to the localization of enzyme activity in the stamens of the tulips. Approximately 95% of the total enzyme activity was present in stamen tissue (Table 27). Fleshy scales possessed from 5 to 6% of the activity. Similar activity in fleshy scales was not detected in the 1968-69 experiments (data not presented).

Shoots provided approximately 73% of the extractable activity with iris while fleshy scales and roots provided 18 and 9%, respectively. Few enzyme activity determinations were possible with iris flowers because of either complete absence or extreme lack of sufficient amounts of tissue during a large part of the experimental period. Determinations which were made were performed after the flowers were visible in the greenhouse. Results from these determinations indicated little or no enzyme activity in extracts of flower or stamen tissue at this late stage of development (data not presented).

Enzyme Activity

The results of the time course studies on enzyme activity of tulip scape, fleshy scales and stamens are presented in Figures 28 and 29 for Ralph and Elmus, respectively. With both cultivars, activity in scape tissue was highest prior to planting. During subsequent

9°C treatment with Ralph, activity decreased and then increased after being subjected to 5°C treatment (Figure 28). Complete loss of activity occurred within 3 days after Ralph was placed into the greenhouse.

Enzyme activity in the fleshy scales and stamens of Ralph correlated with scape activity except for: (1) the first determination with fleshy scales was lower and (2) the rapid activity decline of scales and stamens after being placed into the greenhouse lagged behind the corresponding decrease with scape tissue.

Similar to Ralph, the initial enzyme activity in scape tissue of Elmus declined and then increased during the subsequent 9 and 5°C treatments (Figure 29). Placing the bulbs at 2°C resulted in a steady decline in scape activity. Eventually, activity became too low to note any changes in enzyme activity upon placing the bulbs into the greenhouse.

In contrast to Ralph, enzyme activity in fleshy scales and stamens of Elmus did <u>not</u> correlate with scape activity of this cultivar. There was a definite rise in activity with fleshy scales during the 2°C period which was inversely related to the activity decrease in scape tissue during this same period. A similar rise in activity, after a lag period, was noted with stamen tissue. While no substantial enzyme activity decrease was detected with Elmus scape tissue after being placed into

greenhouse, definite decreases were evident in fleshy scales and stamens of this cultivar during this period.

Enzyme activities with tissues of Wedgwood Iris are presented in Figure 30. Shoot tissue exhibited the lowest activity when the bulbs were vegetative. As the bulbs became reproduction during the 9°C treatment, activity increased. After planting and placing the iris bulbs into the greenhouse, activity decreased and then increased to the highest level just before flowering. Activity decreased again at flowering which corresponded to visible signs of senescence.

Fleshy scales of iris showed a somewhat different enzyme activity pattern compared to iris shoots. There was a similar rise in activity during the 9°C period but scale activity increased earlier than shoot activity. After the earlier activity crest with fleshy scales, activity decreased to a final low level. No further enzyme activity increase was noted with scales.

Pollen Development

Upon arrival with both tulip cultivars, reduction-division of microsporogenesis in stamens had already taken place and the tetrad stage had been reached. Throughout the entire low temperature treatments, no detectable change was noted in pollen development from the initially observed tetrad stage. However, once

placed into the greenhouse, the pollen rapidly developed within 3 to 5 days into, seemingly, mature pollen grains.

It was also observed that a latex-like substance was exuded from the tulip anthers while preparing them for microscopic examination. This latex-like substance was not detected once the bulbs were placed into the greenhouse. Thus, pollen maturation, latex-like substance disappearance and loss of enzyme activity in stamens occurred at the same time.

Identification of ^{14}C-Products

The information known concerning the identification of the ^{14}C-products arising from MVA-2-^{14}C has been presented in Part I of this dissertation for iris and in the 1968-69 Results of Part II for the tulip cultivars.

Inhibitor Study

With the same amount of stamen extract in each assay, increasing levels of the remaining scape extract (scape tissue minus stamens) inhibited the incorporation of MVA-2-^{14}C into neutral terpenes (Figure 31).

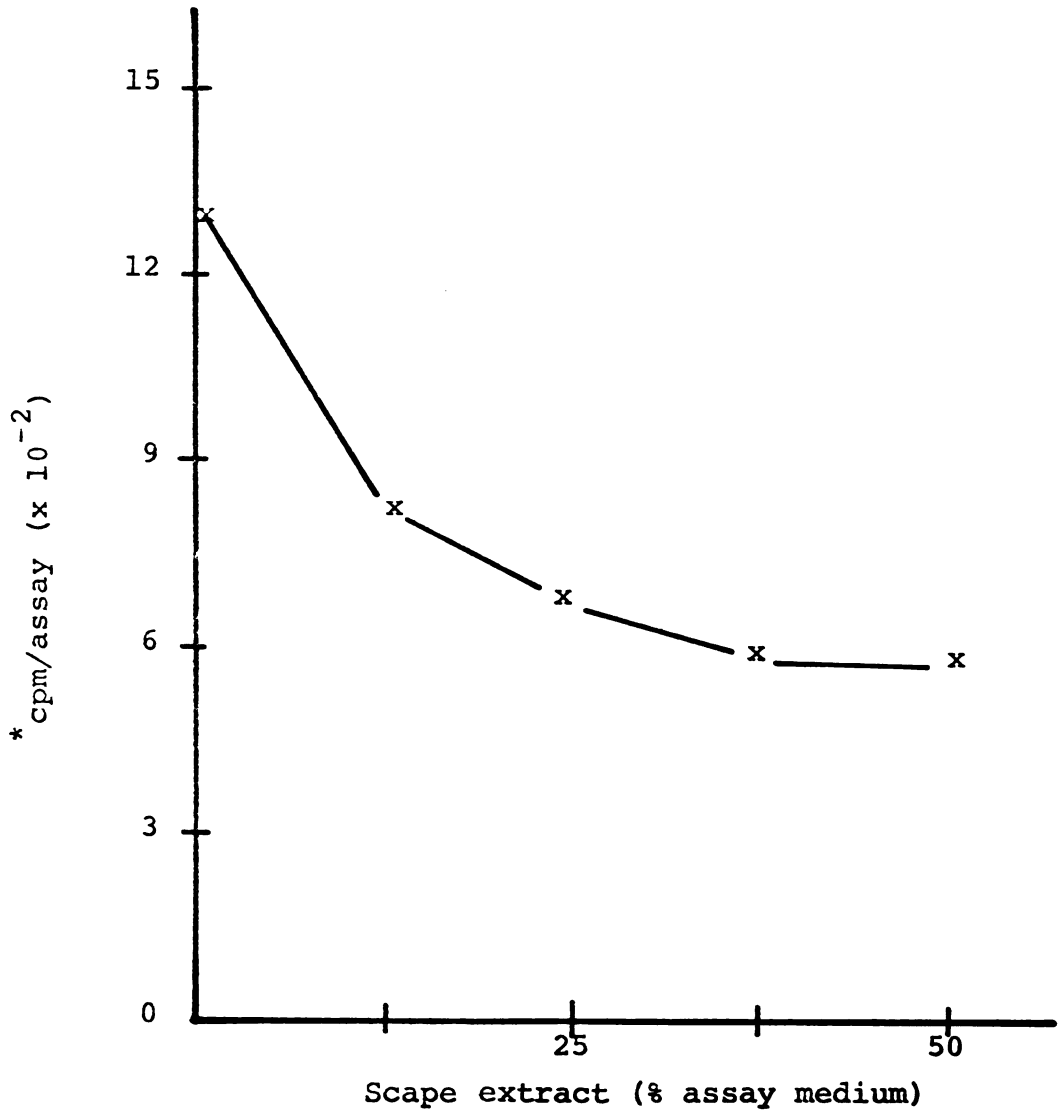

Figure 31.--Effect of scape extract (minus stamens) on the incorporation of MVA-2-^{14}C into neutral terpenes using stamen extracts of T. Gesneriana L. cv. Elmus.

*cpm/assay corrected for scape enzyme activity.

DISCUSSION

The positive identity of the ^{14}C-products of the tulip and iris cell-free systems was not determined, however, the following discussion was written with the assumption that some of the enzyme activity may have reflected gibberellin biosynthesis *in situ*.

Wedgwood Iris

Approximately 73% of the enzyme activity in the iris was located in shoot tissue while scale and root tissue provided 18 and 9%, respectively (Table 27). This high enzyme activity in shoot tissue and moderate to low activity in scales is supported as being possible sites of gibberellin biosynthesis *in situ* by work of Halevy and Shoub (1963) and Rodrigues Pereira (1961, 1964, 1965, 1966). The activity in root tissue was not correlated with other iris GAs data because no such data has been published. However, it has been shown that roots of other plants were sites of hormone biosynthesis including GAs (Butcher, 1963; Jones and Lacey, 1968; Phillips and Jones, 1964; Sitton *et al.*, 1967). Thus, iris roots may also have been a site of gibberellin biosynthesis.

The rise in shoot and scale activity during the 9°C period (Figure 30) corresponded to the most recent findings of Rodrigues Pereira (1966). He found that levels of GAs in isolated scales and bud (shoot) tissue increased during low temperature treatment. The increase in GAs in each tissue was sufficient to induce flowering in non-cold treated buds.

The peak in scale tissue enzyme activity was found to be prior to the peak in shoot tissue during the 9°C period (Figure 30). Again these results were supported by the work of Rodrigues Pereira (1964). He reported that the maximum GAs level in scales was 14 days before the maximum level in bud tissue. Also, his data indicated that there was an approximate 6 fold higher GAs level in bud than scale tissue when compared at their maximum levels. Comparing similar maximum enzyme activity levels in the present study resulted in an approximate 4 fold higher level in shoot compared to scale tissue. Thus, enzyme activity results from the present study supported data in the literature (Halevy et al., 1963; Rodrigues Pereira, 1961, 1964, 1965, 1966) suggesting that at least some of the GAs increase in iris during low temperature treatments was due to biosynthesis in situ.

The increase in enzyme activity during the 9°C period may have also reflected the changing metabolic

state of the bulb. Correlating with enzyme activity increases were increases in: respiration (Halevy et al., 1963; Kamerbeek, 1962; Rodrigues Pereira, 1962), peroxidase and catalase activity (Halevy et al., 1963; Van Laan, 1955) as well as gross quantitative and qualitative changes in carbohydrates (Halevy et al., 1963; Kamerbeek, 1962).

It can be hypothesized that developing stamens were partially responsible for the extractable enzyme activity in the present study and thus were a possible site of gibberellin biosynthesis. There has been evidence presented supporting this hypothesis with other plant species (Greyson and Tepfer, 1967; Marré, 1946; Plack, 1957, 1958). Further evidence supporting the hypothesis that developing stamens are a site of gibberellin biosynthesis has been presented by Rodrigues Pereira (1966). He showed that the most critical time of gibberellin biosynthesis in situ, and of iris bulb response to exogenously applied gibberellic acid, was during stamen or tepal development.

The decrease-increase-decrease pattern of enzyme activity after planting (Figure 30) cannot be related to changes in GAs because no such data on GAs during this period has been published. This activity pattern did correlate with changes in respiration reported for this stage of iris development (Halevy et al., 1963).

The concurrent changes in respiration and enzyme activity may have represented the general metabolic state of the bulb. This enzyme activity increase after planting may also be related to synapsis. Wittwer (1943) showed that approximately 2 weeks after synapsis of microsporogenesis there was a large increase in hormone content in the plants he tested. Unfortunately, the synapsis stage in iris was not determined.

Rodrigues Pereira (1964) concluded that there were at least three classes of compounds required for iris flower formation: (1) gibberellins, (2) flowering hormone and (3) specific nucleic acids. Both GAs (Halevy and Shoub, 1964; Rodrigues Pereira, 1964, 1966) and nucleic acids (Rodrigues Pereira, 1966) have been implicated in iris flowering. However, no information has been published in relation to a flowering hormone and the flowering of iris.

Bonner et al. (1963, Kopcewicz (1970), Sachs (1966), Sironval (1950, 1957) and others have shown that steroids were implicated in flowering and that these terpenes may be a part of the yet unidentified flowering hormone complex. Vitamin E (Sironval, 1950, 1957) and carotene (Heslop-Harrison, 1969), both terpenes, were also thought to be related to flowering.

Data presented in Part I of this dissertation conclusively showed that sterols were present in extracts of

iris shoots (Figures 17-19). Sterols have been shown to be in high concentrations in flower tissue, especially pollen, with many plant species (Amine et al., 1969; Barbier et al., 1960; Devys and Barbier, 1967; Standifer et al., 1968). It would be interesting to know if sterols were involved in iris flowering, especially since sterols are biosynthesized from many of the same intermediates necessary for gibberellin biosynthesis. Thus, changes in enzyme activity reported in the present study with iris may have partially reflected sterol or some other terpene as well as gibberellin biosynthesis in situ.

Tulip

Results of the present study showed that stamen tissue of tulip contained approximately 95% of the total extractable enzyme activity (Table 27). These results suggested that stamens were a major site of terpene (e.g. gibberellin) biosynthesis in situ. There are many lines of evidence to support this suggestion.

Marré (1946) reported that spontaneous atrophy of anthers, including tulips, always resulted in a subsequent inhibition of growth and/or development. He suggested that important growth regulators were either biosynthesized and/or released from anthers. More recently, Plack (1957, 1958) and Greyson and Tepfer (1967) have confirmed these earlier findings of Marré (1946) and

have specifically shown that GAs were biosynthesized and/or released from anthers with many plant species. Other investigators (Barendse, et al., 1970; Coombe, 1960; Lona, 1961) also showed that anthers may be a source of gibberellins.

The high enzyme activity with stamens and the low and/or absence of activity with scale and root tissue was supported by known levels of GAs in tulips. Einert et al. (1970a) showed with the tulip cultivar Elmus that flower tissue contained high levels of GAs. Lower levels of GAs were detected in scales and little or none in roots. Aung and De Hertogh (1967) also showed that shoot tissue, which contained flowers, often exhibited more GAs than scales.

Changes in relative enzyme activities were partially related to changes in GAs from the same tulip cultivars. Aung and De Hertogh (1968) reported with the cultivar Ralph that GAs increased during low temperature storage. Einert et al. (1970) showed similar results with the cultivar Elmus. Correlating GAs levels with enzyme activity data obtained in the present study during low temperature treatments (Figures 21, 22, 23, 25, 28, 29) with both cultivars suggested that some of the increase in GAs levels was due to changes in biosynthesis rates in situ.

The increasingly higher levels of GAs detected in tulip after being placed into the greenhouse (Aung et al., 1969b; Einert et al., 1970) was not supported by results of the present enzyme activity enzyme study (Figures 21, 28, 29 and Table 24). In fact, all enzyme activity rapidly disappeared soon after the bulbs were placed into the greenhouse. Possible explanations for this discrepency are: (1) Enzyme activity results were monitoring the biosynthesis of gibberellin precursors and not gibberellin per se. Once placed into the greenhouse, gibberellin biosynthesis continued from the precursor which was previously biosynthesized from MVA. Such biosynthesis of gibberellins would not be monitored using MVA as the precursor. Results with Corylus avellana (hazel) seeds support this possible explanation (Bradbeer, 1968; Ross and Bradbeer, 1968). They showed with hazel seeds that GAs levels increased substantially only after the seeds were subjected to warm temperatures following a low temperature treatment. (2) Enzyme activity results were not indicating gibberellin biosynthesis in situ, (3) inhibitors of the cell-free system itself resulted in the loss of activity and (4) the gibberellin bioassays used to measure GAs activity (Aung et al., 1969b; Einert et al., 1970a, 1970b) were sensitive to only some of the endogenous gibberellins in tulip and reflected changes in

levels of only certain gibberellins, therefore, not reflecting total endogenous changes in gibberellins.

The earlier increase in enzyme activity noted for precooled bulbs (Figure 21) compared to non-precooled bulbs (Figures 22, 23) may have been related to the 'after-effect' of precooling reported for starch conversion into sugars. Algera (1936, 1947) reported that starch breakdown was enhanced after planting in the fall if the bulbs were precooled compared to bulbs not cooled prior to planting. Thus, effects of precooling in both cases were not monitored until after the bulbs were planted.

Changes in relative enzyme activities among treatments and between years were consistent except for a discrepancy during the initial weeks of the study in each year. The first few extractions made in 1968-69 resulted in little or no enzyme activity detected (Figures 21, 22, 23, 25, 26) while the same period in 1969-70 resulted in very high enzyme activity (Figures 28, 29). One possible explanation for this discrepancy was that the bulbs were at different stages of development upon arrival from The Netherlands between years. Another possible explanation was that the incubation media used for determining enzyme activity differed between years resulting in different enzyme patterns for this early extraction period. For example, the 1969-70 incubation medium contained Mn^{++}

while the 1968-69 medium did not. Similar differences have resulted in marked changes in enzyme activity using other cell-free systems (Anderson and Moore, 1967; Beytia et al., 1969; Graebe, 1968; Jungalwala and Porter, 1967; Loomis and Battiale, 1963; Nandi and Porter, 1964; Upper and West, 1967; and data presented in Part I of this dissertation for the iris cell-free system).

Inhibitors of enzyme activity found in scape tissue minus stamens (Figure 31) suggested that results obtained with stamen tissue may have better reflected terpene biosynthesis in situ. For example, when Ralph bulbs were placed into the greenhouse, scape enzyme activity completely disappeared while stamen activity increased in the 1969-70 experiments (Figure 28). Since scape enzyme activity was due to enzymes present in the stamens (Tables 25, 27), enzyme activity reported for scape tissue was a measure of stamen activity.

Changes in the levels or protein with the cultivar Elmus (Figure 27) was similar to changes reported by Higuchi and Sisa (1967). The reasons for these changes is a matter of conjecture.

SUMMARY

Part II of this dissertation reports a study which attempted to determine: (1) Are changes in the quantity of GAs in Tulipa Gesneriana L. cvs. Ralph and Elmus and Wedgwood Iris during normal forcing treatments a response to changes in their biosynthetic rates in situ? (2) What tissues biosynthesize gibberellins in tulip and iris? The results of this study are summarized as follows:

1. Changes in neutral terpene biosynthesizing terpene enzyme activity correlated with reported changes in GAs during low temperature treatments with both tulip and iris.
2. Changes in terpene enzyme activity after low temperature treatments did not correlate with reported GAs changes in tulip.
3. Terpene enzyme activities determined in iris after planting could not be related to changes in GAs because no such data on GAs during this period have been published.
4. Stamen tissue in tulip accounted for 95% of the enzyme activity with the remaining activity in fleshy scales. With iris, shoot, scale and root tissue provided 73, 18 and 9%, respectively.

5. Enzyme activity in tulip was correlated with pollen development. In iris, enzyme activity was correlated with reported flower initiation and development and respiration during low temperature treatment.
6. Farnesol was tentatively identified as one of the neutral terpenes which was biosynthesized with the tulip cell-free systems. Part I of this dissertation presents data on the identity of the products biosynthesized in the iris cell-free system.
7. Protein levels in iris continually declined throughout forcing while levels in tulip generally remained constant.

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LITERATURE CITED

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