THE INCORPORATION OF MEVALONIC ACID-2-C^{14} INTO TOMATO CAROTENOIDS

By

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1955

Submitted to the faculty of the Graduate School
of the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
August, 1962

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ACKNOWLEDGMENTS

The writer gratefully acknowledges the continual guidance and counsel by his major professor, Mr. George V. Odell.

Sincere appreciation is extended to Dr. LaVell M. Henderson, Dr. Arthur R. Schulz, Mr. George R. Waller and Dr. Howard B. Cordner for their suggestions and encouragement.

The author would like to thank Mr. H. Stanley Homer in Oklahoma City for his generosity in providing the tomato fruit samples.

Finally he is thankful for the facilities and financial assistance in the form of a research assistantship which was provided for him by the Department of Biochemistry of Oklahoma State University.

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CHAPTER I

INTRODUCTION

Carotenoid pigments represent one of the most widespread groups of naturally occuring pigments. They are found in higher plants, algae, fungi, bacteria and in almost all forms of animal life. Carotenoids are formed in all photosynthetic tissue. Speculation as to the role of the carotenoids in plants range from a light filter function to the transfer of light energy to some 'stored' form of chemical energy.

The tomato is a fruit in which ripening results in a marked synthesis of phytoene, phytofluene, certain carotenes and the red pigment, lycopene. It is well accepted that lycopene distribution and content of tomato fruit is related to the quality and consumer acceptance of this product. The synthesis of lycopene is completely blocked at temperatures above 85°F. Since Oklahoma field temperatures very often range above this critical point for extended periods of time, high temperature can markedly affect the quality, composition and value of tomato fruits produced for the fresh market. The availability of certain isotopically labeled precursors of carotenoids and development of procedures for the efficient chromatographic separation of these plant pigments provide techniques whereby the origin and development of carotenoid pigments may be followed in plants and fruits.

The methods of many investigators have been used in this research work, and many results they obtained have been confirmed. Independent studies on the temperature blocked synthesis of carotenoids in tomato

fruits have been made at different stages of maturity. By using radioisotopic tracer techniques, the rate of incorporation of labeled substrate into each carotenoid was determined and some information on the biosynthesis of carotenoids in tomato fruits has been obtained.

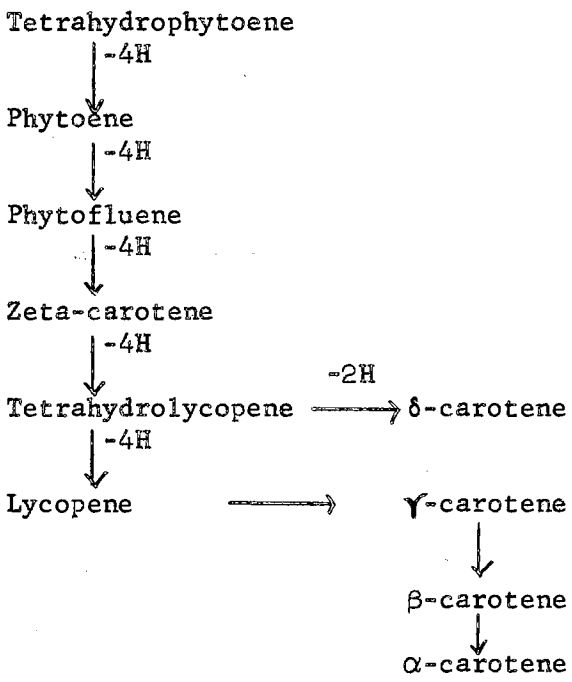
CHAPTER II

HISTORICAL

The problem of the biogensis of the carotenoids in plant tissue has not yet been established. Experimental evidence from various investigators support certain points in the hypotheses proposed, but conclusive evidence for these proposed schemes has not yet been obtained. The evolution of the investigation on this problem can be divided into three stages; 1) morphological and quantitative observations on the pigmentation of ripening fruits and observation on the molecular structure of pigments and genetic relationship between varieties of plants and the differences in the pigmentation, 2) radioisotopic tracer approach, 3) enzymic approach.

Dugger (9) was the first investigator who observed the relationship between temperature and the formation of a red pigment in ripening tomato fruit. He showed that the red tomato pigment is no longer formed at temperatures above 30°C. The agents responsible for the formation of lycopene are not destroyed at 30°C, since the yellow tomato ripened at 30°C acquired a red color due to lycopene on being restored to a lower temperature. The investigation of Dugger was repeated and confirmed by Karrer and co-workers (22). A very detailed description of the morphological changes which take place in fruits during the ripening process can be found in Zechmeister's monograph 'Die Carotinoide' Berlin, 1934.

Phytofluene was first suggested by Zechmeister to be a precursor (41) of lycopene. Bonner also proposed that phytofluene is an intermediate in the biogenesis of carotenoids (4). Porter and Lincoln expanded this concept (29) and proposed on the basis of their experiments with the tomato that a successive dehydrogenation of more saturated polyene leads to the biosynthesis of lycopene and other carotenoids. By examining the data obtained from studies of the structures of the naturally occuring carotenes and colorless polyenes, phytoene, phytofluene, zeta-carotene, tetrahydrolycopene and lycopene form a series which differ from one another only in the number of double bonds in the molecule. This suggested that carotenoids may be formed from one another by either reduction of dehydrogenation. Evidence on this point is supplied by genetic studies. Lincoln and Porter postulated the following scheme for lycopene formation:



The validity of this hypothesis has been questioned on the basis of further genetic studies (12). Goodwin has determined the concentration of some of the postulated intermediates at intervals during the ripening of tomatoes at various temperatures (13) and has concluded that the Porter-Lincoln hypothesis cannot account for the results obtained. Goodwin and Jamikorn (15), Goodwin (11), Mackinney (7) and Jenkins and Mackinney (21) have suggested that the different polyenes might originate from a single precursor by parallel pathways.

Although changes in concentration of substances during the course of metabolic transformations often yield useful information concerning pathways, the interpretation of such data is frequently subject to error because an intermediate may be present in small quantity and may not change in concentration although it may have a high rate of turn over. This objection can be overcome by the use of isotopic techniques. Grob's group reported (20) that in a medium containing ammonium acetate and sodium acetate labeled with C^{14} in the methyl or carboxyl position, 75 per cent of the carbon in the carotenoids of M. hiemalis is derived from sodium acetate. Grob and Butler reported (19) that experiments with acetate-1-C^{14} and 2-C^{14} showed that the methyl side groups of the aliphatic chain of beta-carotene produced by M. hiemalis come exclusively from the methyl group of acetate. The carbon atoms to which these methyl groups are attached are derived from the carboxyl group of the acetate.

In the study reported by Zabin and Shneour (40) acetate-2-C^{14} has been added to ripening tomatoes and polyenes have been isolated at different periods of time after administration of the isotope. Comparison of the specific activity of lycopene to that of some colorless

polyenes indicates that neither the Porter-Lincoln hypothesis nor the reverse of this scheme is operative in tomatoes.

Wolf and co-workers (39) have isolated and identified mevalonic acid as a precursor of cholesterol and their work constitutes an important contribution to the understanding of carotenoid biogensis. Experimental proof for this possibility was presented in reports on the incorporation of mevalonic acid into beta-carotene in carrots (14), M. hiemalis (18) and lycopene in ripening tomatoes (30).

Extracts of carrot root tissue have been obtained (7) which incorporate mevalonic acid-C^{14} into beta-carotene (6). The extent of incorporation is doubled by the addition of coenzyme-A, adenosine triphosphate (ATP), reduced diphosphorpyridine nucleotide (DPNH) and reduced triphosphopyridine dinucleotide (TPNH). Shneour and Zabin (32) have studied the biosynthesis of carotenes in a cell free system. Cell free homogenates of ripening tomato fruit were incubated with mevalonic -2-C^{14} acid (MVA-2-C^{14}). For optimal incorporation of isotope, ATP, pyridine nucleotides, glutathione, Mg++ and oxygen were necessary.

Both Zabin's and Goodwin's experiments showed that ATP was required for the conversion of mevalonate into carotenoids in a cell free system. This observation suggested that in this synthesis phosphorylative steps are involved. The formation of 5-phosphomevalonate (5-P-MVA) from mevalonate and ATP in a yeast enzyme preparation was first reported by Tchen (35), and was also observed in a liver preparation (25). The enzyme responsible for this phosphorylation was partially purified (36) by treatment of a high speed supernatant of yeast autolyzate with protamine, followed by fractionation with ammonium sulfate and calcium

phosphate gel. It was shown by Tchen that the enzyme is a kinase:

$$\text{Mevalonate} + \text{ATP} \xrightarrow[\text{Mg}^{++}]{\text{Mevalonic acid kinase}} \text{5-P-MVA} + \text{ADP}$$

Chaykin has shown that 5-P-MVA is utilized both by the yeast enzyme system (8) and by the liver enzyme system (38) for squalene and cholesterol synthesis respectively but ATP, Mn^{++} or Mg^{++}, still must be added to the preparation. The fact that ATP was needed for the utilization of 5-P-MVA suggested that other phosphorylations might be involved. A second phosphorylated derivative of MVA was found both in yeast and liver (8). The enzyme responsible for this second phosphorylation is distinct from MVA kinase as found in pig liver extract (24,25). MVA kinase purified from pig liver could not catalyze the formation of 5-pyrophosphomevalonic acid (5-PP-MVA). However, protein fractions obtained in earlier stages of purification of the liver enzyme contained a second kinase which converts 5-P-MVA to the pyrophosphate.

$$\text{5-P-MVA} + \text{ATP} \xrightarrow[\text{Mg}^{++}]{\text{liver or yeast enzyme}} \text{5-PP-MVA} + \text{ADP}$$

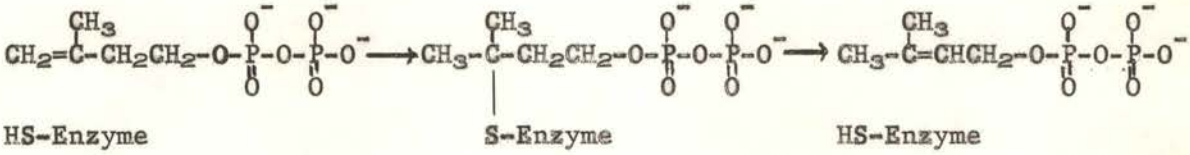
5-PP-MVA was also a better precursor of cholesterol in the liver enzyme system than DL-MVA-2-C^{14}, but like 5-P-MVA it still required the addition of ATP for its utilization (38). Similar results were reported with the yeast system synthesizing squalene from 5-PP-MVA (8).

Chaykin reported that 5-PP-MVA was decarboxylated to isopentenyl pyrophosphate when incubated with a fraction obtained yeast extract and ATP (8). Thus, ATP and Mn^{++} were needed in the decarboxylation. Lynen (26) synthesized isopentenyl-C^{14}-pyrophosphate by chemical means

and found it to be an efficient precursor of squalene in a yeast enzyme system even in the absence of ATP.

$$5\text{-PP-MVA} + \text{ATP} \xrightarrow[Mn^{++}]{} \text{IPP} + \text{ADP} + CO_2 + \text{iP}$$

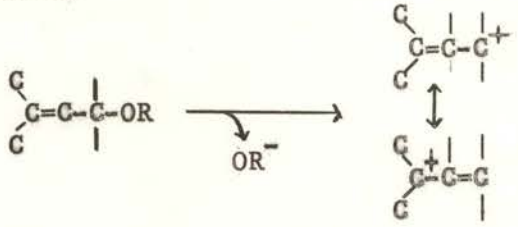
The isomerization of IPP by a yeast enzyme fraction has been described by Agranoff (1) as proceeding as follows:



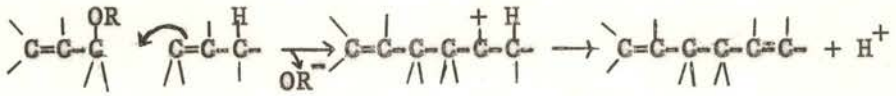
HS-Enzyme S-Enzyme HS-Enzyme

T
The isomerase was completely inhibited by iodoacetamide and by p-chloromercuribenzoate.

Dimethylallylpyrophosphate has properties similar to other substances in which a hydrogen atom, a hydroxyl or an esterified hydroxyl (i.e. esterified with pyrophosphoric acid) is in the alpha position to a double bond. Elimination of such groups may occur readily to form the resulting electron-deficient species that are partially stabilized by resonance.



The product of condensation of two such ionic species carries a positive charge, but stabilization may occur by elimination of a proton:



The head to tail condensation of four of these 5 carbon units yields a 20 carbon tetramer. A 40-carbon precursor in the carotenoid biosynthesis could result from a tail to tail condensation of two 20-carbon units.

In the process of carotenoid biosynthesis, intermediates with different carbon chain lengths (10 to 20 carbons) have been determined by gas-liquid radiochromatographic analysis (2). Phytoene has also been synthesized by incubating 20-carbon polymers with a soluble carrot root preparation. The substrate polymers were obtained by preincubation of MVA-2-C^{14} with rat liver enzymes in the presence of ATP, Mn++, and glutathione.

The biosynthetic interrelationship between the carotenoids, phytoene, phytofluene, the carotenes and lycopene is not yet known. A recent paper published by Jensen (22) supports the Porter-Lincoln postulate. When diphenylamine (DPA) was added to a growing culture of Rhodospirillum rubrum the normal synthesis of carotenoids is arrested and a rapid accumulation of carotenoids more saturated than lycopene was observed. When the DPA was removed and the cells are resuspended in buffer and incubated anaerobically in the light, an endogenous synthesis of normal carotenoids took place at the expense of all the accumulated precursors with the probable exception of phytoene. The kinetic analysis of this endogenous synthesis reveals the sequential relationship between the participating carotenoids.

On the other hand, the results of Krezeminski and Quackenbush on the incorporation of C^{14} labeled compounds into carotenes by Neurospora crassa (23) implied that the individual polyenes are synthesized independently rather than through a stepwise interconversion involving either hydrogenation or dehydrogenation.

As mentioned earlier, Dugger showed in 1913 that the red tomato pigment is no longer formed at temperatures above 30°C and the yellow tomatoes obtained at 30°C can, on being restored to a lower temperature, acquire a red color due to lycopene. The research work reported in this thesis covers certain aspects of the relation between time, temperature and carotenoid biosynthesis in tomato fruits.

CHAPTER III

METHODS AND MATERIALS

Preparation of Labeled Substrate

The mevalonic-2-C^{14} acid was obtained from the Isotope Specialities Company, Burbank, California; as the NN'-dibenzyl-ethylendiamine salt (Lot Number A-39-B-1188, 10 mg, 50 microcuries). A weighed amount of the salt was dissolved in a minimal amount of water and the pH adjusted to 10 with 1N NaOH. The dibenzylethylenediamine was removed by two extractions with an equal volume of ethyl ether. The ether extracts were combined and extracted with 0.5 volume of NaOH solution (pH 10) which was added to the original solution, then diluted to final volume.

Preparation of Optical Hexane

Commercial hexane (Phillips, High purity) was shaken with solid KOH and distilled. The 68°C fraction was collected and purified by passing through a silica gel column (column: 4.6 x 40 cm, silica gel: Davison, grade 12, mesh 28-200). One column would purify 3 to 4 liters of distilled hexane. The absorbance of each 300 ml fraction of the eluate was determined at 210 and 250 mμ. Those portions having an optical density of less than 0 at 250 millimicron and less than 1.0 at 210 millimicron were collected as optical hexane.

Preparation of Chromatographic Columns

Three kinds of chromatographic columns were used in these experiments. The magnesia (Fisher Sea Sorb S-120)-Hyflo super cel (1:1, w/w)

column was tightly dry packed layer by layer, wetted with hexane with the aid of pressure at the top or vacuum from the bottom of the column. The size of the column was selected according to the amount of lycopene and other carotenes or polyenes in the extract. For whole tomato fruit the extract containing a larger amount of carotenoids, the larger size columns (2.4 x 14 cm) were used. For the extracts from tomato sections (about 10 gm in weight) 1.8 x 12mm columns were used.

The calcium hydroxide-Hyflo Supercel (1:1, w/w) column (2.4 x 7cm) used for removing radioactive impurities from carotene solutions was prepared in the same manner as the magnesia-Hyflo Supercel columns.

The alumina (Harshaw, AL-0109p, activated at 300°C for 3 hrs.) column for purification of phytoene was packed as a dry powder to a medium tight condition and washed with three volumes of optical hexane before introducing the sample.

Injection of Labeled Substrate

Tomato fruits selected for use were at green mature slightly turning and medium turning stages (34). The selected detached fruits were supplied with labeled substrate by one of the following methods. In the earlier experiments labeled substrate was injected into whole fruits. In the later experiments the labeled substrate was injected at three points into each section cut from the same fruit. Comparison of these two methods indicated that the second method was better because it resulted in more uniform distribution of the substrate throughout the tissue.

Incubation

In experiments with the whole tomato fruits, the injected fruits

were incubated at room temperature or in a water bath at 38°C for a certain length of time. For experiments with the tomato sections, in longer time studies (i.e. 3-12 days) fruits were sterilized with 70% ethyl alcohol and cut into sections in a sterile hood then transferred into sterile tubes. These sections were incubated at a constant temperature (i.e. room temperature or 38°C) for a definite length of time. In the short time studies, a sterile condition was not necessary, and the injected sections were incubated at the desired temperature for a certain length of time. (i.e. 10 minutes, 30 minutes .....5 hours etc.)

Collection of $C^{14}O_2$

One of the experiments was designed to determine the radioactivity in the CO_2 expired by the tomato fruits after injection of MVA-2-C^{14}. Tomato fruits of green mature stage were injected with MVA-2-C^{14} and placed in a closed system (Figure 1). Incoming air was passed through two tubes of 50 ml of 1N NaOH prior to entry into the incubation chamber. Exhaust air was passed through a dry ice acetone trap, 3 tubes of 10 ml each of 0.5M hyamine, 1 tube 50 ml of 1N NaOH solution, and 1 tube 50 ml of saturated $Ba(OH)_2$ solution. Air was drawn through the system with a water aspirator. At the end of incubation the hyamine solutions were combined, samples were taken and counted in the Tri-Carb liquid scintillation spectrometer.

Extraction of Carotenoids

At the end of the desired time of incubation, the biochemical reactions in the tomato tissue were stopped by homogenizing with an equal weight of methanol. The whole tomato was blended with an equal weight of methanol (for sections 100 ml of methanol was used) and a

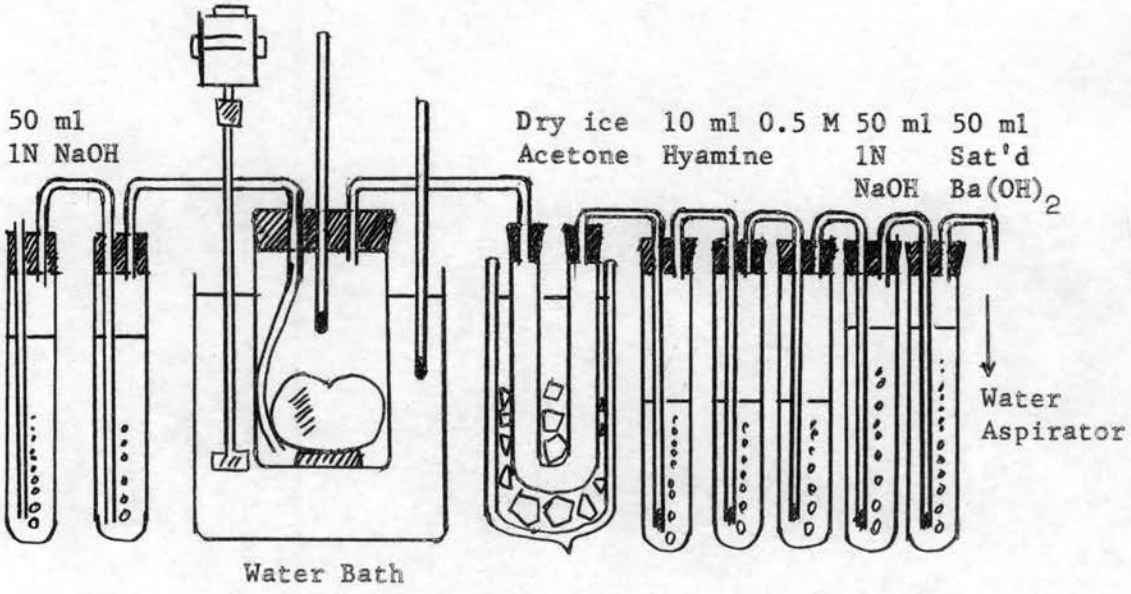


Figure 1. Apparatus for Collecting Respired CO_2 from Tomato Fruits

small amount of Hyflo Supercel. The slurry was filtered to dryness with vacuum in a Buchner funnel. The dried mat was extracted twice by blending with acetone-hexane (1:1) and refiltered. The extracts were combined in a separatory funnel. Water was added and after two layers had formed the lower layer was drawn off. The upper layer was washed free of acetone with water, and saponified by shaking with 1/5 volume of methanol saturated with KOH. After several minutes, the lower layer was drawn off, the upper layer was washed free from alkali with water. The hexane extract was dried by filtering through sodium sulfate. This solution constituted the nonsaponifiable extract.

Chromatography

The nonsaponifiable extract was condensed in vaccuo to the minimum volume possible without crystallization of any component. The concentrated extract was chromatographed on magnesia-Hyflo Supercel column. After the sample was placed on the column, the column was developed with hexane and observed under ultraviolet light (long wave length, Model S.L. 3660). When the fluorescent phytofluene band had progressed about halfway down the column, 2% acetone in hexane was added until phytofluene had nearly reached the bottom of the column. The eluate collected before phytofluene was termed the crude phytoene fraction. The solvent was changed to 2% acetone until phytofluene was removed from the column. The receiver was changed and solvent switched to 5% acetone in hexane until β-carotene was removed. After the bulk of β-carotene was removed from the column zeta-carotene was eluted with 10% acetone in hexane. Ten per cent acetone and 0.5% methanol in hexane were used to remove γ-carotene, then lycopene was eluted with

10% acetone, 3% methanol in hexane.

Purification of Crude Phytoene

The crude phytoene fraction was evaporated to dryness in vaccuo at low temperature. The residue was dissolved in 5 to 10 ml of optical hexane. The solution was placed on an alumina column. After introduction of the sample, the column was developed with the following solvent mixtures in this order, 75 ml of optical hexane, 50 ml. of 0.5% ethyl ether in hexane, 100 ml of 1% ethyl ether in hexane, 100 ml of 2% ethyl ether in hexane, 100 ml of 4% ethyl ether in hexane and 75 ml of 10% methanol in ethyl ether. The eluates were collected in 5 ml fractions with the aid of a fraction collector. The optical absorbance of each fraction was determined at 208, 230 and 286 mμ with a Beckman DU spectrophometer or Cary Model 14 recording spectrophotometer. Elution diagrams were plotted and the contents of all tubes representing a single fraction were combined.

Removal of Radioactive Impurities from Carotenes and Lycopene

Radioactive impurities in phytoene were removed on the alumina column while radioactive impurities in phytofluene, the carotenes, and lycopene were removed by rechromatography on $Ca(OH)_2$-Hyflo Supercel columns (1:1, w/w). The same solvents as used in the magnesia column in the first chromatographic separation were used here.

Determination of Radioactivity

Two methods were used for determining the radioactivity in the carotenoids. In the earlier experiments, samples were plated on aluminum planchets and dried. They were counted under a thin window gas-flow Geiger-Muller tube. In the later experiments, one ml. of the sample of the colorless fractions was transferred to a counting vial, 9 ml.

of counting solvent (1 gm 2,5 diphenyloxazole, 0.05 gm 1,4 bis-2-5-phenyloxazolyl-benzene, 100 ml absolute ethanol and 150 ml toluene) were added and counted in a Tri-Carb liquid scintillation spectrometer. For the colored carotenes and lycopene, 1 ml of the sample was transferred to a vial, a few drops of bromine in CCl_4 were added to decolorize the sample and after a few minutes, the tube contents were taken to dryness in vaccuo. After drying, 1 ml of hexane was added to dissolve the brominated carotene, then 9 ml of counting solvent was added and the sample was counted in the Tri-Carb liquid scintillation spectrometer.

Determination of Carotenoids

Pigments isolated from tomato extract were identified by their ultraviolet or visible absorption spectra and their chromatographic behavior. Quantitative determinations were made by comparing unknowns with the standard curves prepared according to the published $E^{1\%}_{1cm}$ values of each individual carotenoids (Table I, Figure 4).

CHAPTER IV

RESULTS

Experiment I

Absorption Spectra and Standard Curves of Tomato Carotenoids

The carotenoids isolated from tomato fruits were purified by rechromatography and crystallization. The absorption spectra of each carotenoid was determined with the Cary recording spectrophotometer. The observed data were the same as that published by other workers. (Table I, Figure 2 and 3). Standard solutions of each carotene and colorless polyene were prepared from the dry purified carotenoids isolated from the tomato. The absorbancy index of the carotenoids were represented as $E^{1\%}_{1cm}$ (The absorbancy of 1 gm of pigment in 100 ml solution, in an 1cm cell). With the exception of β-carotene, the observed $E^{1\%}_{1cm}$ value of most other pigments were much lower than the published values. These lower $E^{1\%}_{1cm}$ values are in error because of an inadequate drying method. For accurate calculation of the results in all experiments, the published $E^{1\%}_{1cm}$ value of the individual carotenoids (Table 1, Figure 4) were used in the calculation of total carotenoid present.

Incorporation of MVA-2-C^{14} into Tomato Carotenoids

For the purpose of obtaining experience in radioisotopic tracer techniques, certain parts of Purcell's work (30) was repeated. Table II shows a comparison of the data obtained in this experiment with that published by Purcell, Thompson and Bonner. In this experiment 1.78 x 10^6 c.p.m. of MVA-2-C^{14} were injected into a tomato at the pink

TABLE I

WAVELENGTH AND $E^{1\%}_{1cm}$ OF TOMATO POLYENES (IN n-HEXANE)

Polyenes	Wavelength (millimicrons)	$E^{1\%}_{1cm}$ *	References
Phytoene	286	850	Rabourn (1956)
Phytofluene	348	1500	Wallace and Porter (1952)
α-carotene	443	2700	Zechmeister (1944)
β-carotene	450	2590	Zechmeister (1944)
γ-carotene	459	2760	Zechmeister (1944)
ζ-carotene	422	2500	Zechmeister (1944)
Lycopene	469	3460	Zechmeister (1944)

* The absorbancy of 1 gm of pigment in 100 ml solution in an 1cm cell

TABLE II

INCORPORATION OF MVA-2-C^{14} INTO TOMATO CAROTENOIDS

Fractions	Amount per Fruit mg	Total Activity * c.p.m.	Specific Activity * c.p.m./mg	Amount per Fruit mg	Total Activity c.p.m.	Specific Activity c.p.m./mg
Fraction I	4.22	165	37	49	268	5.46
Fraction II	1.00	114,150	114,150	0.2	2,210	11,000
Pyhtoene	0.95	2,645	2,784	0.53	63	118
Phytofluene	0.24	79,970	333,208	0.36	48,000	133,000
α-carotene	0.01	8,551	79,916	---	---	---
β-carotene	0.795	23,505	29,566	1.41	16,940	12,000
γ-carotene	0.039	1,820	46,666	0.075	1,550	20,600
ζ-carotene	0.09	975	10,833	0.37	625	1,680
Lycopene	4.35	26,690	6,136	3.24	645	199

* 1.78×10^6 c.p.m. of MVA-2-C^{14} injected, incubated at 32°C for 48 hours, samples were counted in Tri-Carb counter, corrected for background, not corrected for 8% quenching, efficiency of Instrument was 32%. 1.06×10^6 c.p.m. MVA-2-C^{14} injected, incubated at 35°C for 24 hours, counted in gas-flow Geiger-Muller tube. Reference (30)

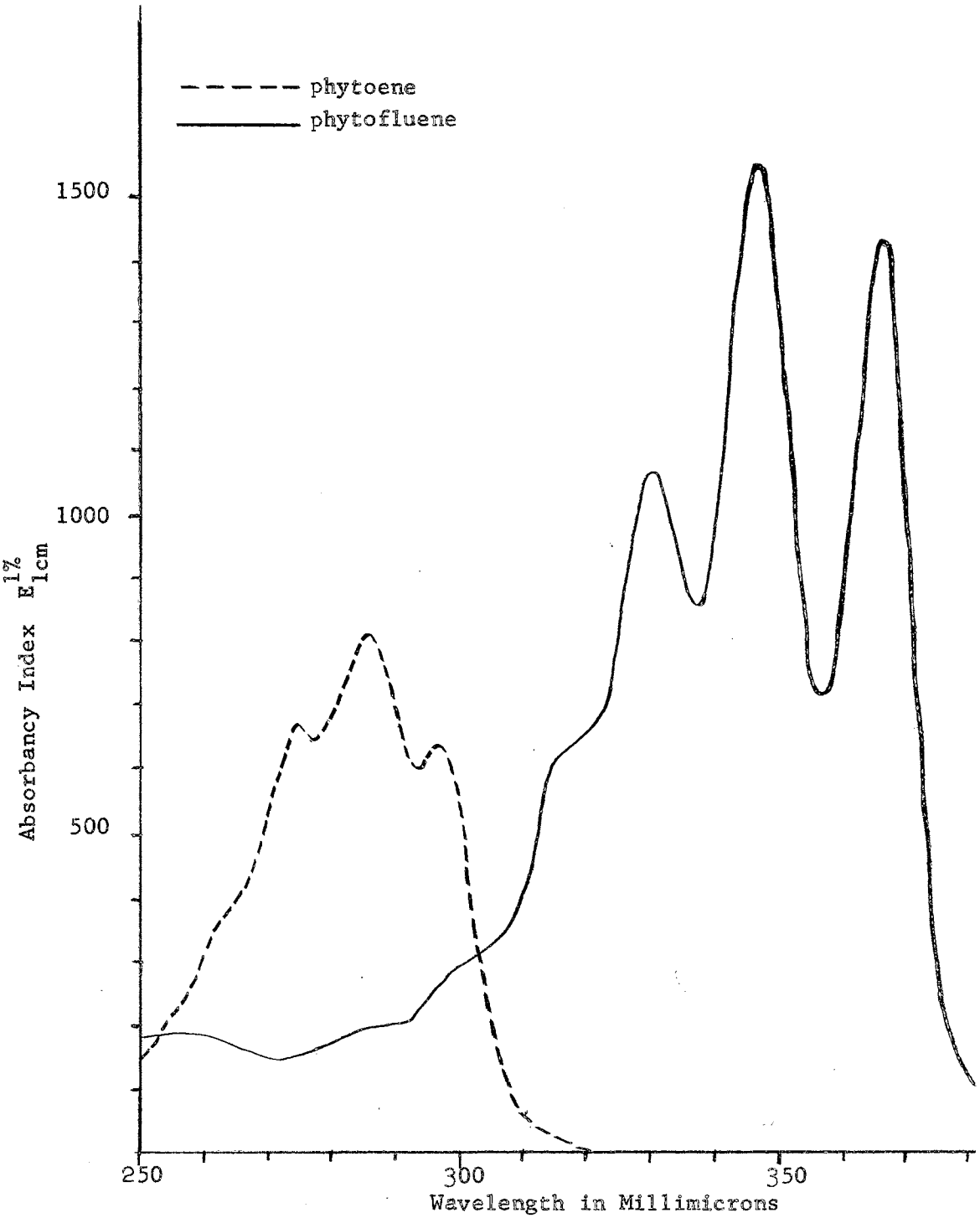


Figure 2. Absorbancy Index of Phytoene and Phytofluene in n-Hexane

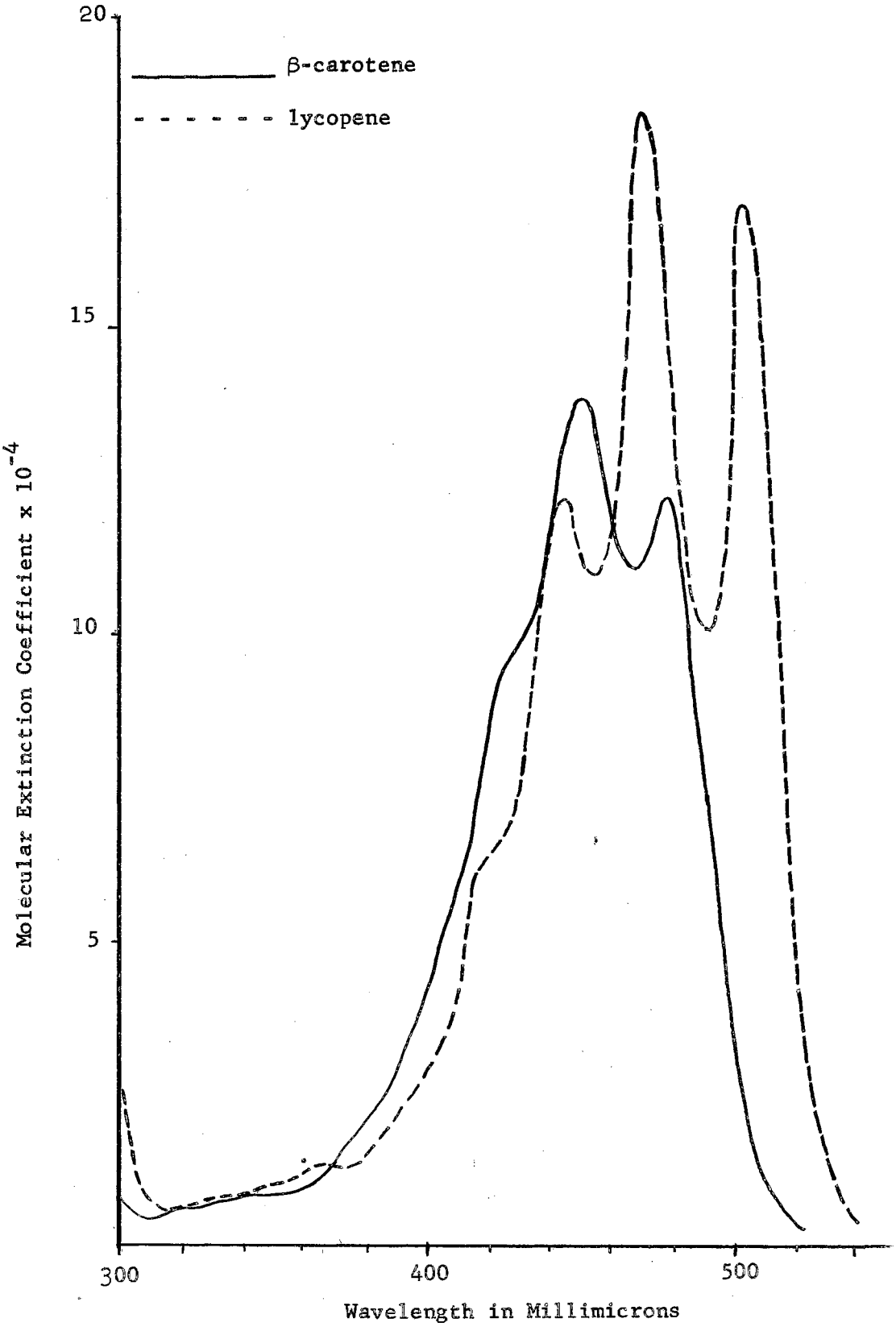


Figure 3. Molecular Extinction Curve of β-Carotene and Lycopene

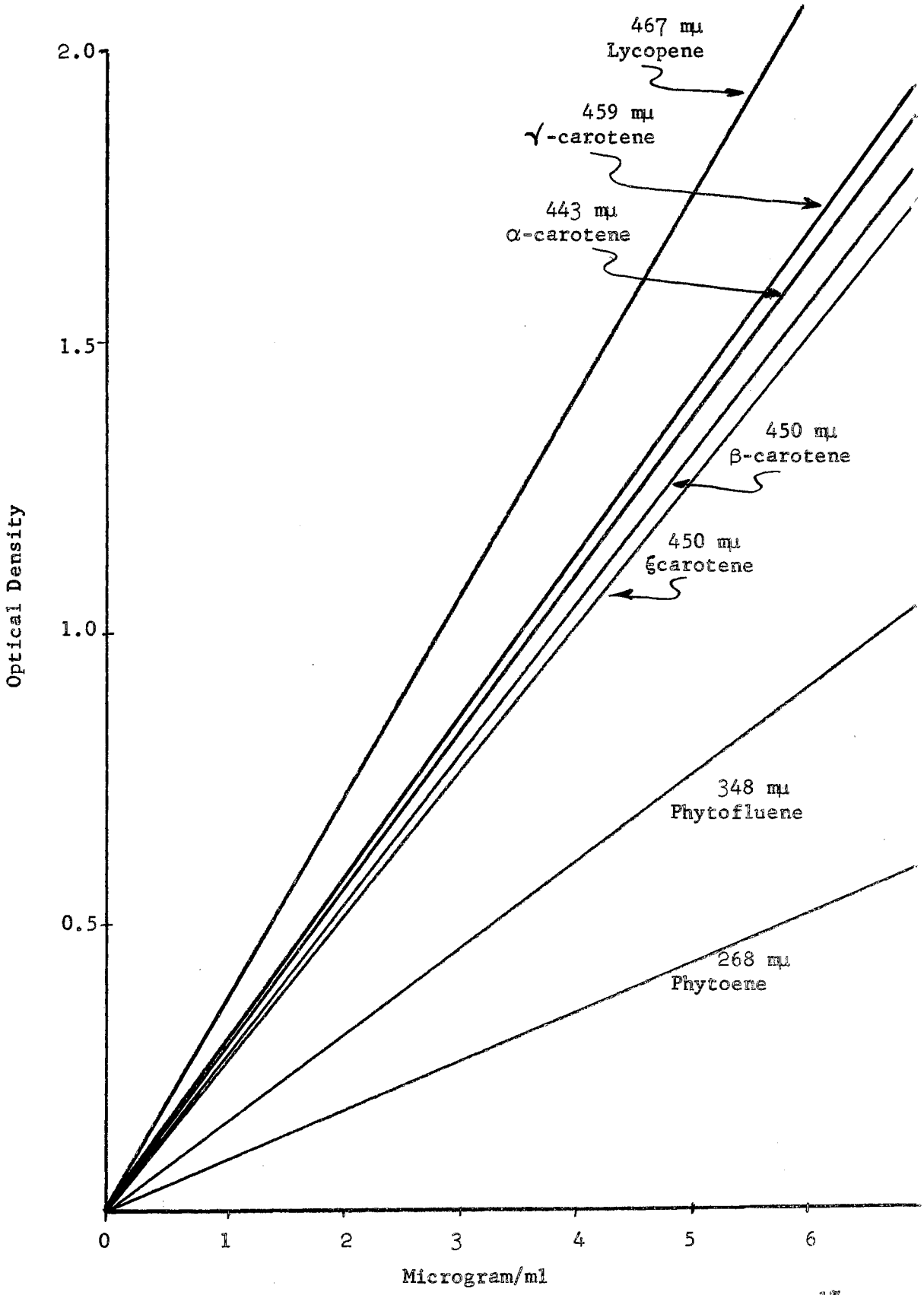


Figure 4. Standard Curves of Carotenoids (From Published $E^{1\%}_{1cm}$ Values)

stage and incubated at room temperature (about 32°C) for 48 hours. The isolated phytoene was purified on an alumina column. Phytofluene, the carotenes, and lycopene were rechromatographed on magnesia-super cel columns. Portions of each sample were decolorized by bromination and counted in the Tri-Carb liquid scintillation counter. The observed specific activities were higher than those (Table II) reported by Purcell; because 1) the Tri-Carb liquid scintillation counter has a higher efficiency than a gas-flow Geiger-Muller tube, 2) in this experiment, the injected tomato was incubated at a lower temperature for a longer time period. The amounts of fraction I and II were obtained by weighting the dried samples since no standard curves or absorbancy indexes are available in the literature. Fraction I has a high optical density below 210 mμ but no absorption peak has been observed. Fraction II has an absorption peak at 230 mμ and high absorbancy below 210 mμ. It was shown in this experiment that MVA-2-C^{14} was efficiently incorporated into tomato carotenoids.

Experiment III

The Conversion of MVA-2-C^{14} into $C^{14}O_2$ Respired from Tomato Fruits

At temperatures higher than 30°C, lycopene formation was arrested. It is possible that at higher temperatures, some of the precursors for carotenoids synthesis (i.e. mevalonate or polymer of MVA) were degraded to acetate and subsequently oxidized to CO_2. An experiment was designed to test this hypothesis by measuring the radioactivity in the CO_2 respired by tomatoes injected with MVA-2-C^{14} and incubated at different temperatures. The apparatus for this experiment is shown in Figure 1. As shown in Table III and Figure 5, CO_2 respired at 20°C has the highest radioactivity. It is known that at lower temperature, more organic acids are used in respiration in plant tissues than carbohydrates. This may partially explain the results from this experiment.

TABLE III

RADIOACTIVITY IN CO_2 RESPIRED FROM TOMATOES INCUBATED AT DIFFERENT TEMPERATURES

Temperature °C	Total c.p.m. in Hyamine Solution	c.p.m. Injected	Per Cent Activity Respired as CO_2
10	18,190	6.63×10^5	2.7
20	32,706	5.6×10^5	5.8
30	57,560	1.27×10^6	4.5
38	14,370	5.6×10^5	2.56

See Footnote page 20, Table II.

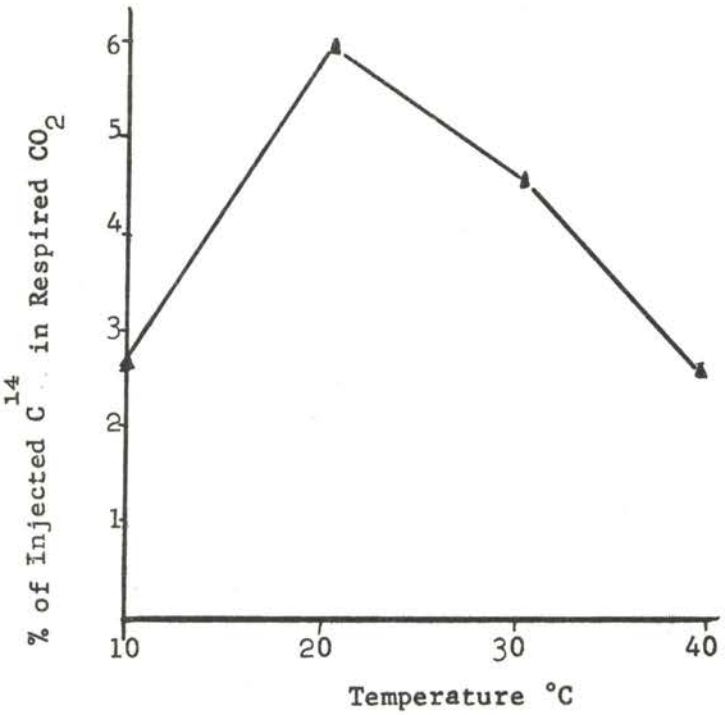


Figure 5. Radioactivity CO_2 Respired from Whole Tomatoes Incubated at Different Temperatures with MVA-2-C^{14}

Experiment IV

Incorporation of Mevalonic-2-C^{14} Acid into Carotenoids in a Tomato Homogenate

Shneour and Zabin reported (33) that the homogenates of tomatoes can synthesize radioactive lycopene from MVA-2-C^{14}. For the purpose of obtaining a uniform starting material for a time study on the carotenoids biosynthesis, Shneour's complete system was adopted in this experiment. A tomato in the pink-red stage was selected. The skin was removed, the tomato was cut into quarters, the placentae was cut off. Thirty gms of this parenchyma of the ovary wall of the tomato were ground for 30 seconds in a Potter-Elvejhem teflon pestle homogenizer with 10 ml of cold medium containing 0.2 M Tris buffer, pH 8.2, 0.2 M nicotinamide and 0.002 M versene. The complete system contained Na-mevalonate-2-C^{14} (3.5×10^6 c.p.m.), 20 μ mole of ATP, 1 μ mole of DPN, 1 μ mole of TPN, 20 μ moles of glutathione, and 50 μ moles of $MnSO_4$ and 9 ml of homogenate prepared with Tris buffer in a final volume of 10 ml. This mixture was incubated in air at 25°C for a definite time period. Three ml portions of the sample were taken at 1 minute, 1 hour, and 24 hours. The carotenoids were extracted, and chromatographed. Samples were plated and counted under a gas flow Geiger-Muller tube as shown in Table IV, with exception of gamma-carotene, all the carotenoids increase in specific activity with increasing time. It should be mentioned here, that in this experiment the colored carotenes and lycopene were not rechromatographed. The specific activities of these compounds were not accurate enough for interpretation since a large part of the radio-activity was present in contaminants. Another problem in this experiment

TABLE IV

INCORPORATION OF 2-C^{14}-MVA INTO CAROTENOIDS IN TOMATO HOMOGENATE

Time	Phytoene	Phytofluene	β-carotene	ζ-carotene	γ-carotene	Lycopene
			Specific Activity (c.p.m./microgram of carotenoid)[1]			
1 min.	33.7	97.4	16.6	86.0	388	3.83
1 hr.	33.9	105.7	32.6	363.0	114	4.0
24 hr.	138.4	141.6	28.4	402.2	81.3	5.82
			Total Activity (c.p.m. in the Carotenoid)*			
1 min.	1,452.8	1,656	201	79.8	400	440
1 hr.	1,855	930	700	400	340	749
24 hr.	3,834	2,577	359.3	591.3	300	838
			Total Concentration (microgram/3 ml Homogenate)			
1 min.	43.2	17	12.2	0.93	1.03	115
1 hr.	54.7	8.8	21.5	1.1	2.98	187
24 hr.	27.7	18.2	12.7	1.47	3.69	144

*Planchet counted in gas-flow Geiger-Muller tube corrected for background, not corrected for self absorption, constant geometry.

was that the plant tissue could not be completely homogenized in the Potter-Elvehjem teflon pestle homogenizer. Some whole cells were found in the preparation which could affect the validity of these results.

Experiment V

Ripening of Green Tomato Sections in Sterile Test Tubes

Green mature tomato fruits (Stokesdale) were sterilized with 70% ethanol, cut into one eighth sections under sterile conditions and transferred into sterile test tubes. This section was supported in the tube by glass beads. Moisture was supplied by a few ml of water in the glass bead column. Sections under these conditions can ripen normally at room temperature, in the same way as the whole fruit (Figure 6). When whole tomato fruits were used for an incorporation study it was impossible to select several fruits at the same stage of maturity so this section technique provides a uniform starting point for a time or temperature series. This also permits the investigator to make several injections of the MVA-2-C^{14} into the parenchyma of the ovary wall of the tomato fruit sections, and bring this material into more intimate contact with the tissue. Sections were incubated at room temperature for 0, 3 and 7 days; at 38°C for 0, 4 and 12 days and at 38°C for 4 days then returned to room temperature. Additional 3 and 6 day incubations were conducted. Samples from different treatments were extracted, the carotenoids isolated and determined quantitatively after chromatography.

Data in Table V and Figures 7 and 8 showed that the synthesis of fractions P230 and P275 were not affected by higher temperatures, but the synthesis of phytofluene, β-carotene and lycopene was evidently

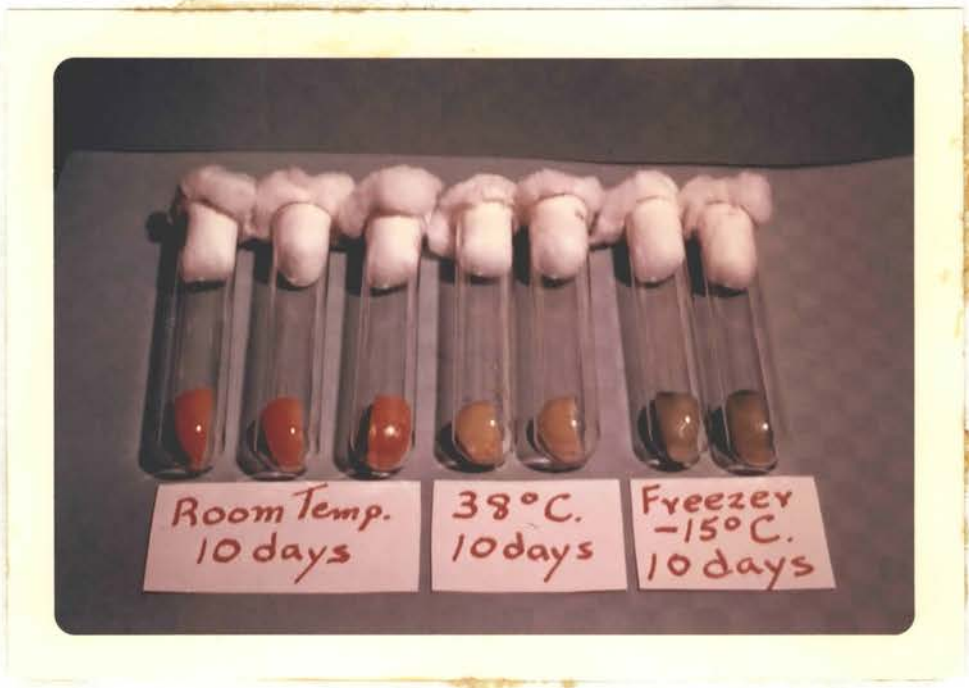


Figure 6. Tomato Sections Incubated at -15°C, Room Temperature and 38°C.

TABLE V

THE CAROTENOIDS FOUND IN RIPENING GREEN TOMATO SECTIONS
(microgram of carotenoid/gm of tissue)

Temperature	Days	P230*	P275*	Phytofluene	β-carotene	Lycopene
R.T.	0	26.9	9.8	0.00	0.476	0.00
R.T.	3	34.1	11.3	0.84	1.02	0.98
R.T.	7	82.7	19.3	3.4	1.743	28.8
38°C	4	60.4	16.6	0.00	0.33	0.00
38°C	12	126.8	36.41	0.00	0.70	0.00
38°C R.T.	4 3	102.3	24.4	1.8	1.016	21.9
38°C R.T.	4 6	113.3	39.4	7.33	1.83	83.3

* P230 and P275 refer to fractions absorbing at 230 mμ and 275 mμ, respectively.

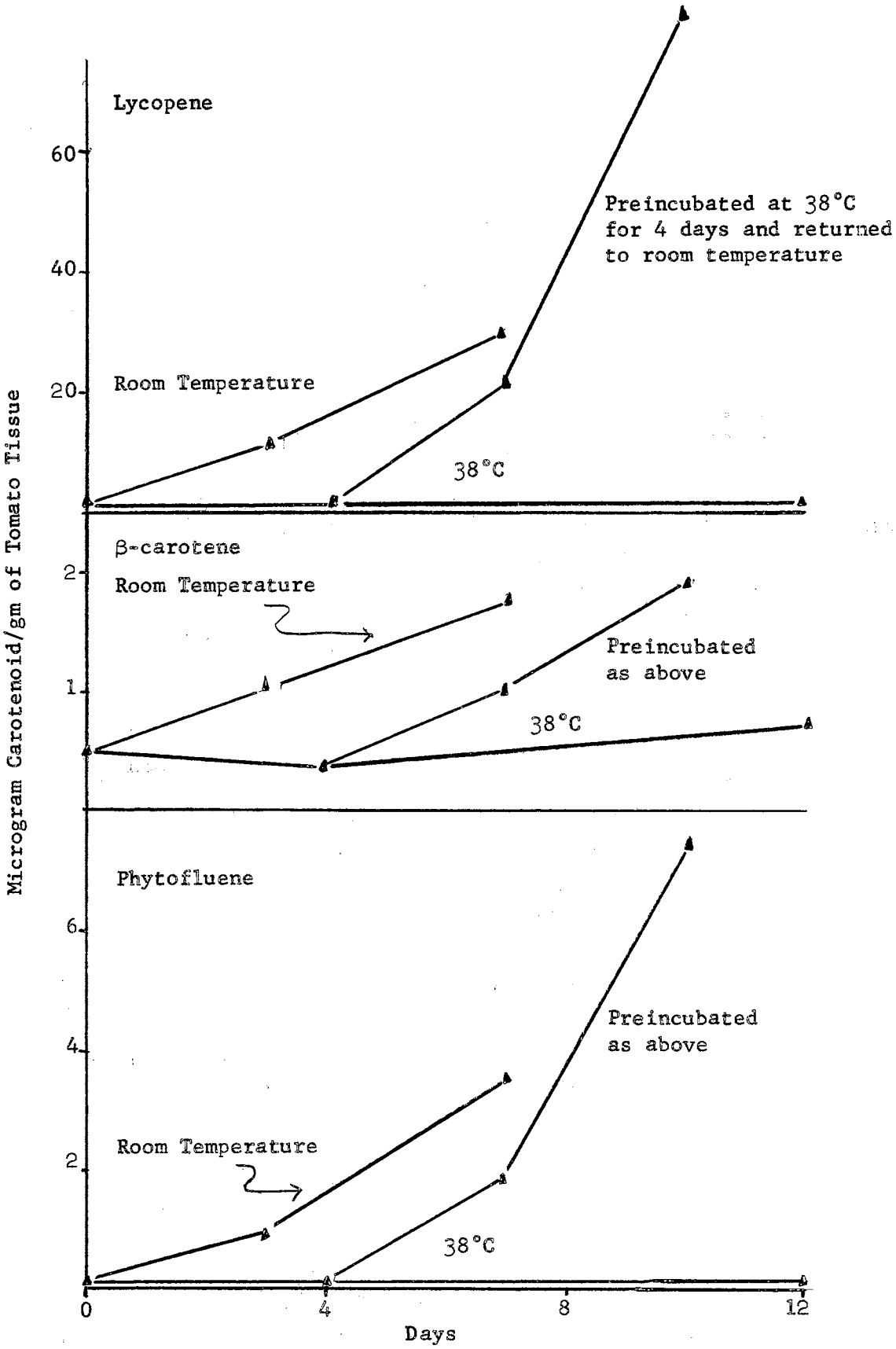


Figure 7. Ripening of Green Tomato Sections in Sterile Test Tubes

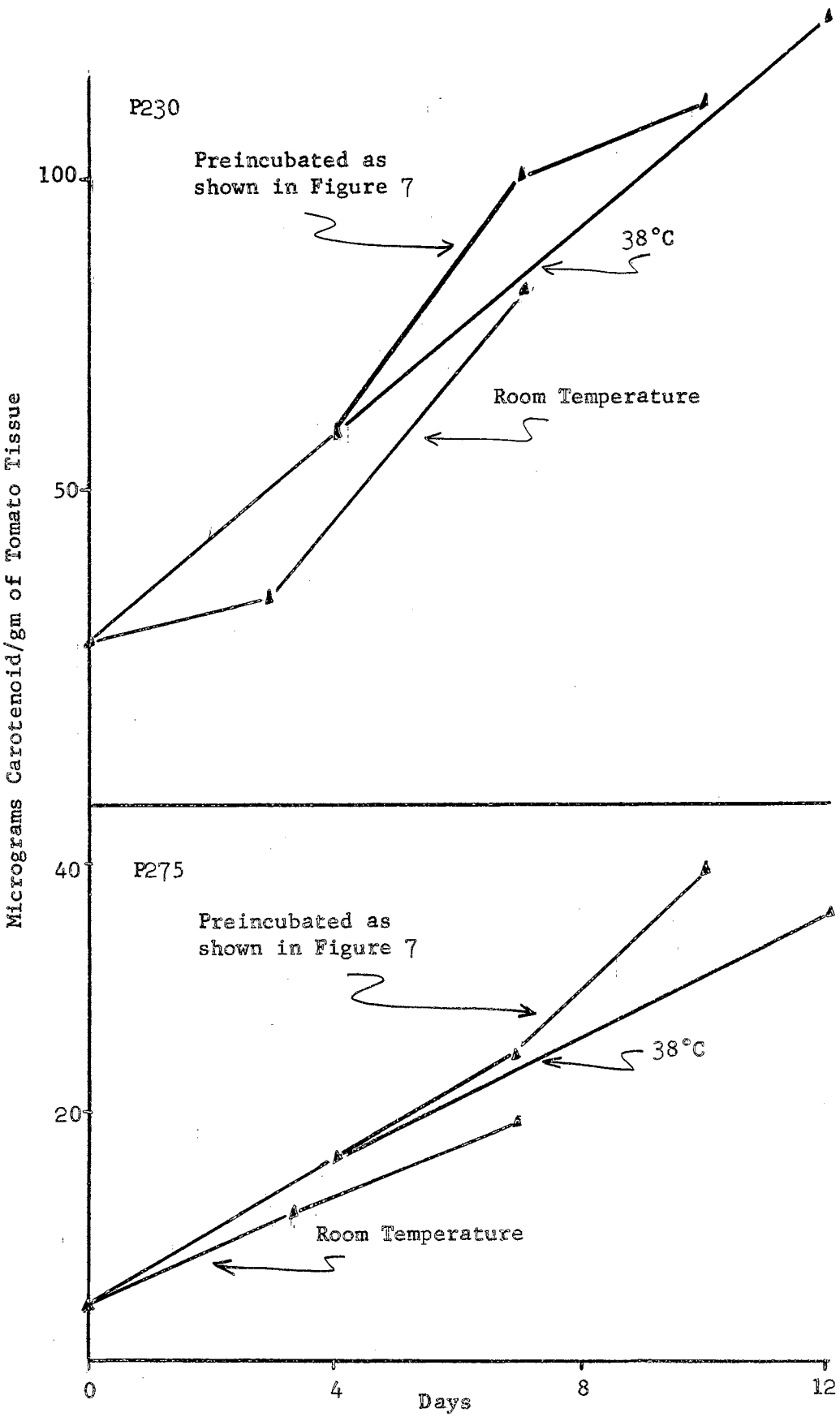


Figure 8. Ripening of Green Tomato Sections in Sterile Test Tubes

arrested at 38°C. Tissue sections in the test tubes were some what desiccated. The data in Table V at higher temperature and for a longer time period were higher than the true value because of this desiccation of the sections. This could account for the apparent rapid synthesis of the preincubated (38°C) sections.

Experiment VI

Effect of Time and Temperature on The Incorporation of MVA-2-C^{14} into Carotenoids in Tomato Sections

This experiment was a revised repetition of Experiment V. It had a similar design and the additional treatments were, first, mevalonate-2-C^{14} was injected into the parenchyma tissues of green mature tomato sections, and second, the fresh weight of the sections was taken as the base for quantitative calculation of carotenoid present. Radioactivity of the isolated carotenoids was determined in a liquid scintillation counter. It should be mentioned that the zero time was actually one and a half hours after the injection of MVA-2-C^{14} into the section. It was found in this experiment that a large portion of radioactivity was incorporated into an unknown fraction within a short time (Table VI). The activity in this fraction decreased rapidly with increasing time. This fraction had the same chromatographic behavior as phytoene, but spectrophotometrically, it was different from phytoene (Figures 9 and 10).It has an absorption peak at 275 mμ and a high absorbancy below 210 mμ (no peak). Another observation in this experiment was that the 230 mμ fraction was essentially free of radioactivity after rechromatography. In Experiment II, this fraction had a high specific activity. This is probably a result of a poor chromatographic separation of radioactive contaminants.

TABLE VI

EFFECTS OF TIME AND TEMPERATURE ON THE INCORPORATION OF 2-C^{14}-MVA INTO CAROTENOIDS IN TOMATO SECTIONS (ROOM TEMPERATURE)

Days	Total µg	µg/gm	Total c.p.m.*	c.p.m./µg*	Incorporation %
			P275		
0	330	10.9	84,362	255.6	37.3
3	331	12.2	39,360	119	35
5	354.8	11.1	1,097.4	3.1	0.97
9	425	16.6	347.8	0.82	0.31
			Phytofluene		
0	0.00	0.00	0.00	0.00	0.00
3	46.8	1.7	21,501	459	19
5	45	1.41	16,401	364.6	14.5
9	138	5.4	18,454	133.7	16.3
			β-carotene		
0	20.4	0.673	513.4	25.2	0.23
3	40.5	1.5	15,689	387.3	14
5	46.25	1.45	14,634	316.41	12.9
9	43.75	1.71	23,688	514.4	20.9
			Lycopene		
0	0.00	0.00	0.00	0.00	0.00
3	69.6	2.75	549.9	7.9	0.49
5	202	6.32	1,097.4	5.4	1.93
9	1947	76.0	1,046.3	0.54	0.93

* See footnote page 20, Table II

TABLE VI (Continued)
(38°C)

Days	Total µg	µg/gm	Total c.p.m.*	c.p.m./µg*	Incorporation %
			P275		
0	330	10.9	84,362	255.6	37.3
3	249.7	15.4	2,328	9.3	2.05
5	594.5	19.8	2,989	5.02	2.6
9	419.2	25.42	1,880	4.5	1.7
			β-carotene		
0	20.4	0.673	513.4	25.2	0.23
3	7.35	0.45	1,012.2	137.7	0.89
5	15.4	0.513	6,202	402.9	5.5
9	7.64	0.463	3,478	455.2	3.07
	(Preincubation at 38°C for 5 days)				
			P275		
0	594.5	19.8	2,989	5.02	2.6
3	481.8	27.1	3,444	7.15	3.04
5	271.1	32.4	843.8	3.1	0.75
9	842	73.5	118.9	0.14	0.11
			Phytofluene		
0	0.00	0.00	0.00	0.00	0.00
3	73.8	4.15	6,221	84.3	5.5
5	73.6	3.89	11,944	162.3	10.6
9	37.4	3.26	8,167.6	218.4	7.2
			β-carotene		
0	15.4	0.513	6,204	402.9	5.5
3	16.5	0.93	2,985	180.6	2.64
5	27.4	1.45	15,162	553.4	13.4
9	14.3	1.26	4,418.7	306.2	3.9

TABLE VI (Continued)
(Preincubated at 38°C for 5 days)

Days	Total µg	µg/gm	Total c.p.m.*	c.p.m./µg*	Incorporation %
			γ-carotene		
0	0.00	0.00	0.00	0.00	0.00
3	1.74	0.1	534.7	307.3	0.47
5	14.98	0.79	531.3	35.5	0.47
9	18.6	1.62	610.8	32.84	0.54
			Lycopene		
0	0.000	0.00	0.00	0.00	0.00
3	105	5.91	777	7.4	0.69
5	425.5	22.5	416	0.98	0.37
9	539	47.1	959.3	1.78	0.85

*See footnote page 20, Table II

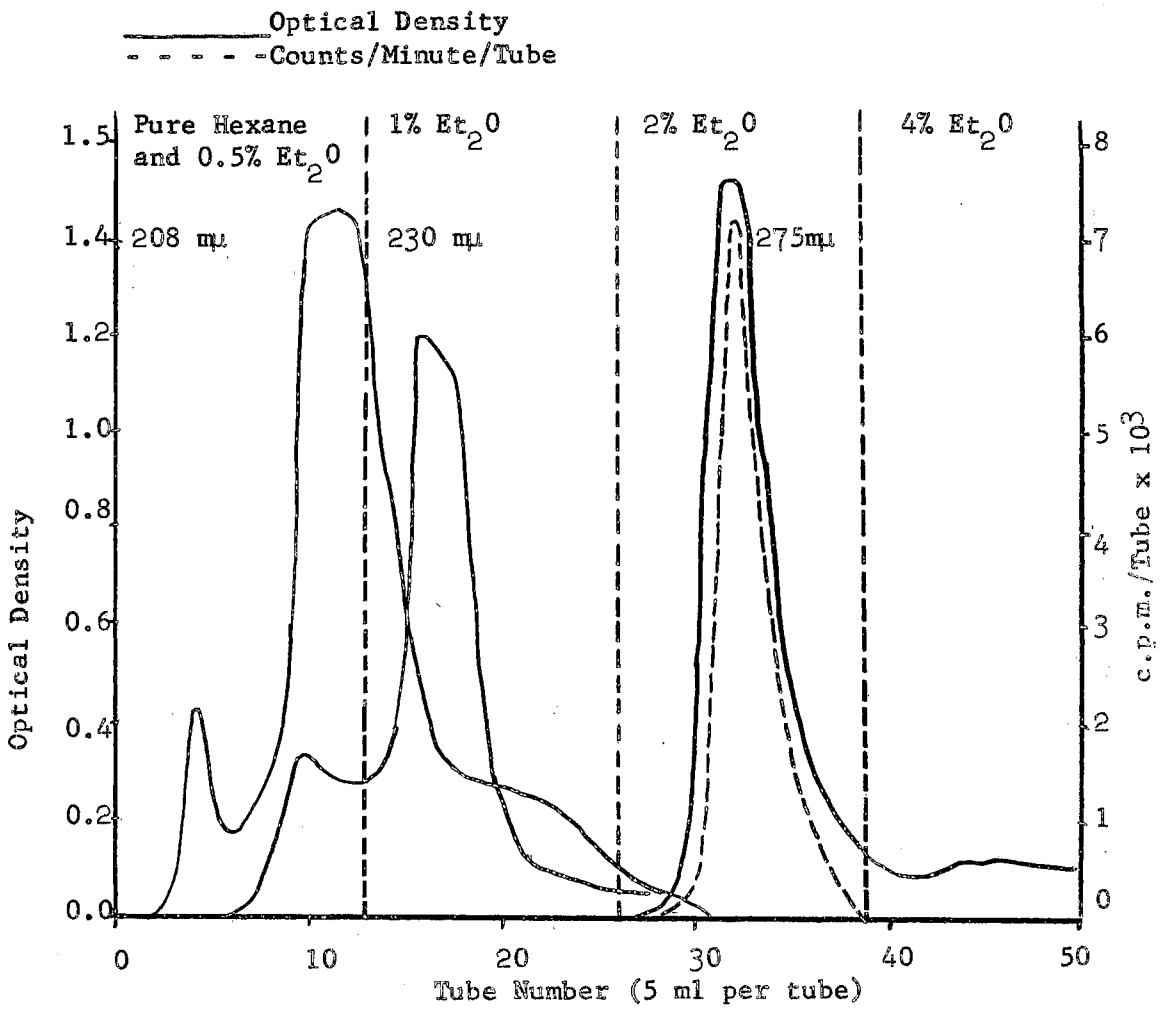


Figure 9. Chromatographic Separation of P275 on Alumina

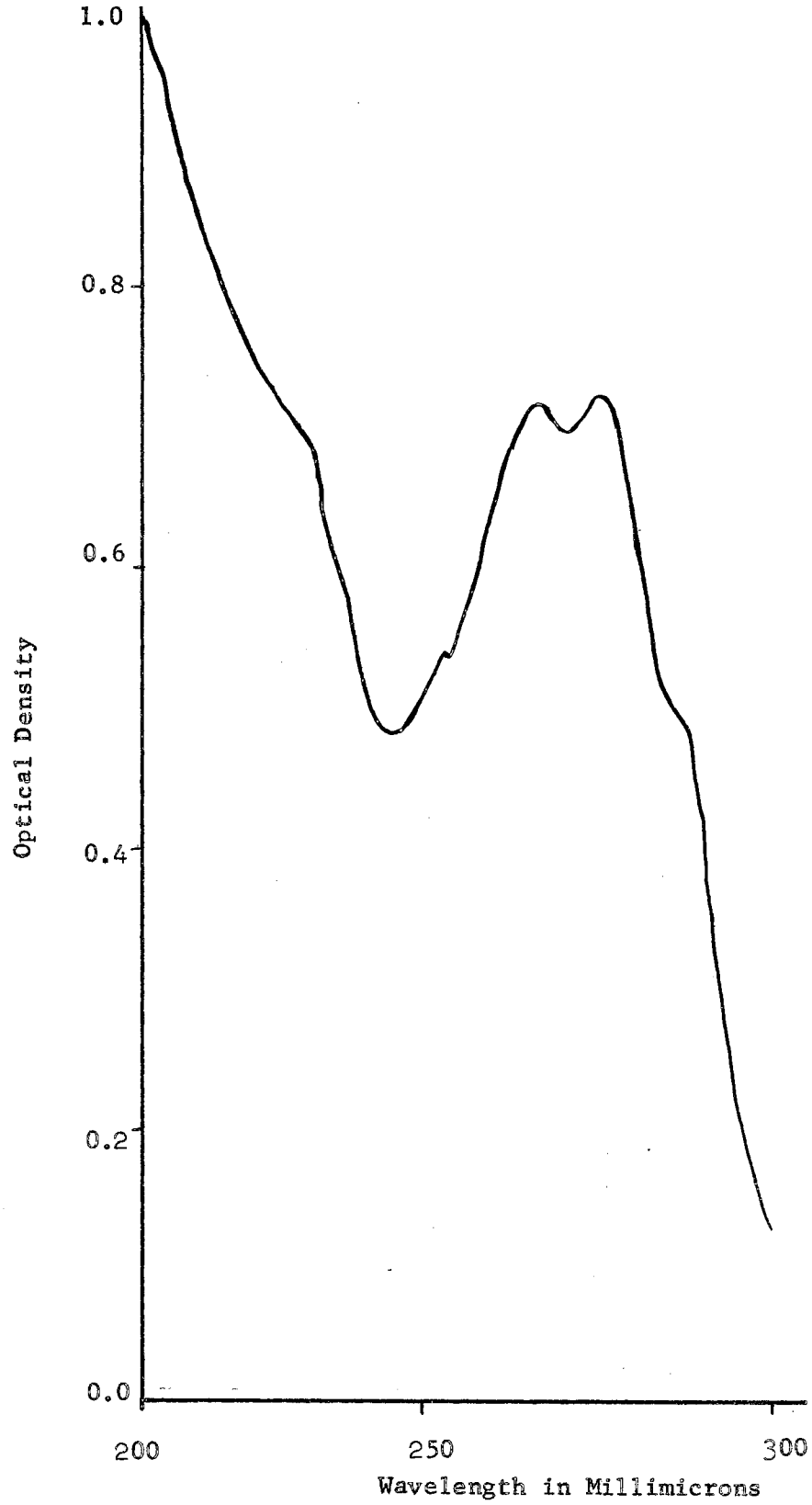


Figure 10. Absorption Spectrum of P275 Fraction

At room temperature, the specific activity of phytofluene also decreased with increasing time, but the rate of decrease was slower than that of P275. The specific activity of beta-carotene increased rapidly with time. The total activity in counts per minute in lycopene increased about 2 fold on the fifth day, but the specific activity decerased as it was diluted by lycopene formed in the rapid synthesis during ripening. (Table VI).

At 38°C the specific activity of P275 decreased as rapidly as that at room temperature. β-carotene also had the same rapid rate of synthesis as at room temperature. No phytofluene, lycopene and other colored carotenes were found at 38°C. Once the samples were brought back to room temperature, the synthesis of phytofluene, lycopene and other colored carotenes were observed (Table VI).

In this experiment, the time intervals were measured in days. For the incorporation study this time period was too long. Many unknown reactions may take place. The radioactivity of the colored carotenes and lycopene was determined on the fractions eluted from the mangesia-super cel column without rechromatographic steps. Radioactivity impurities were without doubt present in these solutions and would account for a large part of the apparent isotope incorporation.

Experiment VII

Effect of time and temperature of the incorporation of MVA-2-C^{14} into carotenoids in tomato sections (Short time study)

Because of the high amount of incorporation of radioactivity into fraction P275 at 0-time in the previous experiment, it was felt desirable to study the rate of incorporation in shorter time periods. All the fractions were purified by rechromatography. Fraction P275

was purified on an alumina column, as in earlier experiments. Phytofluene, beta-carotene and lycopene were purified on the $Ca(OH)_2$-Supercel column.

It was observed in this experiment that the fraction P275 actively incorporates much of the injected radioactivity within the first 60 minutes. The specific activity of this fraction decreases rapidly after the peak value (Table VII, Figure 11). The specific activity data for β-carotene shows that a large amount of radioactive impurities was removed by rechromatography on $Ca(OH)_2$-Supercel column (compare Table VI and Table VII).

Experiment VIII

Effect of time and temperature on the incorporation of MVA-2-C^{14} into carotenoids in tomato sections

A repeat of Experiment VI was conducted. Reproducible results were obtained (Table VIII). The quantities of P275 were lower than those in Experiment VI. This may be caused by the use of a different variety and a chromotographic effect.

The carotenoid quantities and specific activities of this experiment at 38°C shows that a higher temperature stimulates the synthesis of P275 fraction and β-carotene in green mature tomato fruit (Table VIII). In the advanced turning (pink-red ripened) tomato fruit, the picture was different from that in the green mature fruits. The P275 fraction disappeared and phytoene (P286) was found instead. The specific activity of phytoene increased with time (Table VIII). In this stage, phytofluene and locopene were essentially constant in quantity, while the specific activity increased.

Beta-carotene, which incorporated appreciable amounts of radioactivity in green mature fruits, was free from radioactivity in the advanced turning stage. (Table VIII, Figure 12).

TABLE VII

EFFECT OF TIME AND TEMPERATURE OF THE INCORPORATION OF MVA-2-C^{14} INTO CAROTENOIDS IN TOMATO SECTIONS (ROOM TEMPERATURE)

Time (Minutes)	Total µg	µg/gm of Tissue	Total* c.p.m.	c.p.m.* per µg	Incorporation %
			P275		
0	249.7	9.39	287	1.08	0.16
10	547	17.90	6,652	12.16	3.8
30	523.6	18.9	24,539	46.9	14.17
60	189.6	6.7	42,242	222.8	24.4
120	380.2	14.7	28,296	74.33	16.3
300	404.0	11.5	20,995	52.00	8.56
			β-carotene		
0	11.75	0.44	0.00	0.00	0.00
10	12.5	0.41	0.00	0.00	0.00
30	9.5	0.34	0.00	0.00	0.00
60	11.5	0.41	58.8	5.1	0.034
120	8.98	0.35	273.6	30.4	0.16
300	16.85	0.48	3,042	202	1.4

*See footnote page 20, Table II

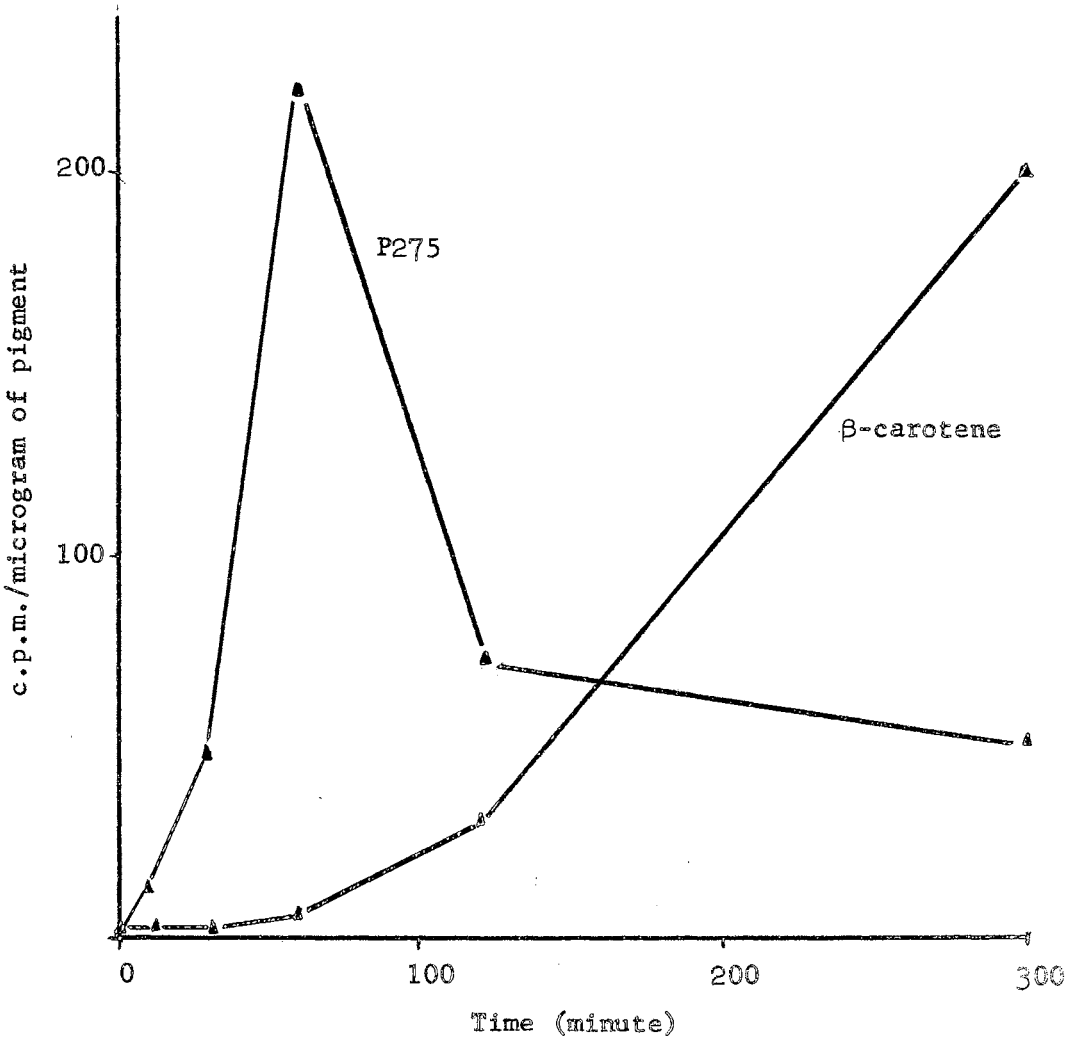


Figure 11. Specific Activity of Fraction P275 and β-Carotene with Time

TABLE VIII

EFFECT OF TIME AND TEMPERATURE ON THE INCORPORATION OF MVA-2-C^{14} INTO CAROTENOIDS IN TOMATO SECTIONS

A, GREEN MATURE

1, Room Temperature

Time (Minutes)	Total µg	µg/gm of Tissue	Total* c.p.m.	c.p.m.* per µg	Incorporation %
			P275		
0	161	5.7	460	2.86	0.2
30	152	5.7	18,880	124.2	8.2
60	137	4.6	28,045	204.7	12.1
200	135	4.5	9,662.5	71.5	4.2
			β-carotene		
0	15.5	0.55	0.00	0.00	0.00
30	21.5	0.81	180	8.4	0.08
60	23.5	0.76	250	10.64	0.11
200	20.0	0.66	720	36.00	0.31
2, 38°C			P275		
60	262	8.9	88,000	355.88	38.1
200	270.3	9.69	18,820	69.63	8.1
			β-carotene		
60	25.5	0.87	980	38.4	0.42
200	23.7	0.85	1,560	65.8	0.68
B, ADVANCED TURNING (PINK-RED RIPEN)					
Room Temperature			Phytoene		
60	76.0	3.38	23,390	307.8	11.3
200	101.4	4.7	39,403	388.6	20.8
			Phytofluene		
60	23	1.02	1,190	51.74	0.63
200	22.5	1.05	6,378	283.5	3.4
			β-carotene		
60	43	1.9	0.0	0.0	0.0
200	34	1.58	0.00	0.00	0.00
			Lycopene		
60	106.5	4.73	880	8.263	0.47
200	102.7	4.77	1,970	19.2	1.04

*See footnote page 20, Table II

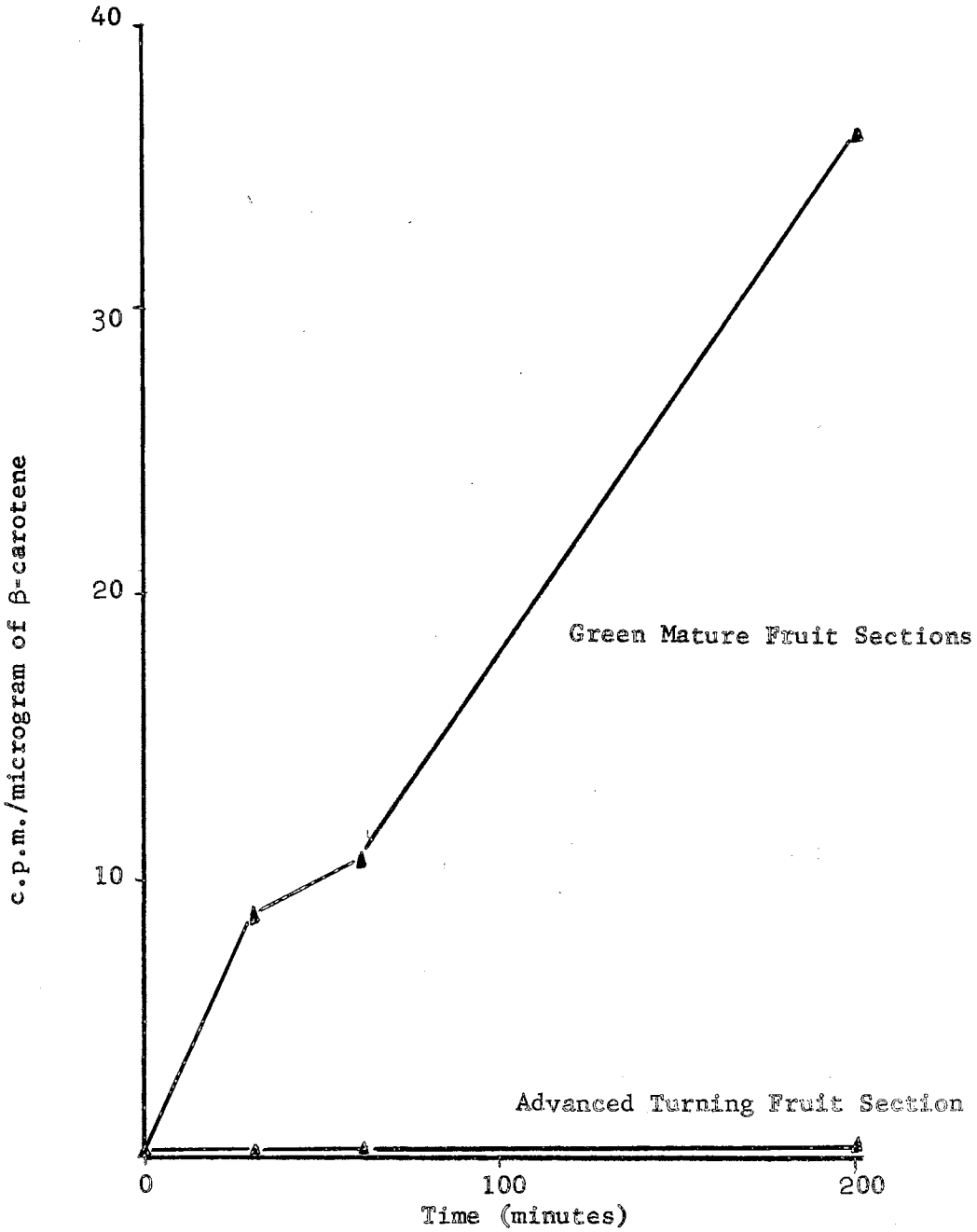


Figure 12. Incorporation of MVA-2-C^{14} into β-Carotene in Green Tomato and Advanced Turning Tomato

CHAPTER V

DISCUSSION

The mechanism by which carotenoids are synthesized in plant tissues has been widely debated. Different hypotheses have been proposed by many investigators. Based on the results in this research work, reproducible results of Purcell's observation (30) have been obtained. This shows that MVA-2-C^{14} serves as a precursor in carotenoid biosynthesis in the tomato. Anderson has shown (3) that 90 per cent of this accompaning radioactivity is not present in the carotenoids. Separation of this contaminating radioactivity may be achieved by rechromatography on a different adsorbent or by crystallization. It is shown in Table VII, compared to Table VI, that a large amount of radioactive impurity in β-carotene was removed by rechromatography on a calcium hydroxide-supercel column. The specific activities of fractions eluted from the first magnesia column are in error and should not be used. In Experiments VII and VIII all of the fractions were purified by rechromatography, and the radioactivity represents true incorporation of MVA-2-C^{14} in to the carotenoids. In Experiment IV the radioactivity of carotenoids was measured with a thin window gas-flow Geiger-Muller tube. Carotenoids are soluble in n-hexane and since this is a solvent with a very low surface tension, a problem of restricting the solution to the bottom of the planchet arose. The solution would flow up the wall of the planchet evaporate at the solvent front and cause a tremendous error in the istope assay.

This trouble was avoided when samples were counted in the Tri-Carb liquid scintillation spectrometer. The carotenes and lycopene are highly colored compounds and phytofluene is a flouresent compound. Radioassay data on the direct determination of C^{14} in these compounds with the Tri-Carb counter should be corrected for quenching. Since carotenes, lycopene and phytofluene are 40 carbon hydrocarbons with different numbers of double bonds, they absorb halogen readily (i.e. chlorine, bromine or iodine) and are thereby decolorized easily. This technique was useful in these studies. Methods to decolorize the carotenes and lycopene were described in 'Methods' (page 17). Table IX shows the effect of bromination on the radioassay data of β-carotene. Quenching reduced the count without bromination of the carotenoids to 84% of the value obtained when correction for quenching was made on the basis of internal standard measurement. The corresponding figure for the brominated carotenoids was 92% of actual value. Examples are as follows:

1. Non-decolorized carotenoid sample (yellow color)

   Activity of 1 ml sample = 2691 c.p.m.

   1 ml sample + 1 ml internal standard (toluene-C^{14}) = 7443 c.p.m.

   1 ml toluene-C^{14} = 5642 c.p.m.

$$\text{corrected c.p.m.} = \frac{\text{c.p.m. of sample} \times \text{c.p.m. of toluene-}C^{14}}{(\text{c.p.m. of sample+toluene-}C^{14}) - (\text{c.p.m. of sample})}$$

$$\frac{2691 \times 5642}{7443 - 2691} = 3159 \text{ c.p.m. .....corrected c.p.m.}$$

$\frac{2691}{3195}$ x 100 = 84.2% of correct value

2. Brominated carotenoid sample (colorless)

1 ml sample = 3023 c.p.m.

1 ml sample + 1ml toluene-C^{14} = 8200 c.p.m.

1 ml toluene-C^{14} = 5642 c.p.m.

$\frac{3023 \times 5642}{8200 - 3023}$ = 3295 c.p.m. . . . . .Corrected c.p.m.

$\frac{3023 \times 100}{3295}$ = 91.8% of correct value

In Experiment VI, a compound was obtained from the green mature tomato which has an absorption maximum at 275 mμ (P275). As shown in Figure 11, this compound incorporated a large amount of radioactivity within a relatively short time. The absorption spectrum of this compound is shown in Figure 10 and it has an absorption maximum at 265 and 275 mμ with a high absorbancy below 240 mμ. Rechromatomgraphy on an alumina column did not change the absorption spectrum of this compound. At this time, it would be premature to say that this fraction is a pure compound of evan a 40 carbon polyene although it appears to be a phytoene like material. Further work on the purification and identification of this compound is needed.

The data shown in Figure 11 indicates, but does not prove, that P275 fraction may be a precursor of certain carotenes although other intermediates may be involved between P275 and these carotenes. If there is no compartmentalization, other intermediates must be involved.

An attempt has been made to determine whether the P275 fraction is

TABLE IX

EFFECT OF BROMINATION OF RADIOASSAY DATA OF RADIOACTIVE β-CAROTENE SOLUTIONS COUNTED BY TRI-CARB LIQUID SCINTILLATION SPECTROMETER

Amount of β-carotene gm/ml	Br_2 Treated c.p.m./ml	Not Treated c.p.m./ml
1.72	204.1	185.2
1.55	261.7	200.1
4.50	357.7	260.7
1.32	116.1	96.4

in fact a precursor of carotenes or lycopene. Radioactive P275 isolated from green tomatoes (incubated with MVA-2-C^{14}) was suspended in water with Tween 40 and injected into tomato sections. After incubation at room temperature for 24 hours. About 98% of the activity recovered was found in the P275 fraction suggesting that the P275 fraction was not metabolized. This could be attributed to its lack of solubility and to its immobility within the fruit section. A cell free system may give different results.

In Experiements VI, VII, and VIII, the specific activity data shows that the synthesis of P275 and β-carotene as measured by the incorporation of MVA-2-C^{14} was not effected by higher temperatures ($38^{o}C$). The synthesis of phytofluene and lycopene was completely blocked at $38^{o}C$. Once the samples were restored to room temperature, the synthesis of phytofluene and lycopene was renewed, but the rate of change of the specific activity of P275 and β-carotene was not effected by changing temperatures from $38^{o}C$ to room temperature. The structural formula of P275 is not known, but the structures of all other carotenoids, studied are well established.

β-carotene, in which the synthesis is not blocked at higher temperatures is a cyclic compound whereas with phytofluene (37) and lycopene, acyclic compounds, the synthesis can be arrested at higher temperatures. With regard to the specific activity data of different carotenoids under different temperature condition and their structural formula, there is reason to suspect that from a common precursor, the cylic carotenoids and the acyclic carotenoids are synthesized by different pathways. Goodwin (16) in his review mentioned this possibility. The dehydrogenation mechanism may be operating between the

acyclic carotenoids. Zechmeister (42) conducted an in vitro conversion of phytofluene and phytoene into the carotenoid pigments with N-bromosuccinimide. The spectrophotometric behavior of the resulting dehydrogenation products were similiar to those of lycopene and ζ-carotene.

As shown in Fig. 12, β-carotene incorporated an appreciable amount of radioactivity in green mature tomato after injection of MVA-2-C^{14} but is free from radioactivity in the advanced turning tomatoes under the same treatment. Based on this observation, it is reasonable to speculate that β-carotene synthesis is closely related to the tomato fruit chloroplast and its fate in this tissue. This concept was also suggested by Bracco and Euler (5).

Purcell in his recent paper (31) reported that uniformly labeled glucose was incorporated into carotenoids less efficiently than mevalonate-2-C^{14} but more efficiently than acetate. Although mevalonate is the best precursor of carotenoids so far found, a large proportion is also incorporated into other nonsaponifiable materials. Only small amounts of glucose are incorporated into other nonsaponifiable materials. This might be explained on the basis that chloroplast fragments have a greater ability to form mevalonic acid from glucose than do other parts of the cell. Mevalonic thus formed would be more immediately available for carotene synthesis than for formation of other isoprenoids which are not formed in the plastid fragment.

It should be emphasized that the observation reported in this study are of a very limited nature on one variety of tomato fruit and if the same type of experiments were conducted on tomatoes with a different genetic background these observed patterns may not hold.

CHAPTER VI

SUMMARY

A procedure for the extraction and chromatographic separation of carotenoids in tomato fruits has been developed which was used in the identification and quantitative determination of carotenoids. Purcell's work (30) of the incorporation of MVA-2-C^{14} into tomato carotenoids has been repeated. The results showed that MVA-2-C^{14} was incorporated into tomato carotenoids and the results are similar to that reported by Purcell's group.

The $C^{14}O_2$ respired by the tomato fruits incubated at different temperatures after injection of MVA-2-C^{14} was collected and the radioactivity in the CO_2 has been determined. Results showed that the highest conversion of MVA-2-C^{14} into $C^{14}O_2$ occurred at 20°C. This conversion reached the 5.8 per cent level.

Sections from the same tomato fruits (green mature and advanced turning) were ripened in sterile test tubes. MVA-2-C^{14} was injected into these sections. The amounts of carotenoids and incorporated radioactivity in sections incubated under different temperatures and for different lengths of time were determined. It was observed that a compound which has absorption peaks at 265 mμ and 275 mμ is found in green mature tomatoes but disappeared in advanced turning tomatoes. This compound has a chromatographic behavior similar to phytoene but is spectrophometerically different from phytoene. Radioassay data showed that this compound incorporated a large amount of MVA-2-C^{14} at a rapid

rate. The maximum incorporation occurred at 60 minutes. Quantitative data showed that the formation of P230 and P275 were not affected at temperatures higher than 30°C, but the formation of phytofluene, β-carotene and lycopene was blocked at 38°C. Specific activity data showed that the incorporation of MVA-2-C^{14} into P275 and β-carotene was not affected at a higher temperature (38°C), but the incorporation of MVA-2-C^{14} into phytofluene and lycopene was blocked at a higher temperature (38°C). Radioassay data showed that in green mature tomatoes an appreciable amount of MVA-2-C^{14} was incorporated into β-carotene but in advanced turning tomatoes, β-carotene was free from radioactivity (after 200 minutes).

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