THE BIOSYNTHESIS OF CAROTENES IN THE TOMATO

Thesis by

Guy Allen Thompson, Jr.

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1959

DEDICATION

This thesis is fondly dedicated to

Louise Chaney Thompson

ACKNOWLEDGEMENTS

This work would not have been possible without the patient guidance and advice of Professor James Bonner. His criticisms of experimental approaches and of the thesis manuscript have been invaluable. Dr. Albert E. Purcell's collaboration on many of the experiments is also acknowledged. I am deeply indebted to him for advice concerning experimental procedures, for furnishing his own unpublished results, and for his help in proofreading the thesis.

Mr. George P. Keyes, Mr. Charles Newman, and the entire staff of the Earhart Plant Research Laboratory are responsible for the survival of a large collection of tomato plants in the face of my own horticultural malpractices.

Dr. W. L. Stanley of the United States Department of Agriculture Fruit and Vegetable Chemistry Laboratory, Pasadena, has unselfishly devoted much time to performing analyses of compounds isolated during the course of the experiments.

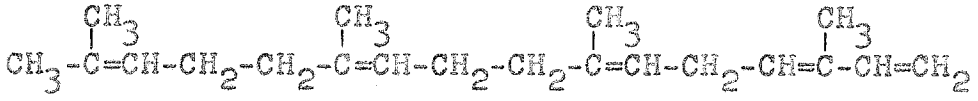
I would like to thank the California Institute of Technology for awarding me a number of generous teaching assistantships, fellowships, and scholarships. I am also grateful to the Du Pont Company, which has supported my research for the past year, and to the Arthur McCallum Fund for financial aid permitting me to carry out research during the summer months.

Mrs. Alethea Miller has offered many useful suggestions in editing this thesis and has typed the manuscript.

ABSTRACT

Ripening tomatoes were injected with 1C^{14}-acetic acid or 2C^{14}-mevalonic(3,5-dihydroxy-3-methylvaleric) acid and, after various incubation times, the carotenes were extracted and purified. The pattern of labeling among the carotenes leads to the conclusion that the more unsaturated pigments are not formed by the dehydrogenation of less unsaturated carotenes, but, rather, that most carotenes are synthesized by independent pathways, although from common precursors.

2C^{14}-Mevalonic acid was found to be incorporated into the carotene fraction with high efficiency. Most of the radioactivity was found by chromatographic separation of the polyenes to be associated with the phytoene fraction. Purification of this fraction yielded phytoene of little radioactivity and an unknown compound of specific activity higher than that of any purified carotene. This compound, designated as fraction II, has been shown to be a 20-carbon-atom isoprenoid hydrocarbon containing a pair of conjugated double bonds. Its probable structure is given below.



Fraction II is rapidly synthesized from mevalonic acid in both ripening and immature tomatoes. A complete pathway for the formation of the carotenes from mevalonic acid is proposed. It is postulated that fraction II is formed

throughout the life of the fruit and is continuously converted to an as yet uncharacterized polyene, which yields carotenes at the time of ripening.

The incorporation into carotenes of $C^{14}O_2$, $1C^{14}$-glucose, uniformly C^{14}-labeled-glucose, and uniformly C^{14}-labeled leucine is described, and the patterns of labeling are compared with those obtained using mevalonate or acetate.

TABLE OF CONTENTS

I. INTRODUCTION

The importance of isoprenoid compounds in animals has been recognized for several decades. The characterization of steroid hormones and of certain vitamins containing isoprenoid structures has led to major advances in medicine. Plant substances constructed of isoprene units have also been described. Since in most instances no useful function could be found for these compounds, the majority of them have been placed in a class termed "secondary plant products." Terpenes, sterols, carotenoids, and rubber fall into this classification.

Whether the plant isoprenoids are of secondary importance and are produced as mere waste products remains to be determined. Our knowledge of the biochemical processes of the plant cell is inadequate to permit any conclusions at the present time. From all appearances the view that rubber and the essential oils are side products is correct. Aside from possible value as a protection against environmental stresses and damages, these compounds seem to be inert. Certain other of the "secondary plant products" may in the future be shown to play important roles in plant metabolism. Sterols are found universally in plants. It is probable that they serve an as yet undiscovered purpose within the cell. Carotenoids have already been implicated as taking part in the photosynthetic processes of higher plants.

The functions of isoprenoids occurring in animals have usually been investigated by first isolating and purifying the compounds and then testing their effects upon the metabolic processes. This method is of limited usefulness in the study of analogous plant compounds because of the inherently slower metabolism found in plants. A more profitable approach lies in the study of isoprenoid formation and localization within the plant.

This thesis presentsstudies that have been made concerning the biosynthesis of a typical class of plant isoprenoids, the carotenes. As will be seen in subsequent sections, all isoprenoids, both plant and animal, appear to have a similar biochemical origin. It is likely that information discovered by these experiments will be useful in the study of this general pathway of biosynthesis. In addition, the evidence reported here may contribute to a clearer understanding of the function of carotenes and related compounds in the plant cell.

II. BACKGROUND FOR PRESENT STUDY

A. Characterization

Since Berzelius (1) in 1837 obtained "Blattgelb" or "Xanthophyll" from autumn leaves a host of chemists and botanists have examined carotenoid-containing organisms for the purpose of elucidating the occurrence, formation, and composition of these pigments. Carotenoids were isolated in reasonably pure form in 1907 by Willstätter (2), who also determined the general structure of the carotenes and realized the existence in nature of the xanthophylls. Some years later Zechmeister (3) and Karrer (4) independently demonstrated the aliphatic nature of the carotenes, and subsequent work, largely from the laboratories of these two men, has elaborated the chemical properties of the carotenoids.

In referring to the polyenes considered in this paper, the term "carotenoid" will be used to include all compounds composed of eight five-carbon isoprene units. The term "carotene" will refer to carotenoid hydrocarbons, including those which contain too few conjugated double bonds to produce color. Oxygen-containing carotenoids will be designated as "xanthophylls."

B. Occurrence

We have today a relatively complete picture of the distribution of carotenoids in various organisms. Detailed evidence compiled by Karrer and Jucker (5) and by Goodwin (6) makes it clear that carotenoids in animals are almost

certainly of plant origin. Many animals, while they lack the ability to synthesize carotenes, can oxidize those ingested to xanthophylls. Moreover, the high concentrations of carotenoid pigments often found in the skin of tropical fish and in the plumage of birds are good evidence that such compounds may be selectively accumulated.

Plants appear to be universally endowed with the ability to synthesize carotenoids. Bacteria, fungi, and other lower plants have been shown to form these pigments. All green parts of plants contain carotenoids in association with chlorophyll. β-Carotene has been implicated in the absorption and transfer of energy in the photosynthetic process (7,8). The same pigment is always present in the leaves of higher plants, as are various xanthophylls. Not uncommonly, one or more carotenoids are accumulated in a particular part of a plant such as the root, blossom, or fruit. β-Carotene is present in high concentrations in carrots and palm oil. Large amounts of lycopene are found in ripe tomato fruits.

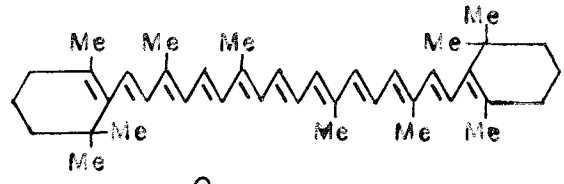
Figure 1 depicts the structure of some of the carotenes which are of most abundant and frequent occurrence in the plant world.

C. Biosynthesis

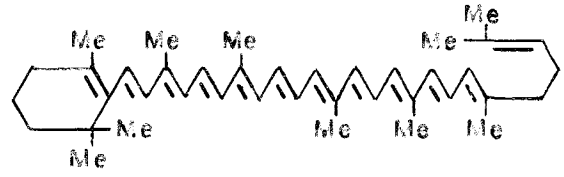
a) General

Several approaches have been used in the study of the biogenesis of carotenoids. The discovery that different organisms or different genetic mutants of a single organism

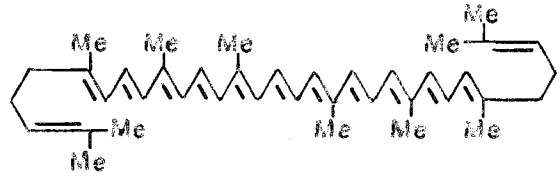
FIGURE I



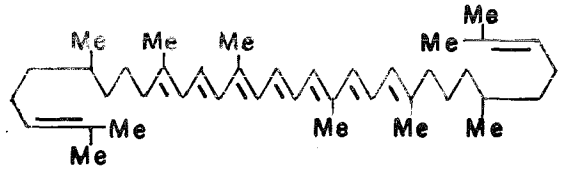
β-CAROTENE



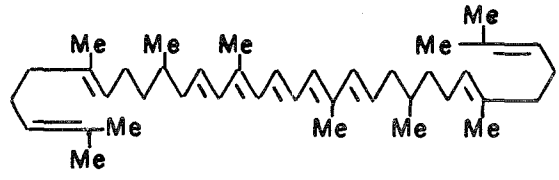
γ-CAROTENE



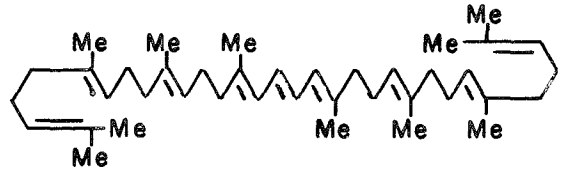
LYCOPENE



ζ-CAROTENE



PHYTOFLUENE



PHYTOENE

may accumulate particular members of the carotene group has led many workers to study such systems. The accumulation of carotenes as a result of chemical treatment or of exposure to high or low temperature has afforded helpful information. Recently, incorporation studies involving the use of radioactive isotopes have been more commonly employed. These various avenues of investigation are thoroughly discussed in recent reviews (9,10). The summary below outlines the experiments that have been carried out on some frequently used organisms.

b) Accumulation of carotenes

1) Neurospora crassa

Neurospora contains a complex mixture of carotenoids, including phytoene, phytofluene, β-carotene, lycopene, and various xanthophylls. Haxo (11,12) analyzed a number of mutants which accumulate different carotenoids and from the results postulated a pathway of biosynthesis that begins with unknown precursors of phytoene and leads through the partly hydrogenated carotenes and highly unsaturated carotenes to the xanthophylls.

The synthesis of xanthophylls and of carotenes in Neurospora is diminished in the dark while that of phytoene and phytofluene is increased (13). If, however, the incubation in the dark is interrupted by illumination of the culture even for one minute, almost normal pigmentation results, provided oxygen is present (14). The amount of all other carotenoids

which appear following illumination can be accounted for by the amount of phytoene which disappears.

2) Phycomyces blakesleeanus

Temperature studies with Phycomyces, which contains essentially only β-carotene, show that carotenogenesis proceeds fastest at 26°C (15). Yet, a culture transferred from 28°C to 31°C produces more β-carotene than one kept at a constant 28°C (16). No explanation has been found for such behavior.

Goodwin discovered in 1952 (17) that diphenylamine (DPA), when added to the culture medium of Phycomyces, inhibits the synthesis of the more unsaturated carotenoids but increases the concentration of the more saturated ones. His suggestion that DPA blocks the enzymatic dehydrogenation of the more saturated carotenoids was made less attractive by later work (18) which showed that adenylic acid can reverse the inhibition of β-carotene synthesis without preventing the accumulation of phytofluene.

Addition of β-ionone increases the synthesis of β-carotene in growing cultures of Phycomyces. This action is undoubtedly indirect since neither 6[C^{14}]-β-ionone nor 9[C^{14}]-β-ionone is incorporated into β-carotene (19,20). Methylheptenone increases the production of phytofluene without affecting β-carotene synthesis (21).

The apparent lack of relation between the synthesis of β-carotene and of the other polyenes led Goodwin, et al. (22) to conclude that there must be two separate pathways and that

the more saturated polyenes are not precursors of β-carotene.

3) Rhodospirillum rubrum

The effect of diphenylamine on R. rubrum has been investigated by Goodwin and Osman. These workers found (23) that DPA-treated cells accumulate small amounts of the more saturated carotenes in place of the normal carotenoids. Such treated cells, when washed and resuspended in buffer, synthesize normal carotenoids although untreated cells in a similar environment show no further synthesis of these compounds. More recent studies (24) have shown that in washed cells pretreated with DPA the production of normal carotenoids is attended by a quantitatively equivalent loss of saturated carotenes, expepting phytoene. This finding is taken as evidence for implicating the more saturated polyenes as intermediates.

4) Rhodotorula rubra

Ultraviolet light irradiated red yeast has produced mutants which, in some cases, contain sub-normal concentrations of the usually predominant carotenes and excesses of phytofluene (25). Here again the possibility that phytofluene may be a precursor of more oxidized carotenoids has been emphasized.

5) Tomato

A great deal of experimentation has been carried out on the genetic control of pigment formation by tomatoes. Unfortunately, no clear-cut determination of genic interrelationships

has as yet been made. The principal genes responsible for carotenoid formation in the tomato are R, T, and B (26,27), all of which are completely dominant. The carotene content of the normal red fruit and of such mutant varieties as yellow and tangerine has shed some light on the function of the genes involved. A tabulation (28) of work done by MacKinney and Jenkins (29,30) and by Tomes (27) shows the chief effects of genes R, T, and modifier a (Table 1). The presence of T seems to yield lycopene, t yielding the ζ-carotene-prolycopene series. R is responsible for lycopene in quantity. Tomatoes containing gene B produce abnormally large amounts of β-carotene, perhaps by diverting precursors from the lycopene pathway.

In 1956 MacKinney, et al. (31) described the pigments of tomatoes which possess the recessive ghost gene (gh). Such fruit, when ripe, are very pale and contain only about 1/100 as much lycopene as do normal tomatoes. However, phytoene is present in amounts equivalent to the lycopene content of normal fruit. A partial block is also imposed on chlorophyll synthesis, giving the immature fruit a milky-white appearance. A more complete discussion of effects brought about by the presence of these genes and of additional modifiers has been published in a recent volume of Advances in Genetics (28).

From the evidence presented above plus other experimental findings, Porter and Lincoln (32) in 1950 proposed a formal model of the biosynthesis of carotenes. Their scheme supposes

Table 1

Carotene composition of fruits of several genotypes (amounts expressed in µgm/gm)

Constituent	Normal red	Yellow r	Tangerine t	Yellow-tangerine rt	Apricot at	Apricot tangerine at t	Yellow apricot r at
Lycopene (including mono-cis isomers)	70-130	0-0.5	10-15	2-5	2-5	6.5-21	---
Prolycopene	---	---	20-40	5-10	---		---
poly-cis-ψ-Carotene	---	---	8-15	---	---	---	---
ζ-Carotene	0-0.1	---	20-50	0-0.1	---	0.6-1.0	---
γ-Carotene	---*	---*	---*	---*	---*	---*	---
β-Carotene	5-10	1-3	3-12	0.5-1.0	6-10	0.7-2.1	tr.-1.2
Phytofluene	3-5	0.1	4-7	0.7-1.0	0.2	0.5-1.1	0.5
TOTAL	80-150	3-7	75-150	15-20	8-15	8-25	0.5-2

*Not detected or not determined.

that a basic C_{40} compound is formed by the combination of two C_{20} fragments, followed by stepwise symmetrical dehydrogenation as shown in Figure 2. Gene R is envisaged as being responsible for α-β dehydrogenation, gene T as causing γ-δ dehydrogenation, and gene B as bringing about ring closure (33). This attractive hypothesis has been tested in a number of different manners, as will be seen below, but as yet evidence confirming either this pathway or others has not been forthcoming.

The effect of temperature on the carotene content of ripening tomatoes has been thoroughly investigated (34,35,36). At temperatures near 0°C or at 30°C or above, lycopene, the predominant carotene of tomatoes, forms only in small quantities. If mature green tomatoes are kept at 33°C for 42 days very little lycopene is present, although tomatoes ripened at 26.5°C for 12 days contain 270 mg lycopene/100 gm dry wt. If fruit is maintained at 33°C and afterward transferred to lower temperatures for 15 days lycopene is synthesized, in some instances in amounts approaching those attained in fruits kept at 26.5°C.

The effects of some inhibitors of carotenogenesis on tomato pigmentation have been analyzed by Francis (37). Diphenylamine, β-ionone, and α-ionone all inhibit lycopene formation in ripening fruit. The synthesis of β-carotene is either unaffected or enhanced. These facts led Francis to the conclusion that β-carotene and lycopene arise through separate pathways from a common precursor.

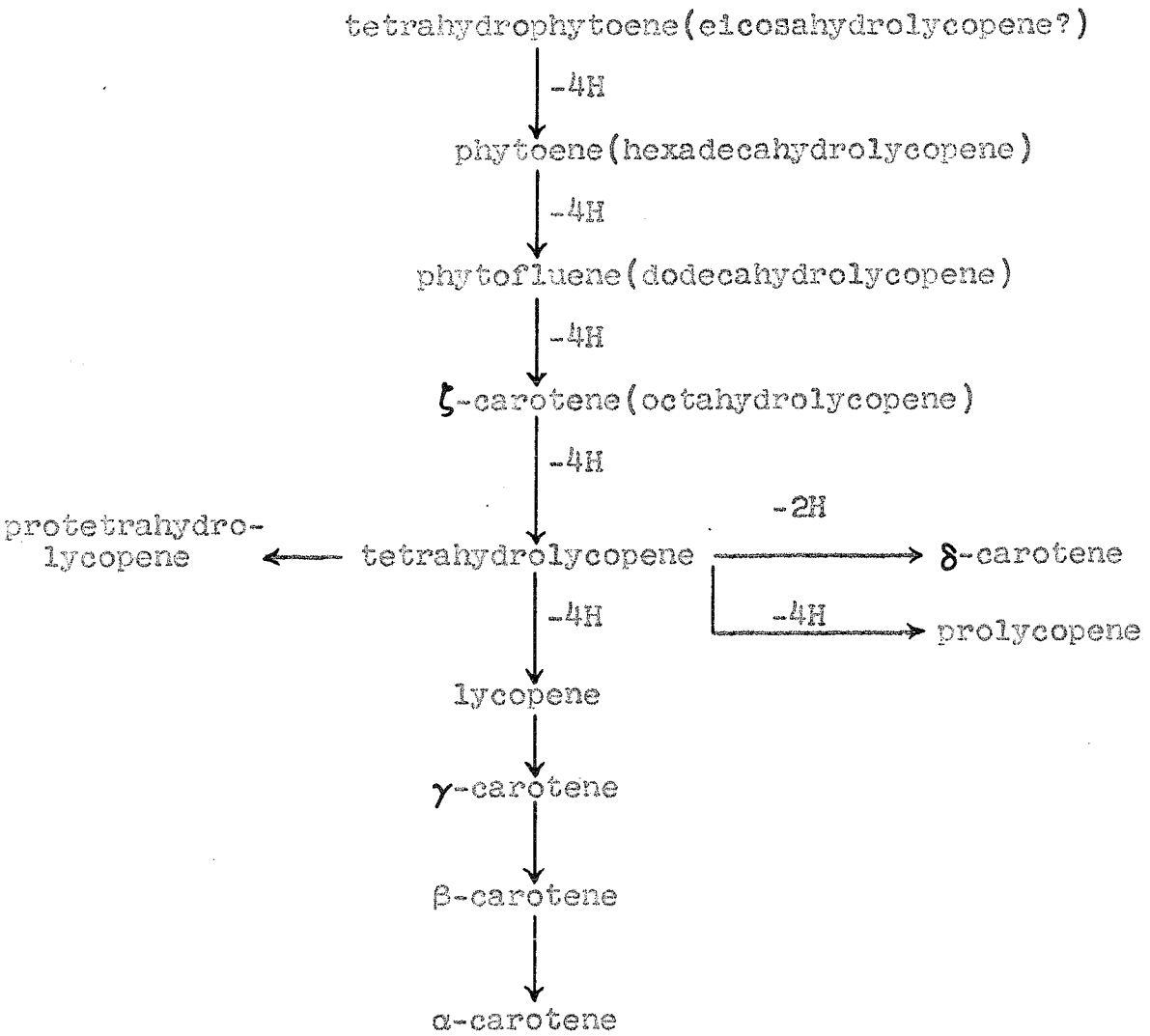


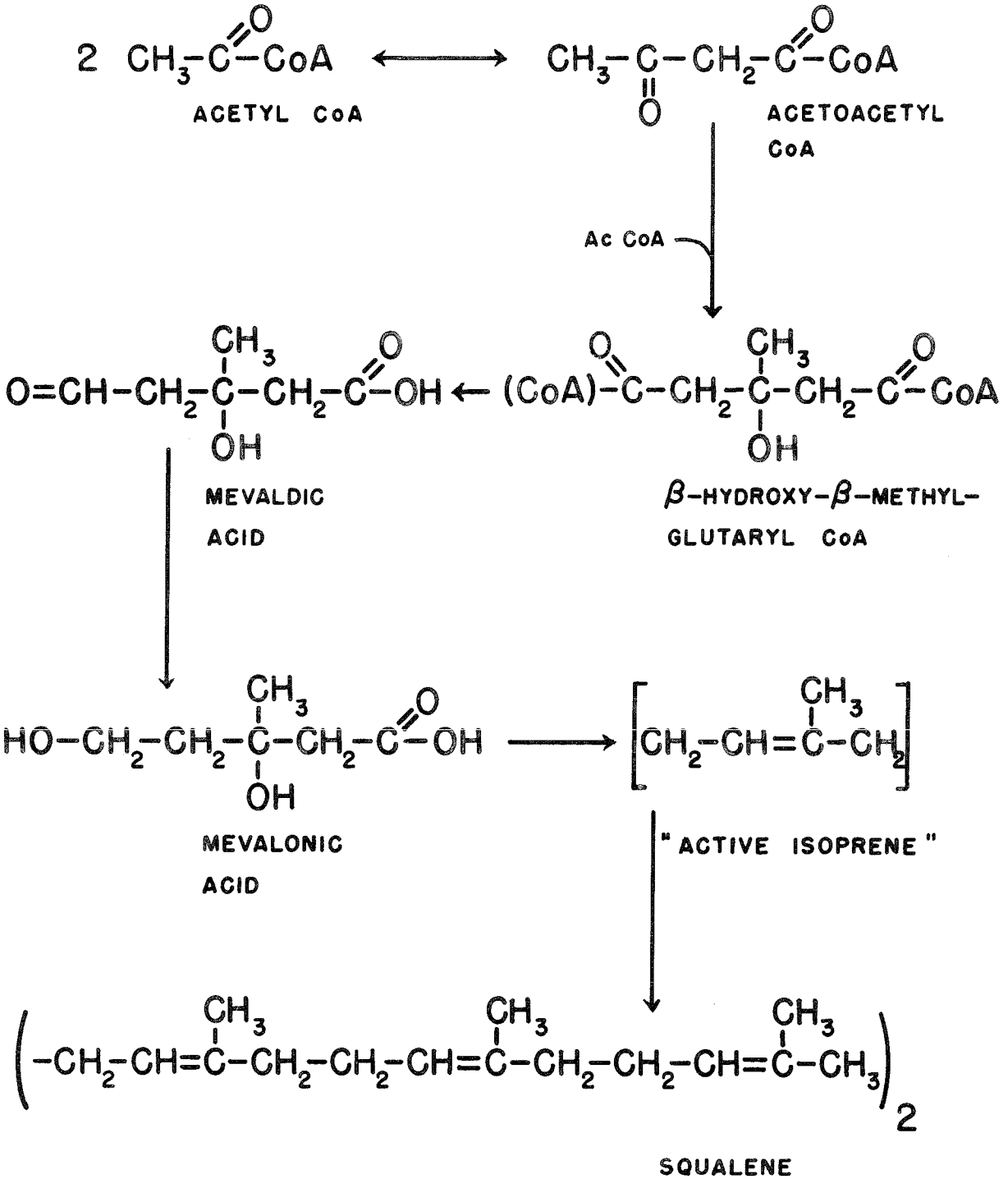
Figure 2. Porter-Lincoln Sequence

c) Isotope studies

1) Isoprenoids other than carotenes

Sterols: Because of their physiological significance, much interest has been focused on the biochemical origin of sterols. In 1950 acetic acid was shown to be the source of carbon atoms for the entire cholesterol molecule (38). A year later the role of acetate as the precursor of ergosterol in Neurospora was firmly established (39). The demonstrated conversion of C^{14}-labeled acetate to β-hydroxy-β-methylglutarate (40) and of β-hydroxy-β-methylglutarate to squalene (41) and cholesterol (40) provided important steps in the pathway. Attention was then drawn to 3,5-dihydroxy-3-methylvaleric (mevalonic) acid when the C^{14}-labeled compound was found to be an excellent substrate for cholesterol formation (42). The scheme outlined in Figure 3 is generally accepted as being the probable sequence for the biosynthesis of the immediate sterol precursor, squalene.

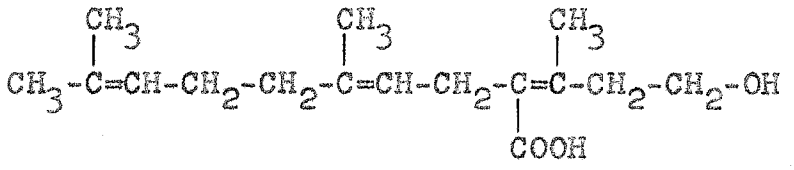
Further data integrating mevalonic acid into the pathway are those which demonstrate the origin of this compound from β-hydroxy-β-methylglutaric acid (43) and its activation by phosphorylation (44,45). An hypothesis concerning the condensation of mevalonic acid units to produce squalene has been discussed by Rilling, et al. (46). It calls for the simultaneous dehydration and decarboxylation of the activated acid, leaving an unsaturated five-carbon unit. Three such units are supposed to then condense to produce a farnesene-like hydro-



BIOSYNTHESIS OF SQUALENE

FIGURE 3

carbon, which in turn reacts head to head with an identical fifteen-carbon atom intermediate to produce the symmetrical squalene molecule. A compound obviously related to the postulated fifteen-carbon atom intermediate has been isolated from an enzymatic system prepared from rat liver (47). Preliminary characterization (48) of this compound indicates the following structure:



Lynen et al. (49,50) have confirmed in part the Rilling postulate by their discovery of a soluble yeast enzyme which will convert the squalene intermediate Δ^3-isopentenol pyrophosphate to γ,γ-dimethylallyl pyrophosphate and to farnesol pyrophosphate. A particulate enzyme will catalyze the formation of squalene from farnesol pyrophosphate.

Rubber: As early as 1949 Bonner and Arreguin found that rubber synthesis in seedlings of guayule (Parthenium argentatum Gray) could be enhanced by the feeding of acetate (51). Two years later in vivo C^{14}-labeled acetate incorporation into guayule rubber was demonstrated (52). Recently, an enzyme system found in the latex of the rubber tree (Hevea brasiliensis) has been shown to utilize acetate for rubber synthesis (53), and a similar enzyme preparation will rapidly incorporate isotopically labeled mevalonic acid into rubber (54). Degradation studies yield the same pattern of labeling as found for

squalene by other investigators.

Terpenes: Tips of Pinus attenuata seedlings have been reported by Stanley (55) to incorporate both $2C^{14}$-acetate and $2C^{14}$-mevalonate into α-pinene as well as into the small amounts of the other terpenes present.

2) Carotenoids

Recent work of Grob and Bütler (56) coupled with their earlier degradative studies of β-carotene from the fungus Mucor hiemalis leaves no doubt that the formation of this compound from acetate proceeds in a manner analogous to the formation of squalene. On the other hand, a different pattern of incorporation has been reported for the lycopene of ripening tomatoes (57). A possible explanation for the latter result is that the incubation times were sufficiently long for quite extensive randomization of labeling to take place.

The C^{14}-acetate labeling of various carotenes of the tomato fruit has been studied in some detail. Shneour and Zabin (58) discovered that in ripening tomatoes a mixture of the more saturated polyenes (phytoene-tetrahydrophytoene-phytofluene) possesses a higher specific activity than lycopene for twelve hours after isotope injection. After twenty-four hours of ripening, the specific activity of lycopene has reached a level higher than ever attained by the polyene mixture. Their interpretation of these data rules out the Porter-Lincoln sequential scheme and favors parallel formation

of carotenes from one or several common precursors. Francis (37) has reached the same conclusion on the basis of his finding that β-carotene of ripening Tangerine-type tomatoes has a higher specific activity than do the colorless polyenes.

As yet few experiments concerning the biosynthesis of carotenoids from mevalonic acid have been reported. Some incorporation of mevalonate into β-carotene by Phycomyces blakesleeanus and by carrot slices has been found (59). Goodwin (60) has followed the incorporation of $C^{14}O_2$, $2C^{14}$-acetate, and $2C^{14}$-mevalonate into non-saponifiable compounds of illuminated etiolated maize seedlings. The total non-saponifiable matter, including sterols, becomes labeled very rapidly, but the β-carotene accumulates little activity. The apparent preferential uptake of $C^{14}O_2$ into carotene may be due to the failure of acetate and of mevalonate to reach the site of carotene synthesis in the chloroplast.

Homogenates of ripening tomatoes incubated overnight with a variety of cofactors show no incorporation of acetate into lycopene and only a small utilization of mevalonate (61).

Thus we see that although much progress has been made towards clarification of the pathway of carotenogenesis, the mechanisms have not been satisfactorily explained. Carotenes may be formed by sequential dehydrogenation of saturated polyenes, by separate pathways from a common precursor, or perhaps even by hydrogenation of unsaturated compounds. All

three possibilities have been suggested; none has been experimentally ruled out. The experiments to be reported on the following pages were performed with the hope of illuminating some aspects of this problem.

III. EXPERIMENTAL METHODS

A. Culture conditions for plants

Fruits of several commercial varieties of tomato were used in these experiments. Much of the early work was carried out with tomatoes obtained by a local wholesaler from the Coachella Valley, Imperial Valley, and San Diego regions of California and from the northernmost part of Mexico. During the winter months occasional low temperature damage could be detected. In order to insure a steady crop of fruits grown under uniform conditions, plants were cultured in the Earhart Plant Research Laboratory under a constant 26°C day temperature and 14°C night temperature. Twelve-inch pots filled with 50% vermiculite and 50% gravel were employed, with Hoagland's solution used as a source of nutrients. Tests involving the standard varieties grown, including Bounty, John Baer, Rutgers, Redskin, and Pearson, showed no difference in ripening or incorporation of substrates. The carotenes were present in these varieties in the same ratios and concentrations. Two mutant varieties, Snowball and Y-13, were raised under conditions identical to those described above. The average size of the fruits was about 150 gm.

Corn seedlings were planted in vermiculite and kept in a humid darkroom at 25°C for two days after sprouting.

B. Judging the stages of ripeness

The method of Workman et al. (Table 2) (62) was utilized to describe the various stages of fruit ripeness. Unless otherwise stated substrates were administered to detached fruit in the breaker or light pink stage of maturity. Previous investigations (36,63) have shown that at normal temperatures the ripening of detached tomatoes is similar to that of non-detached fruit in all physiological and biochemical aspects examined.

C. Preparation of substrates

Samples of $1C^{14}$-sodium acetate were obtained from Tracerlab, Inc. (Lot 31-28a-2, .012 mc/mg). The $2C^{14}$-DL-mevalonic acid was obtained from Merck and Co. (Batch #L-553-233-1-2, 0.1 mc/mM), Tracerlab, Inc. (Lot 471-57-20, 1.1 mc/mM), and Isotopes Specialties Co., Inc. (Lot #A39Ill307, 0.92 mc/mM) as the N,N'-dibenzylethylenediamine salt. It was freed from this stabilizing DBED salt by dissolving in water, raising the pH to 10 with NaOH, extracting three times with ether to remove the salt, and adjusting the pH to 7 with HCl. The solutions of mevalonic acid generally were prepared to contain about 5×10^6 counts per minute per ml. The identity of all samples had been assayed chromatographically and biologically.

The samples of unlabeled mevalonic acid were obtained from Merck & Co. as the DBED salt and from Fluka AG. Chemische Fabrik as the lactone. These were shown to move with radio-

Table 2

Classification of tomato fruits

Class name	Class description
Mature green	Entirely green but mature
Breaker	First appearance of coloring
Light pink	Approximately equal amounts of green and pink
Dark pink	Entirely pink, no green
Table ripe	Fully red colored, minimum eating ripeness
Canning ripe	Intense red coloring, but still firm
Soft ripe	Perceptible softening

active mevalonic acid chromatographically using the following solvent systems:

NH_4OH: 95% EtOH --- 1:100

NH_4OH: EtOH: H_2O --- 5:15:80

Uniformly labeled C^{14}-DL-leucine was purchased from Nuclear-Chicago Corp. (7.95 mc/mM).

Isotopes Specialties Co., Inc., furnished the uniformly labeled C^{14}-D-glucose (Lot #H2S10178, 45.9 mc/mM).

D. Introduction of substrates

Two methods of substrate administration were used. In most cases the compound to be studied was injected with a hypodermic syringe into the locules at several points. The punctures were either covered with small pieces of transparent tape or plugged with tapered bits of wood. No deleterious effects of injection were noticed. The fact that fragments of skin and flesh taken at various spots on an injected fruit after incubation were radioactive indicated that mevalonic acid was transported throughout the tomato.

A vacuum infiltration method similar to that used by McCready and Hassid (64) for leaves and Spencer (65) for tomato fruits was also found to be effective. This procedure involved placing the solution of substrate in about 0.2 ml of water on the stem scar of the fruit, enclosing the fruit in a vacuum dessicator while a vacuum was slowly drawn, and

then gradually releasing the vacuum in order to allow the solution to be pulled into the tomato. The incorporation of mevalonate and acetate into tomatoes by either method of administration was the same.

E. Incubation conditions

Unless otherwise stated the fruits were incubated at room temperature in the light.

The etiolated corn plants were excised at the node connecting root and stem and placed in a small beaker containing the substrate. They were then illuminated by bright fluorescent lights.

F. Procedures for extraction of carotenes

A modification of the method of Zscheile and Porter (66) was followed for the extraction of carotenes from the fruits. The tomatoes were sliced into an equal weight of methanol containing 4 gm Hyflo Supercel/100 gm fresh weight of fruit and ground in a Waring blendor at 100% line voltage for 1 minute. After standing for approximately thirty minutes the mixture was filtered through a pad of Hyflo Supercel in a Büchner funnel and the carotenoid containing residue dried. This residue was again ground in the blendor with a solution of 50% acetone:50% hexane, and the residue was washed with aliquots of this solution until no more color was present in the filtrate. At this point the residue was discarded and the acetone removed from the filtrate by adding about a 1/4

volume of water. The acetone containing hypophase was removed, and the remaining hexane solution was washed several times with water. Saponification was carried out by shaking the hexane solution with a 1/5-1/4 volume of KOH saturated methanol. Xanthophylls were then removed from the hexane by three extractions with 95% methanol. The hexane solution was washed free of alkali with water and finally dried by filtering through anhydrous sodium sulfate. A diagrematic outline of the extraction procedure is shown in Figure 4.

G. Separation and purification techniques

The non-saponifiable fraction of the tomato was concentrated to a small volume by vacuum distillation under nitrogen. For fractions extracted from samples of fruit of less than 400 gm fresh weight columns 2 cm in diameter and about 15 cm in height containing a 1:1 mixture by weight of Fisher Sea Sorb magnesium oxide and Hyflo Supercel were used. The column was packed dry, covered with a layer of anhydrous sodium sulfate, and wet with hexane before use. The various polyenes were eluted with progressively more polar solvent mixtures beginning with hexane and ending with 2% methanol: 10% acetone:88% hexane. From the order of elution as given in Table 3, it can be seen that in general the more saturated polyenes were the first to come off the column, followed by compounds of more unsaturated structure.

The crude phytoene fraction, which was eluted with hexane,

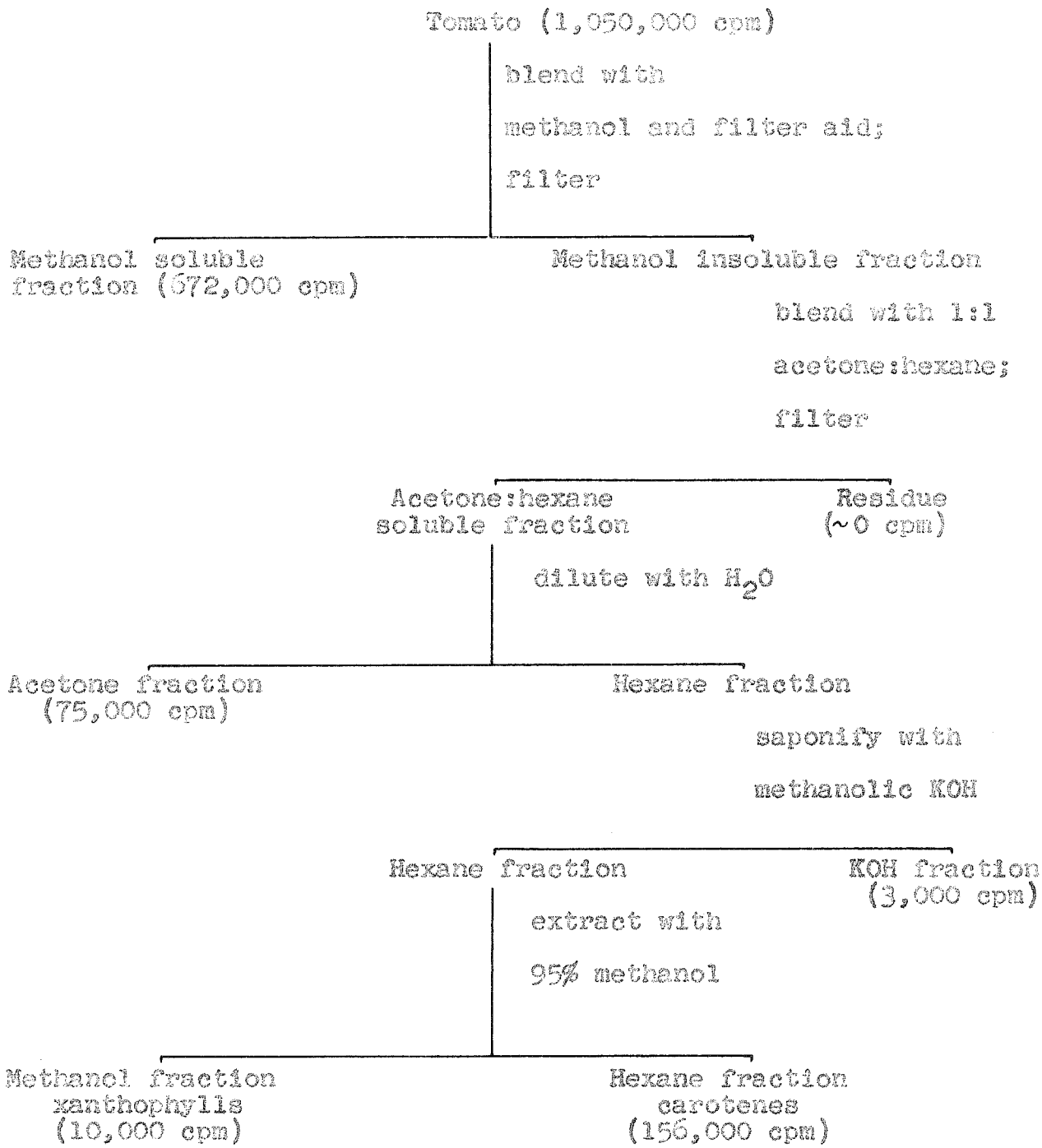


Figure 4. Extraction Procedure

Table 3

Elution sequence

Fraction	Eluent	Abs. max.	Abs. coeff.	Reference
Crude phytoene	hexane	---	---	
(phytoene)		(286)	(.085)	(67)
Phytofluene	2% acetone in hexane	348	.152	68
Neo-β-carotene	2% acetone in hexane	450	.250	66
β-Carotene	5% acetone in hexane	450	.250	66
ζ-Carotene	10% acetone in hexane	400	.220	66
Minor pigment mixture*	10% acetone:1/2% methanol in hexane			
γ-Carotene	10% acetone:1% methanol in hexane	460	.250	66
Lycopene	10% acetone:2% methanol in hexane	502	.320	66

*Probably a mixture of protetrahydrolycopene, δ-carotene, prolycopene, and some unidentified pigments (69).

was further separated into its components by chromatography on 200 mesh Alcoa alumina activated by heating the commercial alumina for three hours at 350°C in a muffle furnace. The crude phytoene fraction was evaporated by vacuum distillation under nitrogen and dissolved in a small volume of "optical hexane," prepared as described below. The solution was placed on a 1.2 x 30 cm column of the activated alumina previously washed with "optical hexane." Elution was carried out by adding successive portions of "optical hexane" diluted as follows: 70 ml pure, 50 ml 0.5% ether, 100 ml 1% ether, 100 ml. 2% ether, 75 ml 4% ether, and 50 ml 95% ether:5% methanol. Five ml fractions were collected with an Autonomos automatic fraction collector. The other compounds eluted from the magnesia: Supercel column were purified by recrystallization and rechromatography.

Routine quantitative spectral measurements in the ultraviolet range were carried out using a Beckman Model DU Spectrophotometer equipped with a photomultiplier. For complete spectral curves a Cary Model 11MS Recording Spectrophotometer was used. Samples were analyzed in hexane solution using fused silica absorption cells. Infrared spectra were obtained with a Perkin-Elmer Model 137 "Infracord" spectrophotometer.

Because commercial hexane was found to contain impurities which exhibited a high absorption at low wavelengths in the ultraviolet range, it was purified before use in analyzing the

fractions obtained from the alumina columns. The method of Potts (69) as modified by Purcell (70) gave satisfying yields of "optical hexane." Phillips hexane was passed through a 5 x 40 cm column of silicic acid and collected in fractions of 100-200 ml. Those fractions having an absorption of 0 optical density units at 250 mμ and less than 1.0 at 210 mμ were saved for use. The used column was sucked dry, flushed with steam, and heated for several hours in a muffle furnace at 400°C. Several liters of "optical hexane" could be obtained from each column.

H. Radioactive assay methods

In order to measure the radioactivity of a sample, a small aliquot was plated on a 1-1/4 inch sand-blasted aluminum planchet. The planchet was then assayed with a Nuclear-Chicago Corp. Model D47 Micromil gas flow counter equipped with a Model 181 scaler and an automatic sample changer. The sensitivity of the counter was 20%. Because of the small amounts of sample plated correction for self absorption was unnecessary.

Paper chromatograms were assayed for radioactivity with a D47 gas flow counter fitted with an Actigraph strip-counter.

I. Preparation and incubation of homogenates

Preliminary studies of the incorporation of mevalonate by tomato homogenates were carried out by first grinding the fruits under nitrogen in a Waring blendor at 4°C. Most of

the liquid portion of the homogenates was removed by filtration or centrifugation. The residue was incubated with mevalonate and cofactors as indicated in the following sections. After an incubation time of three hours the reaction was stopped by the addition of boiling methanol. Methanolic KOH was added and after a three hour saponification the samples were neutralized and filtered, and the residues were extracted as usual.

J. Degradation procedures

The ozonolysis of Fraction II was performed as follows: a stream of oxygen containing approximately 1% ozone was bubbled through the sample in 10 ml of chloroform at 0°C and then through a trap filled with an aqueous solution of KI and boric acid. When no more ozone was being absorbed by the sample, as indicated by the appearance of free iodine in the trap, 0.1 ml of acetic acid was added to the chloroform solution and ozonization was continued for 3 minutes. Following this, 0.2 ml of 30% H_2O_2 was added, the mixture was shaken for 15 minutes, 0.15 ml more of H_2O_2 was added, and after shaking for 15 additional minutes 1.0 ml of H_2O_2 plus 1.5 ml of water were added, and the mixture was refluxed for 7 hours. Washed air was bubbled through the mixture and into a solution of 2,4-dinitrophenylhydrazine reagent to trap any volatile carbonyl compounds that might be present. The re-

maining ozonolysis mixture was made basic with NaOH, extracted with ether, then acidified and extracted again with ether. Both extracts were examined for degradation products with the aid of paper chromatography.

IV. EXPERIMENTAL RESULTS

A. Quantitative changes in carotene content during ripening

With the exception of β-carotene, the immature tomato fruit contains only insignificant quantities of carotenes. As ripening begins, a rapid synthesis of these compounds is initiated, and by the time the fruit is fully ripe it may contain several mg of carotenes per 100 gm fresh weight. Analyses of fruit in various stages of ripeness reveal the carotene distributions shown in Table 4. In many cases ripening is completed within 48 hours. It is obvious, therefore, that the tomato, by virtue of its rapid and massive carotene synthesis, is well suited for the study of isoprenoid biogenesis.

B. Incorporation of acetate into carotenes of normally ripening tomatoes

In order to confirm and expand the results of previous workers (37,58), the utilization of acetate for carotene synthesis was tested by injecting tomatoes with 1 C^{14}-acetate and then allowing the fruit to ripen for a 24-hour period. The carotenes of these fruit contained from 1-2% of the total radioactivity administered. A full tabulation of the distribution of labeling in such a fruit is shown below (Table 5).

Since mevalonic acid has been found to participate in isoprenoid biosynthesis at a step subsequent to that in which acetic acid participates, it would be expected that the incorporation of injected mevalonate would at least equal, and

Table 4

Amounts of polyenes present in tomatoes at different stages of ripeness. Quantities expressed in mg/100 gm fresh weight.

Fraction	Stage			
	Mature green	Breaker	Dark pink	Table ripe
Fraction Ia*	---	16.6	5.9	5.7
Fraction Ib*	---		5.7	11.8
Fraction IIa*	0.345	0.24	0.075	0.029
Fraction IIb*	0.293		0.078	---
Phytoene	0	0.067	0.162	1.36
Phytofluene	0	0.005	0.135	0.54
Neo-β-carotene	0.0038/	0.005	0.0048	0.0018
β-Carotene	0.14	0.43	0.35	0.323
ζ-Carotene	0		0.061	0.027
γ-Carotene	0	0.018	0.094	0.025
Lycopene	0	0.085	1.34	6.89

*See section D for description.

/May be mostly α-carotene.

Table 5

Incorporation of $1C^{14}$-acetate into the polyenes of a detached, ripening tomato fruit after a 24-hour incubation at room temperature. Approximately 1.6×10^6 cpm acetate administered.

Fraction	Total activity (cpm)	Specific activity (cpm/mg)
Crude phytoene	27,006	---
Fraction Ia	3,300	133
Fraction Ib	---	---
Fraction IIa	680	212
Fraction IIb	---	---
Phytoene	72	260
Phytofluene	470	1,740
Neo-β-carotene	135	9,700
β-Carotene	378	182
ζ-Carotene	108	1,010
Minor pigment mixture	65	---
γ-Carotene	33	183
Lycopene	616	158

probably surpass the incorporation of acetate. This expectation has been borne out by studies with 2C^{14}-labeled mevalonic acid.

C. Incorporation of mevalonate into carotenes of normally ripening tomatoes

Analyses of tomatoes incubated for 24 hours with C^{14}-mevalonic acid revealed that, depending upon the physiological condition of the individual fruit, from 6-28% of the total radioactivity was present in the carotene fraction. It is known that only one isomer of the racemic mevalonic acid mixture is biologically active (72). Therefore, of the isotope available for biosynthetic use, 12-56% is incorporated into the carotene fraction. Such preferential utilization of mevalonate, 10-50 times greater than that of acetate, clearly establishes its role in carotene biosynthesis.

The various members of the carotene mixture were separated chromatographically on a column of MgO:Supercel. A very large percentage of the radioactivity was always associated with the phytoene fraction, which is the first to be eluted from the column. Lycopene, though present in a higher concentration than any other polyene, contained relatively little radioactivity. The isotope concentrations in the isolated carotene fractions are shown in Table 6. The distribution of activity derived from mevalonate is identical to that of activity derived from acetate. These data indicate, therefore,

Table 6

Incorporation of 2C^{14}-mevalonate into the polyenes of a detached, ripening tomato fruit after an incubation of 24 hours at room temperature. Approximately 1.0 x 10^6 cpm mevalonate administered. Average of 5 experiments.

Fraction	Total activity (cpm)	Specific activity (cpm/mg)
Crude phytoene	237,000	---
Fraction Ia	58	2
Fraction Ib	23	24
Fraction IIa	65,467	183,667
Fraction IIb	3,212	6,057
Phytoene	514	1,102
Phytofluene	16,461	77,440
Neo-β-carotene	7,032	695,000
β-Carotene	9,453	26,864
ζ-Carotene	4,519	89,500
Minor pigment mixture	838	---
γ-Carotene	821	5,306
Lycopene	10,631	2,065

that the two compounds enter carotenoids via the same pathway.

In further experiments, mevalonate-containing tomatoes were incubated for 170 hours, at which time they had attained full ripeness. If the results of these experiments are compared with those obtained from 24-hour incubation periods (Table 7), it is clear that activity disappears from the crude phytoene fraction and appears in fractions containing carotenes.

Is there an optimal degree of ripeness for mevalonate incorporation? If a tomato is first injected with C^{14}-mevalonate in the light pink stage and then reinjected with equal amounts of labeled substrate on three successive days, during which the fruit passes through the dark pink stage and attains full ripeness, the total C^{14} content of the carotenes is approximately 4 times that resulting from a single treatment. From this experiment it seems that carotenogenesis proceeds at a more or less constant rate during ripening.

Four matched tomatoes were incubated for 24 hours after being supplied with labeled mevalonate according to the following time schedule: fruit #1 (light pink)--0 hours, fruit #2 (light pink)--24 hours, fruit #3 (dark pink)--48 hours, fruit #4 (table ripe)--72 hours. The highest total activity and specific activity of carotenes occurred in fruit #2, followed in order by fruits #1, 3, and finally 4, which had incorporated into carotenes only one half as much activity as fruit #2. It appears that as the ripening process

Table 7

Incorporation of 2C^{14}-mevalonate into polyenes of tomatoes incubated for 24 hours and for 170 hours. Approximately 1 x 10^6 cpm added.

Fraction	24 hours		170 hours	
	Total activity (cpm)	Specific activity (cpm/mg)	Total activity (cpm)	Specific activity (cpm/mg)
Crude phytoene	197,000	---	80,000	---
Fraction Ia	45.6	4	32.2	2.8
Fraction Ib	23.8	2.1	0	0
Fraction IIa	17,420	120,000	5,880	101,500
Fraction IIb	358	2,370	244	---
Phytoene	364	1,160	578	212
Phytofluene	73,100	279,000	60,500	56,000
Neo-β-carotene	29,150	3,100,000	2,350	600,000
β-Carotene	17,580	25,800	15,600	24,200
ζ-Carotene	24,600	206,500	2,580	47,600
Minor pigment mix.	3,210	---	21,800	---
γ-Carotene	2,860	15,700	1,740	35,500
Lycopene	9,060	3,480	15,400	1,120

advances past the light pink stage the synthesis of carotenes from mevalonate reaches a peak and begins to decline.

D. Purification of components

The phytoene fraction is especially interesting due to the high activity found in it. This fraction, unlike the other polyene fractions, was found by examination of its UV spectrum to be quite impure. Purification could be effected by chromatography on activated alumina. However, only one half or less of the radioactivity placed on the column was recoverable, due probably to the recognized deleterious effect of alumina upon some compounds of this type. Phytoene purified in this manner possesses a spectrum identical to that found by Rabourn and Quackenbush (73). The purified compound is essentially free of radioactivity. Four additional compounds are eluted from the alumina column before phytoene. Figure 5 depicts a typical elution diagram. These materials have been designated fractions Ia, Ib, IIa, and IIb. Fractions Ia and Ib each exhibit a single absorption peak in the UV range at 208 mμ. Fraction IIa is characterized by an absorption maximum at 208 mμ with a prominent shoulder at 231 mμ. Two maxima, one at 208 mμ and a higher one at 231 mμ, typify fraction IIb. Representative spectra of fractions I, IIa, and IIb are shown in Figure 6. The last two compounds are of primary interest for, as can be seen from Table 6, they contain considerable amounts of radioactivity and possess a

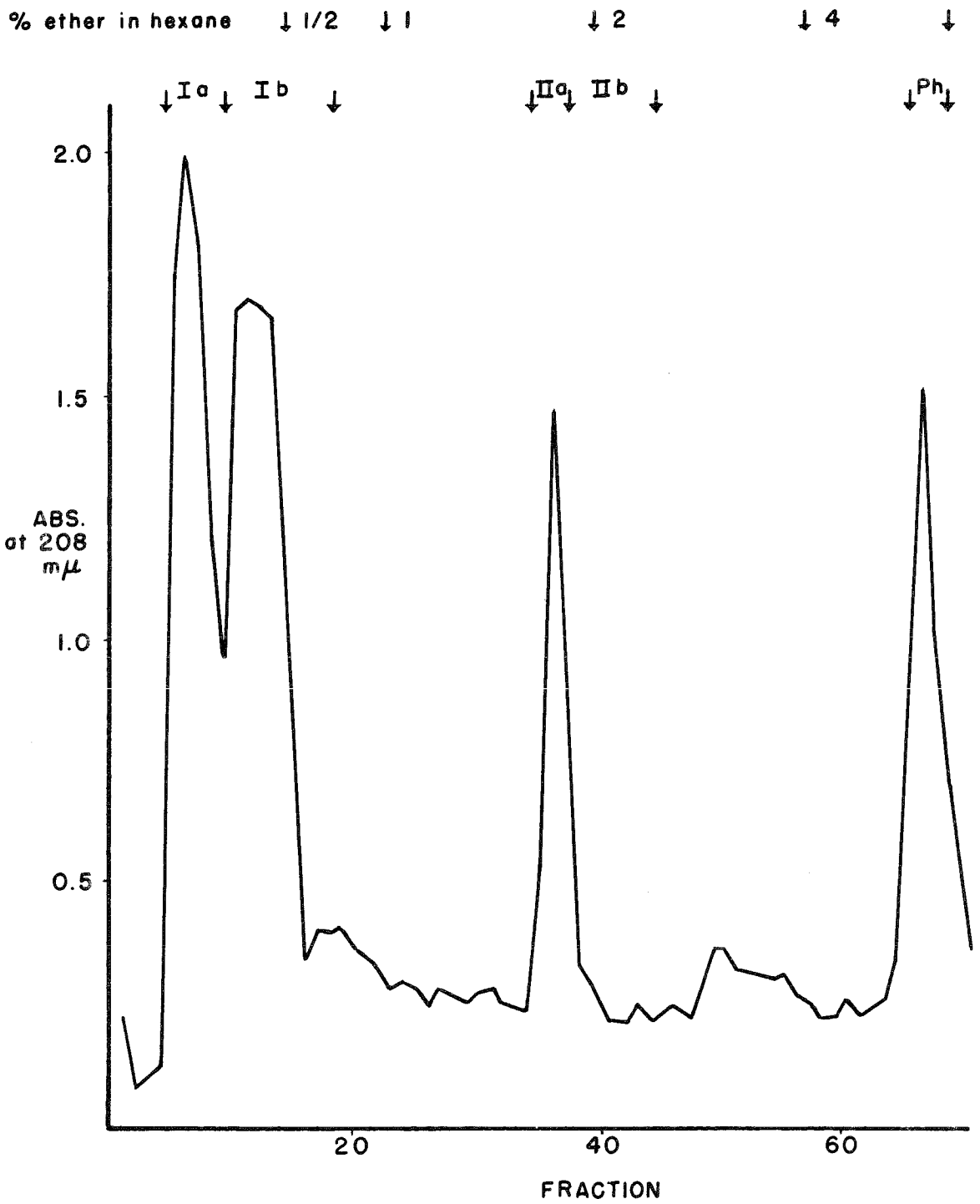
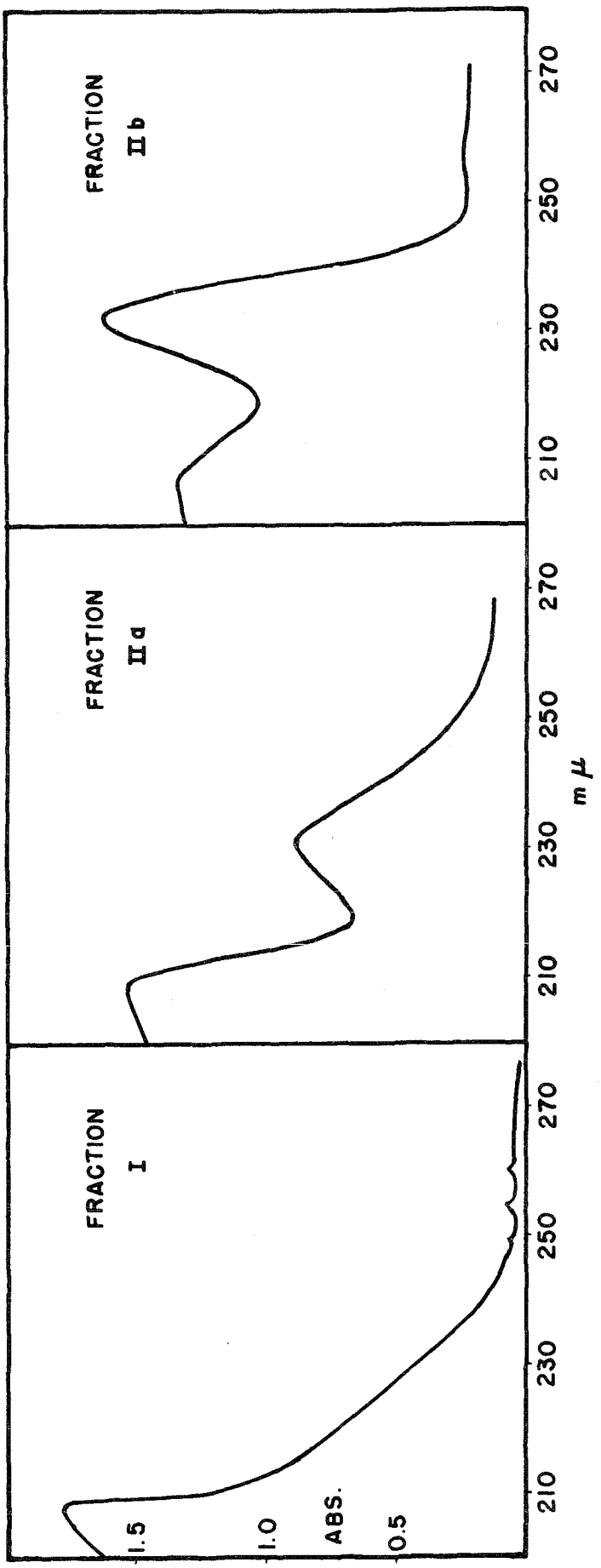


FIGURE 5. ELUTION DIAGRAM OF CRUDE PHYTOENE



SPECTRA OF FRACTIONS I, IIa, AND IIb

FIGURE 6

high specific activity. Following phytoene on the column is a group of compounds, phytol among them, which also contains radioactivity.

The absorption coefficient for a mixture of fractions IIa and IIb was determined to be 0.0554. The corresponding value assigned to fractions Ia and Ib is 0.00445. More details concerning the structures of these compounds will be presented in a subsequent section. Suffice it to say at this point that fractions IIa and IIb seem to be of an isoprenoid nature and of lower molecular weight than carotenoids, while fractions Ia and Ib are probably straight-chain hydrocarbons.

The remaining carotene fractions were also examined for the presence of trace amounts of highly labeled impurities. Phytofluene, when rechromatographed on ignited magnesia, is generally reduced in specific activity to less than 20% of its original value. The neo-β-carotene fraction undoubtedly contains contaminating radioactive substances, probably identical with those found in the phytofluene fraction, but the minute quantities of neo-β-carotene present make recrystallization difficult.

Recrystallization of further pigments was achieved by the use of hot methanol. β-Carotene reached a constant specific activity of approximately 10% of the original value after recrystallization first in methanol, then in hexane. At least one half of the activity present in the ζ-carotene

fraction is associated with contaminating materials. Sterols were found to be eluted from the MgO:Supercel column principally in the ζ-carotene fraction, with further small amounts in the β-carotene fraction. As the tomato begins to ripen, however, sterol synthesis seems to be sharply curtailed, as evidenced by the relatively insignificant incorporation of mevalonate. The remaining pigments, γ-carotene and lycopene, lost little activity upon purification and may therefore be considered to be essentially pure.

E. Mevalonate incorporation under high and low temperature conditions

Because of the reported failure of tomato fruit to form lycopene at temperatures higher than 33°C, as well as the failure of such fruit to ripen normally at low temperatures, a study of the conversion of C^{14}-mevalonate to carotenes at various temperatures was undertaken. Three matched, ripening fruit were injected with mevalonate and incubated for 24 hours at 10, 25, and 35°C, respectively. Although the total carotene fractions of the three fruits contained approximately equal activities, the distribution of activity among the individual carotenes differed. As can be seen from Table 8, low temperature causes an accumulation of radioactivity in fraction IIa. At 35°C a buildup of activity in the phytofluene fraction takes place. Thus it appears likely that a partial block may be imposed at these steps by extremes of temperature.

Table 8

Distribution of radioactivity among the polyenes of tomatoes incubated at different temperatures after being injected with 1.06×10^6 cpm of $2C^{14}$-mevalonate each. Incubation time—24 hours.

Fraction	10°C		25°C		35°C	
	Total activity (cpm)	Specific activity (cpm/mg)	Total activity (cpm)	Specific activity (cpm/mg)	Total activity (cpm)	Specific activity (cpm/mg)
Crude phytoene	111,000		101,000		87,000	
Fraction Ia	319	6	360	7	78	2
Fraction Ib	80	4	13	1	190	19
Fraction IIa	80,000	220,000	1,900	76,000	2,210	11,000
Fraction IIb	1,620	5,300	254	1,270	95	525
Phytoene	302	780	356	420	63	118
Phytofluene	2,430	10,000	16,000	55,000	48,000	133,000
Neo-β-carotene	23,300	2,120,000	6,090	870,000	6,170	247,000
β-Carotene	20,450	20,000	13,800	14,000	16,940	12,000
ζ-Carotene	6,400	114,000	3,820	54,500	1,550	20,600
Minor pigment mix.	478	—	1,300	—	745	—
γ-Carotene	450	2,640	700	3,300	625	1,630
Lycopene	580	132	880	167	645	199

F. Incorporation of mevalonate and of acetate by immature tomatoes

Since the high specific activity of fraction IIa after mevalonate injection suggests that this substance may be a carotene precursor, further information concerning its synthesis was obtained. An analysis of a fruit in the mature green stage revealed that although the carotenes, with the exception of β-carotene (0.14 mg/100 gm fresh weight) are present in only insignificant amounts, fraction II(a+b) is present at a concentration higher than that found in ripening fruit.

Half-grown tomatoes were examined for synthesis of fraction II by incubating them with C^{14}-mevalonate or C^{14}-acetate. In each case the incorporation of label was greater than that obtained with ripening fruit of equivalent size. Further chromatography of the mevalonate-injected tomato carotenes showed that here also the specific activity of fraction II was higher than that of β-carotene, the only carotene detected. Second only to fraction II in its radioactivity content was an unknown compound or group of compounds eluted from the MgO:Supercel column at a location corresponding to phytofluene and neo-β-carotene. This probably is the contaminant found with the latter two polyenes in ripening tomatoes.

If fraction II is a carotene precursor the radioactivity that it accumulates should be eventually transmitted into the

carotenes themselves. Some conversion was found in the 170-hour experiments reported above, but a large percentage of the activity remained in the crude phytoene fraction. Since the green tomato can utilize mevalonate, a long term experiment was designed to determine the metabolic fate of fraction II. Young fruit were injected at various stages of immaturity while still attached to the vine. They were allowed to ripen normally and were extracted when they had reached the dark pink stage of ripeness. Table 9 details the results of this experiment. Concurrently a young tomato was injected and then, after 6 days, was harvested at a still immature stage and analyzed. In the last instance almost all of the carotene radioactivity appeared in 3 fractions: crude phytoene fraction--1490 cpm, fraction between crude phytoene and β-carotene--45,700 cpm, β-carotene fraction--6350 cpm.

This evidence coupled with that of Table 9, indicates that mevalonic acid is converted first to fraction II, then to the compound associated with phytofluene and neo-β-carotene, and finally to the carotenes proper.

C. Studies with mutants

In the past, mutants blocked at a particular step in carotenoid biosynthesis have provided information of value. Several colorless varieties of tomato have been described. One of these, the ghost tomato, has already been mentioned as accumulating phytoene. Seeds of another carotene-less variety,

Table 9

Distribution of radioactivity among polyenes of tomatoes injected with 4.8×10^5 cpm of $2C^{14}$-mevalonate while immature and allowed to ripen on the vine.

Fraction	Injected 12 days before ripening		Injected 17 days before ripening	
	Total activity (cpm)	Specific activity (cpm/mg)	Total activity (cpm)	Specific activity (cpm/mg)
Crude phytoene	751	---	1,600	---
Phytofluene	10,200	35,700	2,715	16,000
Neo-β-carotene	1,120	320,000	1,390	211,000
β-Carotene	6,190	29,400	14,500	40,200
ζ-Carotene	5,120	85,500	7,640	182,000
Minor pigment mixture	486	---	345	---
γ-Carotene	60.6	566	212	1,725
Lycopene	0	0	163	71.5

Snowball, were obtained through the generosity of Dr. A. E. Thompson of the University of Illinois. This mutant has been assigned the genotype rrTThp$_1$+, where hp$_1$ is one of two recessive genes necessary for extensive pigment formation (74). A ripening Snowball tomato incorporates a normal amount of radioactivity into the carotene fraction. Almost all of the labeling is present in the fraction corresponding to crude phytoene. A small amount of activity is associated with β-carotene, which is present in about 1% of the normal amount, and with an unidentified compound eluted from the column by relatively highly polar solvents. Purification of the crude phytoene fraction discloses an absence of phytoene and a slightly higher than usual concentration of radioactive fraction II.

The effect of large amounts of unlabeled mevalonate was tested on the Snowball mutant. Forty mg of unlabeled mevalonate were injected and, after 24 hours, a mixture of 0.323 mg (1.3×10^6 cpm) of C^{14}-mevalonate and 40 mg more of unlabeled mevalonate were administered. Surprisingly, both the concentration of constituents and the distribution of radioactivity were approximately the same as found when only labeled substrate was fed, except that a slightly higher percentage of activity remained in the crude phytoene.

A high-pigment-content variety of tomato, also supplied by Dr. Thompson, was tested for incorporation of mevalonate. This tomato, Y-13, contains 2 to 3 times the normal content of carotenes, but the various pigments are present in roughly

the same ratio as found in normal fruit. The incorporation is correspondingly greater; however, the specific activities are typical of commercial varieties.

H. Dilution experiments

A knowledge of the maximum rate of mevalonate incorporation into the polyenes might be helpful in elucidating the biosynthetic pathway. Accordingly, experiments were carried out in which C^{14}-mevalonate was diluted by various methods with several times the quantity of unlabeled mevalonate.

A ripening tomato was provided with a mixture of 0.323 mg (1.3×10^6 cpm) of C^{14}-mevalonate and 8 mg of non-radioactive mevalonate. An analysis after 24 hours revealed that the 25-fold dilution neither depressed the incorporation of activity nor influenced its distribution among the carotene fractions.

In a separate experiment injection of the usual quantity of radiomevalonate was preceded by the administration one day earlier of a 25-fold larger amount of unlabeled substrate. Uptake of activity into the carotene fraction was depressed to one fourth of normal, but the distribution among the carotenes was not altered.

Next, a slightly more elaborate experiment was devised in which the time of dilution was varied. The essential information is summarized in Table 10. The results indicate that a large amount of unlabeled material following a small addition of C^{14}-mevalonate has no marked effect. There does

Table 10

Distribution of radioactivity among polyenes of tomatoes fed unlabeled and/or 2c^{14}-mevalonate according to the following time schedule:

Fruit #	0 hours	24 hours	48 hours
I	10^6 cpm(.323 mg)	harvest	---
II	10^6 cpm	8 mg unlabeled	harvest
III	10^6 cpm	---	harvest
IV	8 mg unlabeled	10^6 cpm	harvest

Fraction	I		II	
	Total activity (cpm)	Specific activity (cpm/mg)	Total activity (cpm)	Specific activity (cpm/mg)
Crude phytoene	108,000	---	98,000	---
Phytofluene	1,940	55,800	14,400	80,000
Neo-β-carotene	9,790	2,650,000	13,900	200,000
β-Carotene ζ-Carotene	27,900	85,000	21,600	49,900
Minor pigment mixture	2,540	---	4,430	---
γ-Carotene	2,025	20,000	2,325	16,200
Lycopene	3,170	1,480	6,550	1,020

Table 10 (continued)

Fraction	III Total activity (cpm)	III Specific activity (cpm/mg)	IV Total activity (cpm)	IV Specific activity (cpm/mg)
Crude phytoene	119,000	---	148,000	---
Phytofluene	18,480	132,000	10,650	8,720
Neo-β-carotene	13,670	74,000	8,450	322,000
β-Carotene ζ-Carotene	21,900	27,300	6,480	6,630
Minor pigment mixture	16,400	---	1,210	---
γ-Carotene	1,790	8,690	1,630	3,450
Lycopene	4,480	1,018	9,020	860

appear to be some acceleration in the exodus of activity from the crude phytoene fraction into later fractions. Quite the opposite effect is noted when the diluent precedes the tracer. The incorporation is not depressed, but the activity proceeds no farther than the crude phytoene fraction.

From the data of Table 10 it might be inferred that there is an increase in the lycopene content as a result of large doses of mevalonate. The variation among different fruit, even when visually well matched, is probably enough to account for the apparent increase. This pattern of increasing lycopene concentrations was not found in another similar experiment.

I. Use of tomato homogenates

Preliminary attempts to obtain carotene synthesis in cell free homogenates have been made during the course of the present work. Shneour and Zabin (61) have reported on a cell free system which shows some activity in carotene synthesis. The present experiments have been directed towards increasing the activity of such homogenates.

Fifteen ml of a crude homogenate prepared from ripening tomatoes was incubated with 2.5 ml of 0.02 M potassium phosphate buffer (pH 7.2) containing 5×10^5 cpm of mevalonate. After 3 hours the reaction was stopped and the carotenes extracted. There were 3700 cpm present in the carotene portion. Chromatographic separation yielded the following information: 60% of the activity was found in crude phytoene, 3% in the

lycopene fraction, and the rest in a pool of the remaining fractions. This incorporation is low in quantity, but if allowances are made for the amount of material used, the time of incubation, and the amount of substrate, the uptake is of the same magnitude as that found in whole tomatoes.

An attempt was made to improve and concentrate the enzymatic activity in a homogenized tomato by isolating the crude chloroplast-chromoplast fraction. Only a small incorporation of radioactivity was achieved.

Incubation mixtures containing adenosine triphosphate, reduced diphosphopyridine nucleotide, triphosphopyridine nucleotide, Mg^{++}, phosphate buffer, and acetone powder prepared from ripening tomatoes also failed to incorporate mevalonate.

J. Incorporation of other substrates

Naturally, the most convincing method of showing that one compound is a precursor of another is to demonstrate its enzymatic conversion to the supposed product. Experiments of this type with fraction II have proved unsuccessful. Labeled samples were emulsified with Tween 80 and injected into a ripening fruit. In two similar trials a large percentage of the activity remained in the crude phytoene after an incubation period of 167 hours. When crude phytoene from a C^{14}-mevalonate treated tomato was injected as an emulsion into a ripening fruit, results were similar to those obtained when purified fraction II was employed, i.e., no demonstrable

conversion to carotenes. However, in considering these negative results, one must be mindful ofthe expectation that a compound of this nature, completely insoluble in aqueous media, would permeate plant tissue with great difficulty if at all.

According to the Porter-Lincoln scheme, lycopene serves as an intermediate in β-carotene formation. Of 11,000 cpm of C^{14}-lycopene supplied to a tomato, 98% was recovered in the lycopene fraction. In this case also, impermeability of the cell for the substrate makes any results of doubtful value.

If the pigments of a tomato are synthesized from soluble precursors at or shortly before the time of ripening, those carotenes which are being most rapidly produced should draw heavily from the precursor pool. Although the rate of lycopene synthesis greatly exceeds that of all other pigments as a tomato begins to color, the administration of labeled mevalonate or acetate yields lycopene containing relatively little radioactivity. It has been shown that isoprenoid compounds are actively synthesized prior to maturity. The possibility that such compounds are deposited for subsequent conversion to carotenoids will be discussed in the following section. However, another alternative may be suggested to explain the low specific activity of lycopene. There may be an entirely separate pathway contributing to the formation of one or more carotenoids. This admittedly unlikely hypothesis does receive some support from the experimental finding that

uniformly labeled glucose gives a distribution of activity among the carotenes that differs in some respects from that found in mevalonate- or acetate-fed fruit. The data of Table 11 show that in fruits supplied with C^{14}-glucose, lycopene contains 34% of the total activity, as compared with approximately 5% when mevalonate or acetate are administered. After 24 hours the specific activity of lycopene is higher than that of fraction II. Synthesis from glucose would normally be expected to proceed through the glycolytic formation of acetate. This is not clearly so in the present case. It may be that the radioactivity of glucose enters the chromoplast as $C^{14}O_2$ formed by the metabolism of glucose. Preliminary experiments have demonstrated that tomatoes incubated in an atmosphere containing $C^{14}O_2$ do show an unusually high incorporation of labeling into lycopene.

Metabolic products of leucine are known to enter the biosynthetic pathway of carotenoid formation after being carboxylated (75). In view of the finding that some enhancement of color formation is brought about by CO_2, uniformly labeled C^{14}-leucine has been examined as a possible substrate. Conversion proved to be very low, with less than 1% of the injected leucine converted to carotenes in a 24-hour period.

K. Localization of labeling within the cell

Although carotenes are known to be localized within the chromoplasts of a cell, the site of carotene synthesis has not been determined. Whole tomatoes were allowed to ripen

Table 11

Incorporation of uniformly labeled C^{14}-glucose into the polyenes of 6 detached, ripening tomato fruit after a 24-hour incubation at room temperature. Approximately 3.8×10^6 cpm glucose administered.

Fraction	Total activity (cpm)	Specific activity (cpm-mg)
Crude phytoene	17,700	---
Fraction I	450	---
Fraction IIa	192	345
Fraction IIb	0	0
Phytoene	1,066	374
Phytofluene	2,780	3,480
Neo-β-carotene	660	3,660
β-Carotene	1,790	726
ζ-Carotene	430	1,260
Minor pigment mix.	280	---
γ-Carotene	340	630
Lycopene	12,400	630

for 24 hours in the presence of C^{14}-mevalonate. They were then homogenized and strained through cheesecloth. A pellet greatly enriched in chromoplasts was obtained by ultracentrifugation at 20,000 x g. The specific activity of the chromoplast mixture was 67,400 cpm/gm dry weight, or approximately 10 times that found in the residual cell wall preparation, which still contained considerable chromoplasts. Thus the indication is that fraction II is laid down in the chromoplast, ready for conversion to carotenes.

L. Fraction II in other organisms

A survey of carotene-producing organisms for the presence of Fraction II would be of great interest. However, limitations of time have ruled out a detailed search. A compound similar in spectral characteristics has been found in carrot oil. Because of Goodwin's report of highly labeled unidentified lipids being formed from mevalonate in etiolated maize seedlings (60) his system was examined. Of 1.9×10^6 cpm C^{14}-mevalonate fed to 9.5 gm of illuminated etiolated seedlings, 354,000 cpm (37.4% of the active isomer) were incorporated into the non-saponifiable fraction. Only 36,700 cpm appeared in the crude phytoene, while the fractions corresponding to phytofluene and neo-β-carotene in the tomato contained 221,500 cpm, and the β-carotene fraction yielded 29,400 cpm. Purified fraction II contained a total of 11,000 cpm. In conjunction with this work pink grapefruit and watermelon were assayed for fraction II by Purcell. It was found in both

fruits (71).

M. Identity of fraction I and fraction II.

Fractions Ia and Ib were grouped together for study because of their similarity in physical characteristics and of the difficulty in securing a clean chromatographic separation of one from the other. It has seemed likely since early in the study that fraction I is not an isoprenoid compound. Although it is present in large quantity in both immature and ripe tomatoes, it does not become labeled with C^{14}-mevalonic acid. The fact that C^{14}-acetate is incorporated indicates that fraction I is a straight chain hydrocarbon derived from a fatty acid. Ozonolysis of purified fraction I causes no detectable degradation; therefore, the molecule is completely saturated. A molecular weight determination carried out by Mr. G. Swinehart using the Rast method gave values on duplicate samples of 329 and 325. This would correspond to a C_{23} or a C_{24} straight chain molecule. A carbon-hydrogen analysis made by Dr. A. Elek yielded the following figures for duplicate trials: C = 82.02%, H = 12.96%; C = 81.90%, H = 13.05%. The 5% unaccounted for is sufficiently large to allow an oxygen atom to be present, but there is no indication from infrared spectra or from chromatographic behavior that an oxygen-containing group is part of the molecule.

It might be imagined that fraction I is a constituent of the waxy cuticle of the tomato. This possibility was

tested by analyzing separately the peeling and the peeled fruit. Fraction I was found to be present in the body of the fruit as well as in the skin. It is concluded that this compound is distributed throughout the tomato and that it plays some as yet unidentified role in the structural makeup of the fruit.

Fraction II, because of its high specific activity, has been studied in somewhat greater detail. The ultraviolet spectrum, which shows a characteristic maximum at 231 mμ, indicates with reasonable certainty the presence of a pair of conjugated double bonds. The theoretical value for a compound of this type is, using Woodward's rule (76), 227 mμ, but shifts of several mμ have been found by Woodward for some substituted dienes. O'Conner and Goldblatt (77) have found maxima as high as 235 mμ for terpenes containing two exocyclic conjugated double bonds. Non-conjugated systems do not exhibit appreciable absorption above 210 mμ, and trienes may be distinguished by their three maxima in the 260 mμ to 280 mμ region.

The infrared spectrum of fraction II furnishes additional evidence that it is of an isoprenoid nature. In the fingerprint region of 4000 cm^{-1} to 1000 cm^{-1} the peaks characteristic of conjugated diene terpenes are matched very closely in position and intensity by the maxima of the fraction II spectrum. Two areas of the spectrum are worthy of special mention. Absorption by carbon-carbon double bonds is generally found as a weak band in the range 1680-1620 cm^{-1}. Substitution

at one of the carbon atoms further reduces the intensity. When conjugation is present the band is often split into two absorbing peaks, with the one showing the higher intensity being shifted to about 30 cm^{-1} lower wave number (78). Fraction II shows in this region a single rather wide band at 1600 cm^{-1}. While other interpretations are possible, this pattern might be expected from a compound possessing both conjugated and non-conjugated double bonds.

The other area of particular interest is that extending from 900-800 cm^{-1}. Unsaturated isoprenoids commonly contain the isopropylidine group ($R-CH=\overset{CH_3}{\overset{|}{C}}-R$), known to absorb at 840-800 cm^{-1}. This peak is prominent in the fraction II spectrum. From degradative studies discussed below the possibility exists that the grouping

$$R-CH_2-\overset{CH_2}{\overset{\|}{C}}-R \qquad \text{or} \qquad CH_2=CH-\overset{CH_3}{\overset{|}{C}}-R$$

might be present. Although the absorption of a $CH_2=CH<$ group is quite intense in the region of 890 cm^{-1}, fraction II shows only a diffuse band with less intensity than would be expected if the above-mentioned group were involved. However, the fact that some absorption does take place at this frequency prohibits the exclusion of the $CH_2=CH<$ group as a possibility.

If a concentrated hexane solution of the crude phytoene mixture is allowed to remain in the light at room temperature for several weeks there is extensive aggregation and destruc-

tion of fraction II, although fraction I and phytoene remain unchanged. A gray precipitate is formed which lacks the absorption peak at 231 mμ and cannot be eluted from alumina. This behavior was first noticed when a sample of fraction II was submitted to Truesdail Laboratories, Inc. for a molecular weight determination. The returned sample contained the precipitate in large quantities. The figure for the molecular weight was elevated to 1380 ± 100 because of polymerization or aggregation.

Later a freshly prepared sample was examined by the same firm, and duplicate figures of 297 and 284 were obtained by the Signer isothermal distillation method. The average weight of 290.5 is in close agreement with that expected for a C_{20} isoprenoid compound (expected = 272).

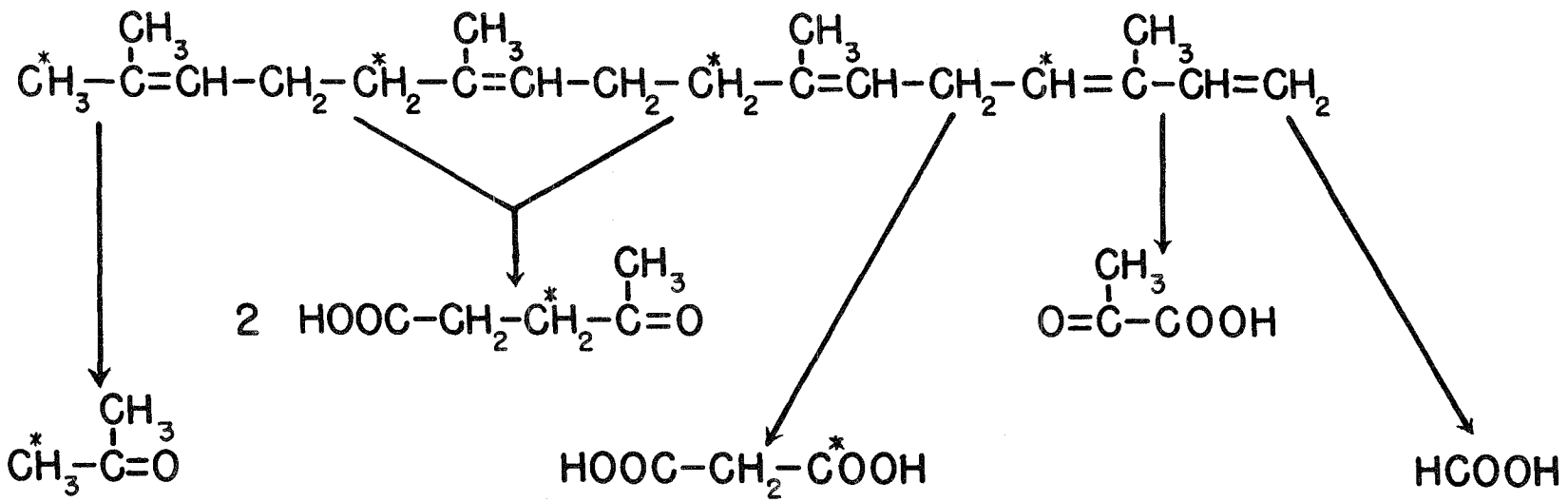
Fraction II can be rechromatographed on activated alumina without significant loss of radioactivity. The activity lost from the crude phytoene on the first passage through alumina may belong to a labile isomer of fraction II or to an entirely different compound. Alteration of this material, whatever its identity, by the alumina is firmly established, since the radioactivity that may be reclaimed from the column contents by extraction will no longer pass through even the MgO:Supercel column.

A sample of purified fraction IIa was examined chromatographically by W. L. Stanley of the United States Department of Agriculture Laboratory in Pasadena. Using the chromato-

strip method developed in that laboratory (79), the R.F. of the sample on a coated glass strip developed with 10% ether in hexane was slightly lower than that found for phytoene, squalene, and a compound tentatively identified as farnesene. Fraction II was degraded by ozonolysis. A sample containing 19.8 mg (342,000 cpm) of fraction IIa and a small amount of fraction IIb was ozonized. A radioactive volatile fragment was trapped in 2,4-dinitrophenylhydrazine reagent. The derivative was isolated and chromatographed, using the solvent systems: 5% ether: 95% hexane, and 80% ethanol: 20% hexane. It was found to have an R.F. identical to that of authentic 2,4-dinitrophenylhydrazone prepared from acetone. Chromatography of the acids in the ozonolysis residue strongly indicated the presence of levulinic acid, malonic acid, and perhaps two others.

A structure which yields radioactive acetone, levulinic acid, and malonic acid upon ozonolysis and also possesses the other properties of fraction II is shown in Figure 7. A comparison of the theoretical yield of radioactivity in the fragments with that found by the semiquantitative method of measuring the area under the radioactive peaks as recorded by the Model D47 strip counter is presented in Table 12(a). The yields are low, especially in the case of acetone.

Unfortunately, degradative products of the reactive end of the molecule would not be detected in this experiment. The fraction II degraded was prepared by incubating 2C^{14}-mevalonate-



OZONOLYSIS FRAGMENTS OF FRACTION II

FIGURE 7

Table 12

Recovery of ozonolysis fragments

	Expected		Found	
	cpm	% total	cpm	% total
(a) Sample of fraction II containing 342,000 cpm.				
Acetone	86,000	25	9,100	2.7
Levulinic acid	172,000	50	63,800	22.4
Malonic acid plus unknown	86,000	25	30,100	8.8
(b) Sample of fraction II containing 239,000 cpm.				
Acetone	60,000	25	4,074	1.7
Levulinic acid	120,000	50	51,516	21.6
Malonic acid plus unknown	60,000	25	46,000	19.2

containing tomatoes for only 24 hours to assure that no randomization of labeling would take place. The labeling pattern in Figure 7 shows that the expected pyruvate and formate will not be radioactive. It has not as yet been possible to accumulate sufficient fraction II to yield fragments in quantities large enough to detect with spray reagents after chromatography. Nor has fraction II labeled in other positions been obtained in amounts large enough to permit degradative studies.

In a second degradation experiment, a smaller amount of fraction II containing 239,000 cpm was used. In this case 5 mg of levulinic acid, 5 mg of malonic acid, and 10 μ liters of acetone were added after the ozone treatment. Even with the added carrier acetone, only a small amount of 2,4-dinitrophenylhydrazone precipitate was formed. Obviously acetone is lost during some prior step in the isolation of the ozonlysis products. C^{14}-Levulinic acid and C^{14}-malonic acid were identified chromatographically by their behavior in 5 solvent systems. The chromatographic data for these acids and for an unidentified acid also present are listed in Table 13. The yield of fragments is given in Table 12(b).

It has not as yet been possible to identify the additional one or two acids present. Whether they originate from contaminants in the fraction II preparation or from the migration of double bonds before or during ozonization cannot be ascertained. There is evidence that contaminating compounds of lower molecular weight may exist in relatively high concentration under

Table 13

Chromatography of acid fragments of fraction II ozonolysis (presented as R.F. values)

System	Malonic		Levulinic		
	Known	Unknown	Known	Unknown	Unknown
n-propanol -- 6 NH_4OH -- 4	.20	.15	.56	.51	.47
ethanol -- 80 water -- 15 NH_4OH -- 5	.095	.08	.48	.45	.34
ether -- 13 acetic acid -- 3 water -- 1	.70	.74	.90	.89	.30
n-butanol -- 1 pyridine -- 1 water -- 1	.57	.55	.72	.70	.19
water sat'd n-butanol -- 95 formic acid -- 5	.64	.65	.81	.80	---

certain conditions. In some dilution experiments and with preparations from the colorless mutant, a partial loss of radioactivity in samples of fraction II occurred due to the evaporation of a volatile component. Degradative studies involving larger quantities of fraction II are advisable.

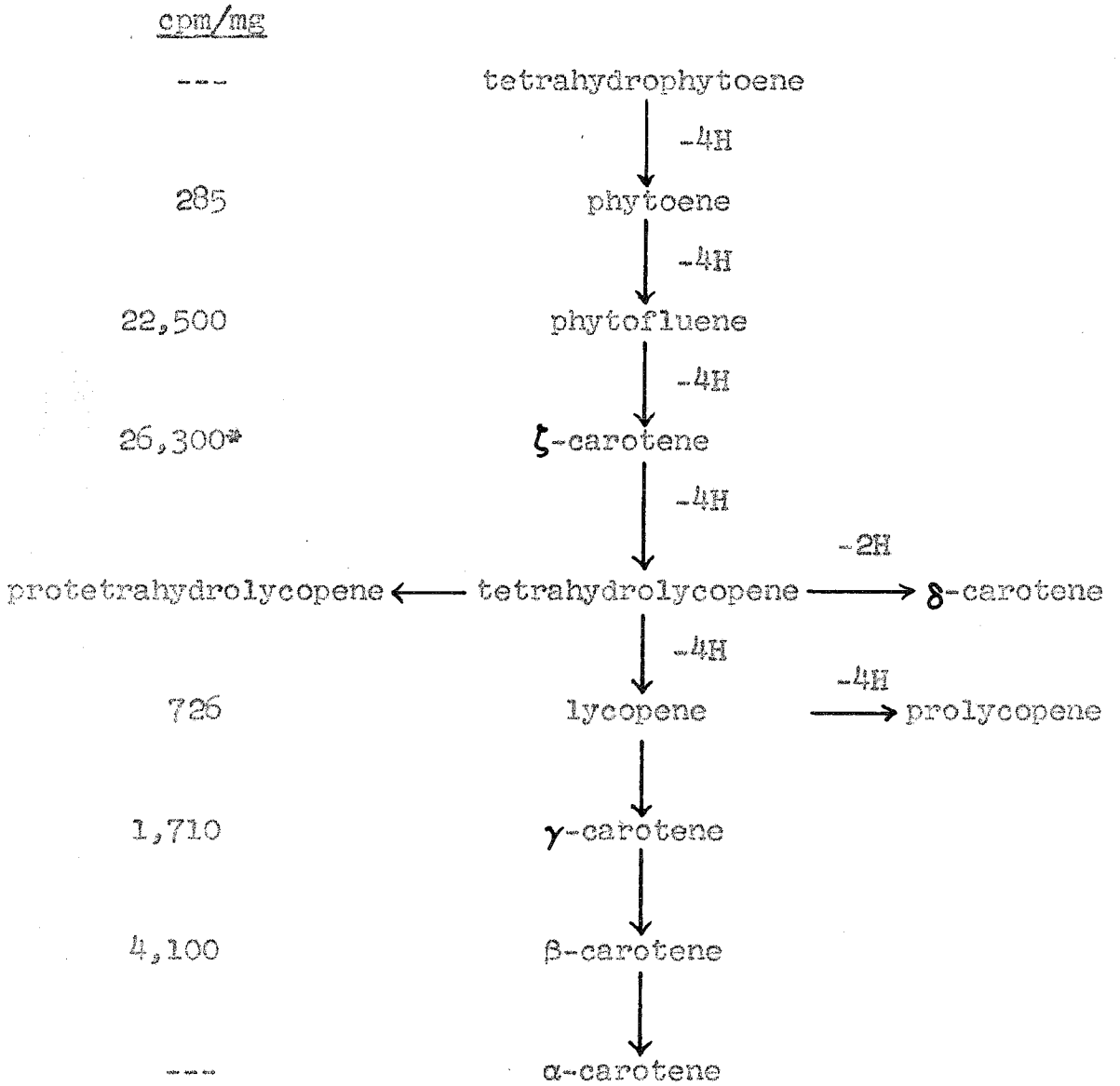
V. DISCUSSION

A. Incorporation of acetate and mevalonate

It has been shown that acetic acid and mevalonic acid are incorporated into the carotenes of the tomato and into other isoprenoid hydrocarbons as well. The conversion of these acids to carotenes in the ripening fruit is appreciable but not as rapid as would be expected from the rate of pigment synthesis at this stage of development.

B. Sequence of labeling in carotenes

Typical data on the specific activity of purified carotenes prepared from C^{14}-mevalonate-injected fruits is presented in Figure 8 together with an outline of the sequence of carotene formation as proposed by Porter and Lincoln (32). If the Porter-Lincoln proposal were correct, the specific activities of the individual carotenes should decrease at each step toward the final products. The data for specific activity are not in agreement with the Porter-Lincoln scheme but rather, suggest the hypothesis that some at least of the carotenes are synthesized from one or more common precursors by non-related pathways. The low activity of phytoene eliminates it as a possible precursor of most of the carotenes. According to the Porter-Lincoln scheme, β-carotene would be expected to have a lower specific activity than it in fact does, especially since at least half of the total amount is present in the fruit before isotope injection. In none of the long-term experiments or dilution experiments here reported



* May contain traces of contaminants

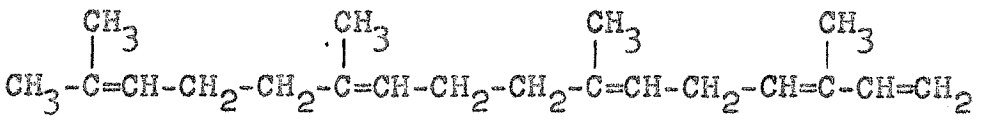
Figure 8. Porter-Lincoln Sequence

has there appeared any suggestion of a marked "pulse" of radioactivity through the carotene series, as would be expected from a single pathway of sequential dehydrogenation. It may therefore be concluded that the carotenes are not synthesized by the Porter-Lincoln pathway. In all likelihood they originate by independent mechanisms involving fraction II and/or closely related compounds.

C. The role of fraction II

Despite an intensive search, intermediates of more than one isoprene unit in size have eluded workers in the field of isoprenoid biosynthesis until quite recently. A preliminary report of the present work was first made in 1958 (80). Within the last few months evidence has been presented which shows the existence of a 3-isoprene-unit intermediate in squalene synthesis (48,49,50). The symmetrical nature of both the squalene molecule and the carotene molecule suggests the possibility that fragments containing 15 carbon atoms and 20 carbon atoms respectively may be formed and subsequently condensed with a similar intermediate to yield the final product.

The data obtained in these experiments support the following structure for fraction II:



This proposed structure is in agreement with the molecular weight determinations, the requirement for a pair of con-

jugated double bonds, and the results of degradation by ozonolysis.

Fraction II may be formed by the condensation of Δ^3-isopentenol pyrophosphate units in a manner analogous to that proposed by Lynen et al. (50). The terminal configuration could result from the loss of the pyrophosphate moiety, followed by a simultaneous migration of the double bond and dehydration of the hydroxyl group. Figure 9 illustrates this suggested mechanism. It is suggested here that the condensation of fraction II with its parent phosphorylated C_{20} compound yields the basic carotene skeleton, which may then be transformed by various dehydrogenases into the several carotenes.

This suggested pathway is similar in many respects to that proposed for squalene by Rilling, Tchen, and Block in 1958 (46). The evidence for their hypothesis includes data which show that during the formation of squalene from mevalonic acid in D_2O medium, 3 to 4 atoms of deuterium are taken up by the squalene molecule. Two deuterium atoms are expected to be in the terminal methyl groups which originate from the 2-positions of mevalonate. The other 1 or 2 atoms of deuterium found in the molecule were postulated to result from a shift of double bonds away from the active methylene group or groups during the condensation of 2 sesquiterpenoid intermediates. The absence of appreciable deuterium associated with the 2 central carbon atoms of squalene provides good evidence that a carbonyl group is not involved in the coupling process.

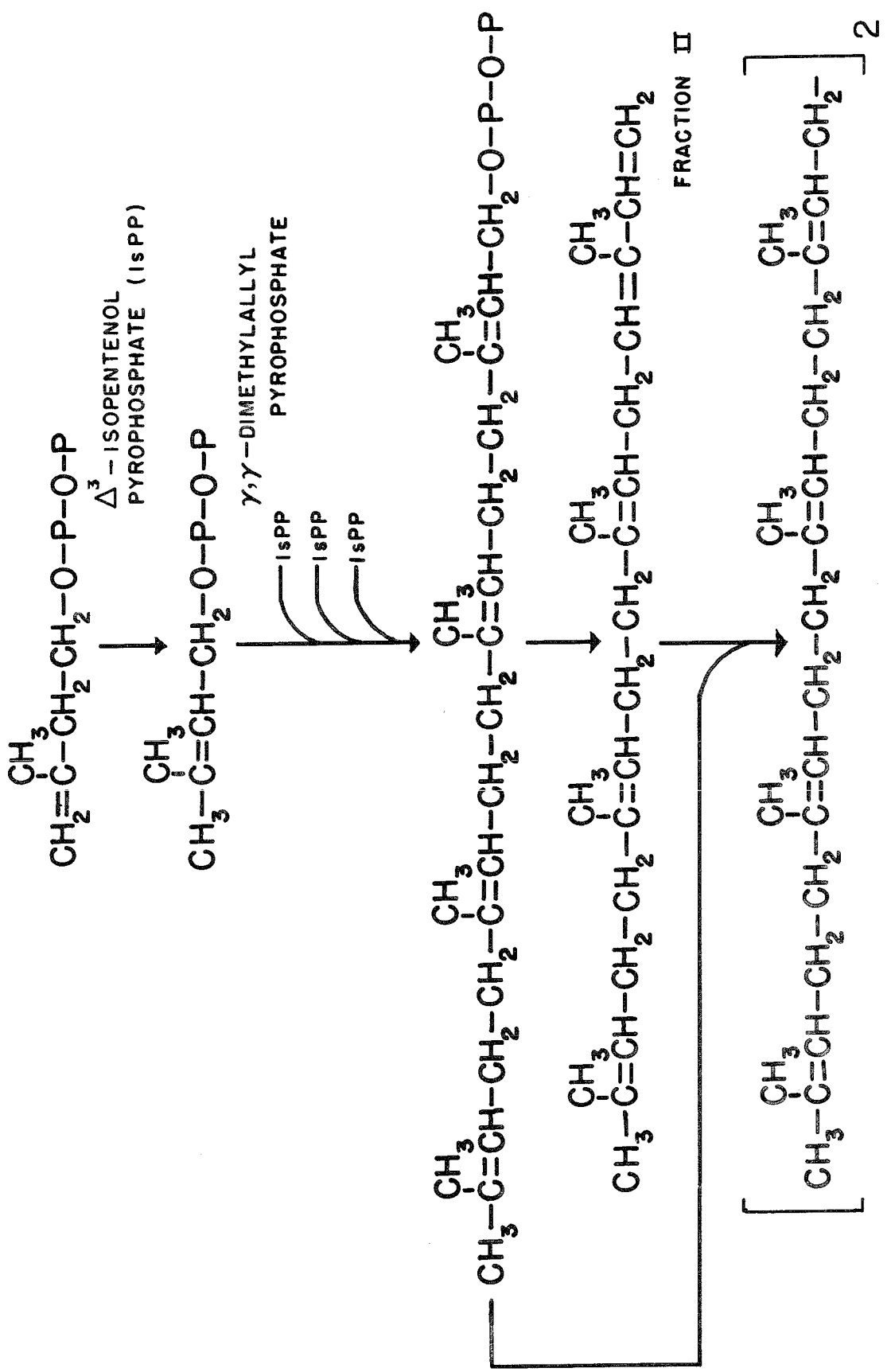


FIGURE 9. FORMATION OF CAROTENE INTERMEDIATES

The first 40-carbon-atom product of coupling as presented in Figure 9 differs from squalene only by the additional isoprene unit on each end. Three atoms of deuterium should be taken up in the synthesis of each molecule from mevalonic acid if the condensation proceeds by the mechanism here proposed. Even if this first condensation product differs from the one drawn by having a centrally located double bond, as may well be the case, the deuterium uptake would not be affected. The experiment awaits the development of an active cell-free system.

The present experiments have shown that the incorporation of C^{14}-mevalonic acid into the carotenes of the ripening tomato falls far short of that expected if the carotenes are synthesized from this intermediate alone during the period of color formation. Yet, no other substrate tested is utilized as well as mevalonate. The interpretation of these findings is derived from studies concerning incorporation of mevalonate into immature fruit. The data indicate that fraction II is formed initially and is converted to another colorless precursor, which remains in the chloroplast until ripening is initiated. At this time the transformation to the carotenes takes place.

The hypothesis draws support from the studies of Frey-Wyssling and Kreutzer (81). These workers examined chloroplasts of developing red pepper (Capsicum annuum L.) fruit with the light microscope and with the electron microscope. It was

found that as the fruit begins to ripen the characteristic grana-stroma structure of the chloroplast becomes disorganized. As fruit maturity is approached the chloroplasts are transformed into chromoplasts. It appears likely that this mode of chromoplast formation is widespread among plants.

Our knowledge of the chemical composition of chloroplasts is far from complete. The dry weight of chloroplasts has been reported to be made up of 50% protein, 30% "lipids," and up to 10% pigments (82). Straus finds that carrot chromoplasts contain an average of 58% lipids, exclusive of pigments (83). In both cases the lipids have not been characterized. The chloroplast models postulated by Wolken and Schwertz (84) and by Steinmann and Sjöstrand (85) contain ample space in the lipoid layers between the grana lamellae to accomodate a large amount of lipid in addition to the carotenoids thought to be present there. It may well be that these layers contain other isoprenoid substances in addition to the carotenoids and the phytol tails of the chlorophyll molecules. These substances would serve a structural purpose and would under certain conditions, e.g., chromoplast formation, be mobilized for the synthesis of additional carotenes.

If the above hypothesis is correct, one would expect fraction II and the other isoprenoids to be universally present in chloroplasts and chromoplasts. Only a few plants other than tomatoes have been assayed. Carrot oil contains a compound similar or identical to fraction II. Maize seedlings rapidly

incorporate C^{14}-mevalonate into a substance resembling fraction II and also into unidentified isoprenoids. Pink grapefruit and watermelon contain fraction II. However, a much more detailed survey must be made to determine the distribution of these compounds in the plant kingdom.

It will be of greatest interest to obtain more information concerning the substances that lie in the pathway between fraction II and the carotenes. The compounds associated with phytofluene and neo-β-carotene have not yet been fully purified although they are present in quantities much larger than those of fraction II. As soon as satisfactory methods of purification are devised, the molecular weight of the principal compound must be determined. Once it is known whether this intermediate contains 20 or 40 carbon atoms it may be placed in the pathway with more confidence.

A promising variation of the D_2O experiments described above might be employed to determine the time at which condensation of 2 20-carbon intermediates is carried out in the tomato fruit. Phytoene first appears as the ripening process begins. However, no labeled substrate yet added has been found to be appreciably incorporated into this polyene. It is reasonable, therefore, to assume that phytoene is formed from a precursor (perhaps containing 20 carbon atoms) that has been previously synthesized and is available for phytoene synthesis under suitable conditions. If an average of 1 atom of deuterium per molecule of phytoene were found by

analysis of a D_2O-injected, ripening tomato, it might be concluded that the "storage form" of carotene precursors is a compound of 20 carbon atoms or less, since the presence of deuterium would signify a condensation.

The existance of a more highly hydrogenated carotene than phytoene has not been satisfactorily demonstrated. Porter and Lincoln (32) reported the discovery of tetrahydrophytoene, a substance with an absorption maximum at 220 mμ, in tomatoes. No other characterization studies have been published, and some doubt has been cast (86) on the identity of the compound. No other C_{40} acyclic polyenes more saturated than phytoene have been reported.

The discovery that C^{14}-glucose yields a pattern of labeling in carotenes different from that found with acetate and mevalonate is unexpected. The per cent incorporation of uniformly labeled glucose into the total carotene fraction is about the same as that of acetate, but the activity is found to reside principally in the crude phytoene fraction and in lycopene. Just why the radioactivity of glucose is accumulated preferentially by lycopene in this case is not evident. Perhaps the carotenes are synthesized at different locations in the chromoplast, with lycopene being made near the center. If the chromoplast were not exceedingly permeable to acetate and mevalonate, these compounds might not reach the innermost parts in high concentrations. However, it is difficult to imagine many products of glucose metabolism which should be more lipid soluble than acetate. CO_2 must enter the similarly

constructed chloroplast with ease; it may therefore be the metabolite responsible for the highly labeled lycopene. This hypothesis is supported by the fact that $C^{14}O_2$ is incorporated into carotenes and yields a pattern of labeling with a rather large percentage of activity in lycopene.

Purcell, working in this laboratory, has found that the incorporation of the labeling into carotenes from 1 C^{14}-glucose is less than that expected on the basis of studies with uniformly labeled glucose (71). This finding further strengthens the CO_2 hypothesis. Radioactivity in the 3 and 4 positions of glucose is released as $C^{14}O_2$ after a single passage through the glycolysis pathway whereas 1 C^{14}-glucose produces no $C^{14}O_2$ until its metabolites have circulated through the citric acid cycle.

The low specific activity of C^{14}-glucose-derived fraction II gives an indication that its turnover rate is more rapid than has been found with acetate and mevalonate. If the labeled precursor permeates more freely to the site of maximum reactivity, this would be expected. A comparison of specific activities after shorter incubation periods has not yet been completed.

Nevertheless, no added substrate is utilized to a degree sufficient to account for the carotene synthesis that occurs. A higher molecular weight intermediate must be involved. Furthermore, the intermediate must be manufactured and

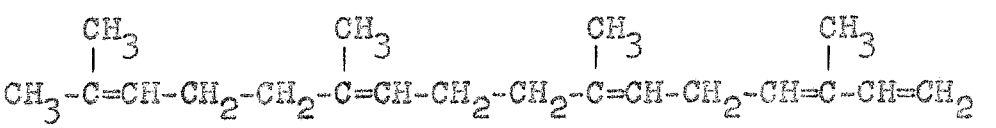
deposited in the chloroplasts continuously throughout the life of the fruit. Only such a formulation can provide for the massive carotene synthesis which characterizes the ripening fruit.

SUMMARY

The incorporation of various C^{14}-labeled substrates into the carotenes of ripening tomatoes has been studied. Of all the compounds examined, mevalonic acid is the most efficient precursor of tomato isoprenoids, as evidenced by incorporation often exceeding 50% of the substrate administered.

The pattern of labeling among the several carotenes present in the tomato has been studied with the aim of detecting any relationship that may exist. According to the widely accepted Porter-Lincoln hypothesis, the more saturated members of the series are synthesized first and are transformed into the highly unsaturated carotenes by stepwise dehydrogenation. The present experiments have led to the conclusion that the Porter-Lincoln scheme cannot account for pigment formation in ripening tomatoes. The specific activities of the various carotenes indicate that they are formed by separate pathways from lower molecular weight intermediates.

A high percentage of the radioactivity found in the carotene fraction after C^{14}-mevalonate injection was found to be associated with a previously undescribed, colorless polyene. Purification and characterization of this compound have shown it to be a 20-carbon-atom, isoprenoid hydrocarbon containing two conjugated double bonds. Degradative experiments have yielded evidence for the following structure:



This compound, termed fraction II, has been examined as a possible carotene precursor. Despite the rapid incorporation of radioactive mevalonate into fraction II in both ripening and immature tomatoes, no accumulation of the compound appears. Nor does carotene formation take place at the expense of newly synthesized fraction II alone. All evidence indicates that fraction II is made throughout the life of the fruit and is converted to another carotene precursor which is bound in the chloroplast until the ripening process is initiated. It has been experimentally observed that the radioactivity of fraction II synthesized in an immature tomato is in time transformed into an unknown polyene, and finally, upon ripening, into carotenes themselves. Purification of the unknown intermediate has not yet been achieved. However, the seemingly large amounts present could easily account for the total carotene production which occurs within a few days of color formation.

The hypothesis has been developed that fraction II and the additional polyene precursor are laid down in the lipid fraction of the chloroplast. Their conversion to carotenes during the development of chromoplasts from chloroplasts is likely.

REFERENCES

1. Berzelius, J. Ueber die gelbe Farbe der Blätter im Herbst. Ann. 21, 257-262 (1837).
2. Willstätter, R., and W. Meig. Untersuchungen über Chlorophyll; IV Ueber die gelben Begleiter des Chlorophylls. Ann. 355, 1-28 (1907).
3. Zechmeister, L., L. Cholnoky, and V. Vrabély. Über die katalytische Hydrierung von Carotin. Ber. 61, 566-568 (1928).
4. Karrer, P., and H. Salomon. Über die Safranfarbstoffe II. Helv. Chim. Acta 11, 513-525 (1928).
5. Karrer, P., and E. Jucker. Carotenoids. Amsterdam, Elsevier (1950) p. 66.
6. Goodwin, T. W. Carotenoids. New York, Chem. Pub. Co. (1954) p. 6.
7. Griffiths, M., W. R. Sistrom, G. Cohen-Bazire, and R. Y. Stanier. Function of carotenoids in photosynthesis. Nature 176, 1211-1214 (1955).
8. Whittingham, C. P. Chloroplast structure and energy conversion in photosynthesis. Progress in Biophys. Butler, J. A. V., and B. Katz, eds., New York, Pergamon Press, vol. 7 (1957) pp. 320-343.
9. Goodwin, T. W. Carotenoids. Ann. Rev. Biochem. Luck, J. M., ed., Palo Alto, Ann. Revs., Inc., vol. 24 (1955) pp. 497-522.

10. Goodwin, T. W. Carotenoids, Handbuch der Pflanzenphysiologie, Ruhland, W., ed., Berlin, Springer-Verlag, vol. X (1958) pp. 186-222.

11. Haxo, F. Carotenoid formation in mutant strains of Neurospora crassa. Biol. Bull. 103, 286 (1952).

12. Haxo, F. Some biochemical aspects of fungal carotenoids. Fortshr. Chem. org. Naturstoffe, Zechmeister, L., ed., Vienna, Springer-Verlag, vol. 12 (1955) pp. 169-197.

13. Zalokar, M. Studies on biosynthesis of carotenoids in Neurospora crassa. Arch. Biochem. Biophys. 50, 71-80 (1954).

14. Haxo, F., in Goodwin, T. W. Carotenoids, Handbuch der Pflanzenphysiologie, Ruhland, W., ed., Berlin, Springer-Verlag, vol. X (1958) p. 201.

15. Friend, J., and T. W. Goodwin. The effect of temperature and thiamine concentration on carotenogenesis by Phycomyces blakesleeanus. Biochem. J. 57, 434-439 (1954).

16. Kuppers, J. R., in Goodwin, T. W. Carotenoids, Handbuch der Pflanzenphysiologie, Ruhland, W., ed., Berlin, Springer-Verlag, vol. X (1958) p. 200.

17. Goodwin, T. W. The identification of the minor polyene components of Phycomyces blakesleeanus and a study of their synthesis under various cultural conditions. Biochem. J. 50, 550-558 (1952).

18. Goodwin, T. W., M. Jamikorn, and J. S. Willmer. Further observations concerning the action of diphenylamine in inhibiting the synthesis of β-carotene in Phycomyces blakesleeanus. Biochem. J. 53, 531-538 (1953).

19. MacKinney, G., C. O. Chichester, and P. S. Wong. Carotenoids in Phycomyces. J. Am. Chem. Soc. 75, 5428 (1953).

20. Engel, B. G., J. Würsch, and M. Zimmermann. "Über die Einfluss von β-Ionon auf die Bildung von β-Carotin durch Phycomyces blakesleeanus. Helv. Chim. Acta 36, 1771-1776 (1953).

21. MacKinney, G., C. O. Chichester, and P. S. Wong. On carotenoid synthesis in Phycomyces blakesleeanus. Arch. Biochem. Biophys. 53, 479-483 (1954).

22. Goodwin, T. W., L. A. Griffiths, M. Jamikorn, and J. S. Willmer. Carotenogenesis in Phycomyces blakesleeanus. Congr. Intern. Biochem. Resumés communs., 2^{e} congr. Paris (1952) p. 210.

23. Goodwin, T. W., and H. G. Osman. Spirilloxanthin synthesis by washed cells of Rhodospirillum rubrum. Biochem. J. 56, 222-229 (1954)

24. Jensen, S. L., G. Cohen-Bazire, T. O. M. Nakayama, and R. Y. Stanier. The path of carotenoid synthesis in a photosynthetic bacterium. Biochim. Biophys. Acta 29, 447-498 (1958).

25. Bonner, J., A. Sandoval, Y. W. Tang, and L. Zechmeister. Changes in polyene synthesis induced by mutation in a red yeast (Rhodotorula rubra). Arch. Biochem. 10, 113-123 (1946).

26. Le Rosen, A. L., F. W. Went, and L. Zechmeister. Relation between genes and carotenoids of the tomato. Proc. Nat. Acad. Sci. (U.S.) 27, 236-242 (1941).

27. Tomes, M. L., F. W. Quackenbush, and M. McQuistan. Modification and dominance of the gene governing formation of high concentrations of β-carotene in the tomato. Genetics 39, 810-817 (1954).

28. Rick, C. M., and L. Butler. Cytogenetics of the tomato. Adv. in Genetics, New York, Academic Press, Demerec, M., ed., vol. 8 (1956) pp. 267-393.

29. MacKinney, G., and J. A. Jenkins. Carotenoid differences in tomatoes. Proc. Nat. Acad. Sci. (U.S.) 38, 48-52 (1952).

30. Jenkins, J. A., and G. MacKinney. Carotenoids of the Apricot tomato and its hybrids with Yellow and Tangerine. Genetics 40, 715-720 (1955).

31. MacKinney, G., C. M. Rick, and J. A. Jenkins. The phytoene content of tomatoes. Proc. Nat. Acad. Sci. (U.S.) 42, 404-408 (1956).

32. Porter, J. W., and R. E. Lincoln. I. Lycospersicon selections containing a high content of carotenes and colorless polyenes. II. The mechanism of carotene biosynthesis. Arch. Biochem. 27, 390-403 (1950).

33. Porter, J. W., in Goodwin, T. W. Carotenoids, Handbuch der Pflanzenphysiologie, Ruhland, W., ed., Berlin, Springer-Verlag, vol. X (1958) p. 195.

34. Sayre, C. B., W. B. Robinson, and T. Wishnetsky. Effect of temperature on the color, lycopene, and carotene content of detached and vine ripened tomatoes. Proc. Am. Soc. Hort. Sci. 61, 381-387 (1953).

35. Goodwin, T. W., and M. Jamikorn. Biosynthesis of carotenes in ripening tomatoes. Nature 170, 104-105 (1952).

36. Went, F. W., A. L. LeRosen, and L. Zechmeister. Effect of external factors on tomato pigments as studied by chromatographic methods. Plant Physiol. 17, 91-100 (1942).

37. Francis, F. J. Some observations on carotenoid biosynthesis in tomatoes. Proc. Am. Soc. Hort. Sci. 71, 349-355 (1958).

38. Little, H. N., and K. Bloch. Studies on the utilization of acetic acid for the biological synthesis of cholesterol. J. Biol. Chem. 183, 33-46 (1950).

39. Ottke, R. C., E. L. Tatum, I. Zabin, and K. Bloch. Isotopic acetate and isovalerate in the synthesis of ergosterol by Neurospora. J. Biol. Chem. 189, 429-433 (1951).

40. Rabinowitz, J. L., and S. Gurin. Biosynthesis of cholesterol and β-hydroxy-β-methyl glutaric acid by extracts of liver. J. Biol. Chem. 208, 307-313 (1954).

41. Dituri, F., F. A. Cobey, J. V. B. Warms, and S. Gurin. Terpenoid intermediates in the biosynthesis of cholesterol. J. Biol. Chem. 221, 181-189 (1956).

42. Tavormina, P. A., M. H. Gibbs, and J. W. Huff. The utilization of β-hydroxy-β-methyl-δ-valerolactone in cholesterol biosynthesis. J. Am. Chem. Soc. 78, 4498-4499 (1956).

43. Ferguson, J. J., I. F. Durr, and H. Rudney. Enzymatic reduction of β-hydroxy-β-methyl glutaryl coenzyme A to mevalonic acid in yeast. Federation Proc. 17, 219 (1958).

44. Tchen, T. T. On the formation of a phosphorylated derivative of mevalonic acid. J. Am. Chem. Soc. 79, 6344-6345 (1957).

45. Chaykin, S., J. Law, A. H. Phillips, T. T. Tchen, and K. Bloch. Phosphorylated intermediates in the synthesis of squalene. Proc. Nat. Acad. Sci. (U.S.) 44, 998-1004 (1958).

46. Rilling, H., T. T. Tchen, and K. Bloch. On the mechanism of squalene biogenesis. Proc. Nat. Acad. Sci. 44, 167-173 (1958).

47. Ogilvie, J. W., Jr., and R. G. Langdon. The enzymatic conversion of mevalonic acid 2C^{14} to an olefinic acid. J. Am. Chem. Soc. 81, 754-756 (1959).

48. Ogilvie, J. W., Jr. The degradation and structure of a terpenoid acid enzymatically synthesized from mevalonic acid. J. Am. Chem. Soc. 81, 756 (1959).

49. Agranoff, B. W., H. Eggerer, U. Henning, and F. Lynen. Isopentenol pyrophosphate isomerase. J. Am. Chem. Soc. 81, 1254-1255 (1959).

50. Lynen, F., J. Knappe, H. Eggerer, U. Henning, and B. W. Agranoff. Biosynthesis of terpenes. Fed. Proc. 18, 278 (1959).

51. Bonner, J., and B. Arreguin. The biochemistry of rubber formation in the guayule. I. Rubber formation in seedlings. Arch. Biochem. 21, 109-124 (1949).

52. Arreguin, B., J. Bonner, and B. J. Wood. Studies on the mechanism of rubber formation in the guayule. III. Experiments with isotopic carbon. Arch. Biochem. Biophys. 31, 234-247 (1951).

53. Teas, H. J., and R. Bandurski. The enzymatic synthesis of rubber. J. Am. Chem. Soc. 78, 3549-3550 (1956).

54. Park, R. B., and J. Bonner. Enzymatic synthesis of rubber from mevalonic acid. J. Biol. Chem. 233, 340-343 (1958).

55. Stanley, B. G. Terpene formation in pine from mevalonic acid. Nature 182, 738-739 (1958).

56. Grob, E. C., and R. Bütler. Über die Biosynthese des β-Carotins bei Mucor hiemalis. Helv. Chim. Acta 39, 1975-1980 (1956).

57. Zabin, I. The formation of radioactive lycopene in ripening tomatoes. J. Biol. Chem. 226, 851-859 (1957).

58. Shneour, E. A., and I. Zabin. On the biosynthetic relationship between lycopene and colorless polyenes in tomatoes. J. Biol. Chem. 226, 861-866 (1957).

59. Braithwaite, G. D., and T. W. Goodwin. Biosynthesis of β-carotene from DL-β-hydroxy-β-methyl-δ-2C^{14}-valerolactone by Phycomyces blakesleeanus and carrot slices. Biochem. J. 67, 13p-14p (1957).

60. Goodwin, T. W. The incorporation of $C^{14}O_2$, [2C^{14}]acetate, and [2C^{14}] mevalonate into β-carotene by illuminated etiolated maize seedlings. Biochem. J. 70, 612-617 (1958).

61. Shneour, E. A., and I. Zabin. Formation of radioactive lycopene in tomato homogenates. Abst. of Papers 133rd ACS Meet., April, 1958, p. 48c.

62. Workman, M., H. K. Pratt, and L. L. Morris. Studies on the physiology of tomato fruits. I. Respiration and ripening behavior at 20°C as related to date of harvest. Proc. Am. Soc. Hort. Sci. 69, 352-365 (1957).

63. Spencer, M. S. Ethylene metabolism in tomato fruit. I. Relationship of ethylene evolution to fruit respiration and ripening. Can. J. Biochem. Physiol. 34, 1261-1270 (1956).

64. McCready, R. M., and W. Z. Hassid. Transformation of sugars in excised barley roots. Plant Physiol. 16, 599-610 (1941).

65. Spencer, M. S. Ethylene metabolism in tomato fruit III. Effect of 2,4-dinitrophenol on respiration, ethylene evolution, and ripening. Can. J. Biochem. Physiol. 37, 53-59 (1959).

66. Zscheile, F. P., and J. W. Porter. Analytical methods for carotenes of Lycospersicon species and strains. Ind. Eng. Chem. (Anal.) 19, 47-51 (1947).

67. Rabourn, W. J., and F. W. Quackenbush. The occurrence of phytoene in various plant materials. Arch. Biochem. Biophys. 44, 159-164 (1953).

68. Wallace, V., and J. W. Porter. Phytofluene. Arch. Biochem. Biophys. 36, 468-472 (1952).

69. Trombly, H. H., and J. W. Porter. Additional carotenes and a colorless polyene of Lycopersicon species and strains. Arch. Biochem. Biophys. 43, 443-457 (1953).

70. Potts, W. J. Purification of hydrocarbons for use as solvents in far ultraviolet spectroscopy. J. Chem. Phys. 20, 809-810 (1952).

71. Purcell, A. E. Unpublished data.

72. Wolf, D. L., C. H. Hoffman, P. E. Aldrich, H. R. Skeggs, L. D. Wright, and K. Folkers. β-Hydroxy-β-methyl-δ-valerolactone (divalonic acid), a new biological factor. J. Am. Chem. Soc. 78, 4499 (1956).

73. Rabourn, W. J., and F. W. Quackenbush. The structure of phytoene. Arch. Biochem. Biophys. 61, 111-118 (1956).

74. Thompson, A. E. Inheritance of high total carotenoid pigments in tomato fruits. Science 121, 896-897 (1955).

75. Coon, M. J. The metabolic fate of the isopropyl group of leucine. J. Biol. Chem. 187, 71-82 (1950).

76. Woodward, R. B. Structure and absorption spectra III. Normal and conjugated dienes. J. Am. Chem. Soc. 64, 72-75 (1942).

77. O'Conner, R. T., and L. A. Goldblatt. Correlation of ultraviolet and infrared spectra of terpene hydrocarbons. Anal. Chem. 26, 1726-1737 (1954).

78. Bellamy, L. J. The infrared spectra of complex molecules. 2nd ed., London, Menthuen (1958) pp. 37-41.

79. Miller, J. M., and J. G. Kirchner. Chromatostrips for identifying constituents of essential oils. Anal. Chem. 25, 1107-1109 (1953).

80. Purcell, A. E., and G. A. Thompson, Jr. Investigations on the biosynthesis of carotenoids in the tomato. Plant Physiol. 33, Supp. Proc. Meet. Am. Soc. Plant Physiologists, Bloomington, Ind., Aug. 1958, p. xlv.

81. Frey-Wyssling, A., and E. Kreutzer. The submicroscopic development of chromoplasts in the fruit of Capsicum annuum L. J. Ultrastructure Res. 1, 397-411 (1958).

82. Rabinowitz, E. I. Photosynthesis and related processes. New York, Interscience, vol. 1 (1945) p. 382.

83. Straus, W. Properties of isolated carrot chromoplasts. Exptl. Cell Res. 6, 392-402 (1954).

84. Wolken, J. J., and F. A. Schwertz. Chlorophyll monolayers in chloroplasts. J. Gen. Physiol. 37, 111-120 (1953).

85. Steinmann, E., and F. S. Sjöstrand. The ultrastructure of chloroplasts. Exptl. Cell Res. 8, 15-23 (1955).

86. MacKinney, G. Carotenoids. Ann. Rev. Biochem., Luck, J. M., ed., Palo Alto, Ann. Revs., Inc., vol. 21 (1952) pp. 473-492.