CAROTENOID BIOSYNTHESIS IN *NEUROSPORA CRASSA*

Thesis by

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ABSTRACT

As a means of inducing a reproducible amount of carotenoid synthesis in large quantities of mycelium, a new illumination-incubation technique was developed and is described. In addition, procedures are presented for obtaining phytoene and each of the carotenoid pigments spectroscopically and radiochemically pure when 2-C^{14}-mevalonate is used as a labelled precursor. The criteria of purity which were employed are discussed.

Using these and other basic procedures, data have been obtained which were all consistent with one particular pathway of carotenoid synthesis. The pathway involves conversion of phytoene to the carotenoid pigments by a series of sequential reactions which include dehydrogenation, cyclization, and oxidative cleavage of a C=C bond.

The proposed pathway is supported by labelling data, time course measurements of each carotenoid in mycelium illuminated and incubated under various physiological conditions, studies with color mutants, and the chemical structures of the compounds involved. In addition, the carotenoid beta-zeacarotene, which had not been previously reported in Neurospora, was shown to be present in mycelium under certain conditions, and this fact added additional support to part of the proposed pathway.

Some information was also obtained about the mechanism of induction of carotenoid synthesis in Neurospora by light. It was confirmed that under well aerated conditions the initial light reaction occurs very rapidly (30 seconds or less). Using cycloheximide, an inhibitor of protein synthesis, evidence was obtained which strongly suggests that one effect of light is to induce the de novo synthesis of an enzyme (or enzymes) which is required for the synthesis of carotenoids and is absent in dark-grown mycelium. Possible mechanisms for such an induction by light are discussed.

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INTRODUCTION

The investigation of the carotenoid pigments has continued over a long period of time as indicated by the fact that they were the first compounds used in adsorption column chromatography (1).

There are about 80 naturally occurring carotenoids which have been characterized (2), and they are found distributed among a wide range of organisms including higher plants, algae, fungi, lichens, and photosynthetic bacteria (3-8). In addition, food provides the carotenoid, beta-carotene, to many animals which can enzymatically convert it to vitamin A (9), an essential compound for vision (10).

The carotenoids are yellow, orange, red, or violet colored compounds, made up of units of isoprene, and contain 7 to 15 conjugated C=C double bonds (2). Most carotenoids contain 40 carbon atoms (8 isoprene molecules) and include hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids, epoxides, furanoid oxides, and various combinations of these groups (2).

Two colorless compounds, phytoene and phytofluene, are generally present in the carotenoid extracts of plants (2). They are 40-carbon hydrocarbons, structurally similar to the carotenoids except that phytoene contains

only 3 conjugated double bonds and phytofluene, which fluoresces strongly in ultraviolet light, contains 5. The chemical structures of these compounds as well as those of 8 carotenoid pigments are shown in Figures 11-20.

There are several other groups of isoprenoid compounds besides the carotenoids found in nature. These include the monoterpenes, sesquiterpenes, diterpenes, triterpenes, ubiquinones, plastoquinones, and rubber. A large portion of the literature concerning each of these classes of compounds has been presented and discussed by Richards and Hendrickson (11) and by Pridham (12).

The suggestion that all such compounds were synthesized from the same basic 5-carbon unit was first made by Ruzicka (13, 14) and is known as his "isoprene rule." The discovery of mevalonic acid by Folker's group (15) was the initial experimental step in confirming Ruzicka's proposal, since a series of subsequent investigations showed that mevalonate was converted to isopentenyl pyrophosphate (16), a compound which met the requirements of the active 5-carbon isoprene unit.

The generally accepted pathway for the incorporation of mevalonate and isopentenyl pyrophosphate into each class of isoprenoid compounds is shown in Figure 1. This scheme is based largely on two types of experiments:

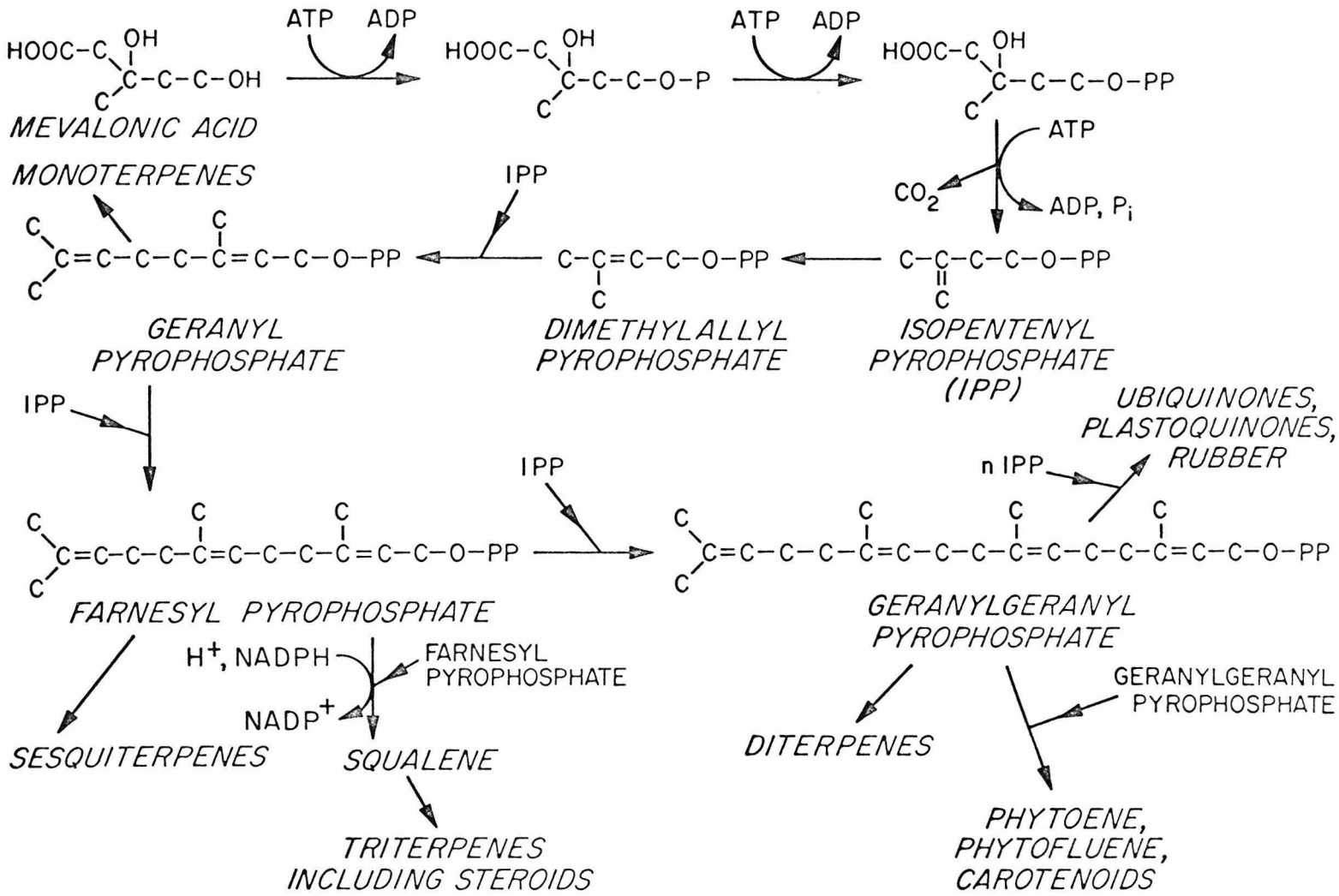

Fig. 1 Pathway from mevalonic acid to isoprenoid compounds.

1. Incorporation of a radioactive intermediate by several chemical steps into a particular isoprene compound or compounds either *in vivo* (for mevalonic acid) or in cell-free experiments.
2. Incorporation of a radioactive compound into the next intermediate in the pathway in a cell-free system using a purified or partially purified enzyme.

Much of the literature involving incorporation of radioactive intermediates of this pathway into isoprenoid compounds is cited by Richards and Hendrickson (11) and by Pridham (12). As summarized in the recent review article on carotenoid biosynthesis by Porter and Anderson (16), incorporation of label into phytoene or the carotenoid pigments has been shown using the intermediates 2-C^{14}-mevalonic acid (17-33), 2-C^{14}-5-phosphomevalonic acid (34), 2-C^{14}-5-pyrophosphomevalonic acid (35), 4-C^{14}-isopentenyl pyrophosphate (36, 37), and 4,8,12-C^{14}-farnesyl pyrophosphate (36). The biological systems used in such studies have included *Phycomyces blakesleeanus*, *in vivo* (21, 22) and *in vitro* (31, 32, 38), *Blakeslea trispora*, *in vivo* (19), *Neurospora crassa*, *in vivo* (23-26), *Staphylococcus aureus*, *in vitro* (28, 29, 34, 35), *Sporobolomyces shibatanus*, *in vitro* (39), *Euglena gracilis*, *in vivo* (27), tomato fruit (17, 19, 20, 30), tomato

homogenates (18, 36, 37), tomato plastids (40, 41) carrot root, *in vivo* (33) and *in vitro* (40), and excised etiolated maize seedlings (42). In addition the individual steps from mevalonic acid to geranylgeranyl pyrophosphate have been investigated and verified in various cell-free systems (16, 43, 44), and cell-free incorporation of farnesyl pyrophosphate plus isopentenyl pyrophosphate into phytoene has been demonstrated (36).

There is disagreement about whether geranylgeranyl pyrophosphate dimerizes to form phytoene or whether the dimerization is reductive in analogy to the synthesis of squalene and leads to the formation of the 40-carbon compound, lycopersene. The detection of this compound in a *Neurospora crassa* culture (45) and its synthesis from geranylgeranyl pyrophosphate by an extract from this organism (46) has been reported;however, in both cases identification of lycopersene was based on co-chromatography. Furthermore, there are several reports of failures to detect the formation of this compound by carotenoid synthesizing systems (36, 40, 47-49) including that of *Neurospora crassa* (50). It has also been suggested that the reason 40-carbon, steroid like compounds are never found in nature is due to the presence of the double bond in the center of phytoene formed by dimerization of geranylgeranyl pyrophosphate (51). Such a double bond

is absent in squalene which readily undergoes cyclization to steroids. Thus, at present most evidence suggests that lycopersene is not an intermediate in carotenoid synthesis.

Additional evidence that the carotenoids and steroids are derived from the same pathway has been obtained using a chemical degradative analysis of labelled carotenoids. It was found that the distribution of label was the same as that found in squalene (27,52).

As already mentioned, phytofluene and the carotenoids contain more carbon to carbon double bonds than phytoene. A fundamental question in carotenoid synthesis is at what point in the pathway does dehydrogenation take place. In 1950, Porter and Lincoln (53) extended previous proposals (54-56) concerning this question and suggested that the carotenoids are synthesized by sequential dehydrogenation from the colorless polyenes. Porter and Anderson later modified details of the earlier pathway to take into account new information which had become available (43). The preponderance of data which have been collected by investigators in this field have been interpreted as supporting this concept of dehydrogenation at the 40-carbon level; however, an alternative proposal that the carotenoids are synthesized in parallel without interconversion and from precursors other than phytoene has also been made in a few cases (17, 23-26, 30, 31).

Several review articles have been written which summarize the information which has been obtained concerning the pathway of carotenoid biosynthesis (3-5, 16, 57-63). The types of experiments which have been carried out include genetic studies (29, 64-81), time course measurements of the carotenoids with or without inhibitors (80, 82-86), and labelling experiments *in vivo* (17, 19, 23-26, 30) and *in vitro* (40, 41).

The most direct information concerning the question of whether the carotenoids are synthesized by dehydrogenation at the 40-carbon level has been obtained by studying reactions in cell-free systems; however, success along these lines has been limited so far. A cell-free preparation of *Sporobolomyces shibatanus* converted phytoene to beta-carotene (39, 87), and a *Staphylococcus aureus* extract was found to convert phytoene to delta-carotene (88). In addition it has been shown that tomato plastids can synthesize phytofluene from phytoene (89). Recently a preparation from tomatoes was shown to synthesize phytoene of high specific radioactivity from farnesyl pyrophosphate plus isopentenyl pyrophosphate (36), and it is likely that the labelled phytoene obtained in this system will prove useful for cell-free studies of its conversion to the carotenoids (36).

Another important aspect of carotenoid synthesis is regulation by the environment, and light is one important factor in this respect in several organisms. In certain cases, for example in Phycomyces blakesleeanus (90) or Penicillium sclerotiorium (91), light increases the amount of pigment synthesized as compared with that made in darkness. In Neurospora crassa (92-96), Fusarium aquaeductuum (97-102), and Mycobacterium sp. (103-105), there is little or no pigment production in the dark, and light triggers carotenoid synthesis. The action spectrum of the light effect in these organisms has been determined, and was found to resemble closely the visible absorption spectrum of flavoproteins (92, 100, 105, 106).

In several cases, it has been shown that addition of inhibitors of protein synthesis immediately after illumination blocked carotenoid production (101, 105). On this basis and from the action spectra, it was suggested that the initial light reaction involves absorption of light by a flavoprotein which then catalyzes the photo-oxidation of a compound to form an inducer; as a result, the inducer initiates the functioning of a gene, and a missing enzyme in the carotenoid pathway is synthesized (101, 105).

In Neurospora crassa, phytoene has been shown to accumulate in the dark (94, 96). There is disagreement as to whether it is a precursor of the carotenoids in this organism (96), or whether unknown precursors are involved (23, 25). If the former is true, then a plausible explanation for the block in the pathway would be that the enzyme used in dehydrogenating phytoene is inactive or missing in dark-grown cultures and is activated or synthesized de novo as a result of illumination. For these reasons, the experiments in this investigation were designed to learn more about the carotenoid biosynthetic pathway and more about the mechanism of light regulation.

MATERIALS AND METHODS

A. STRAINS

The colonial mutant col-4 (70007A) was used in most experiments. Previous workers have used this mutant because it forms distinct colonies on plates (107). It was used in this study because its colonial character permitted the uniform illumination and aeration of large quantities of mycelium in liquid medium.

Mycelial pads formed by wild type Em 5297a, grown in flasks, were used in some experiments. Carotenoid biosynthesis in this wild type strain has been previously studied by Zalokar (93).

Three color mutants were obtained from the Fungal Genetics Stock Center, and the carotenoid composition of each was investigated. These were peach (L-pe-4a), stock number 567, yellow-1 (Y30539yA), stock number 513, and golden (70007gA), stock number 557. The only published information about the carotenoids of any of these mutants is found in a paper by Garnjobst and Tatum (108), where it is reported, by way of a personal communication, that a yellow mutant (Y30539a) does not synthesize the acidic pigment.

Some experiments were carried out with two albino mutants, aur (34508A) and al-2 (15300A). For a recent

study of the genetics of these and other albino mutants, see the paper by Huang (77).

B. CULTURE METHODS

Vogel's minimal medium N (109) was used for all cultures. In most experiments, the conidia from two slants were suspended in 10-20 ml of sterile water and added to six liters of medium in a 2.5 gallon carboy. The carboy was placed inside an empty yeast barrel and aerated aseptically in the dark for 3 days at an air flow rate of 12 liters/minute.

In experiments with Em 5297a, a few drops of a conidial suspension in water were added to 20 ml of medium in 125 ml Erlenmeyer flasks. The cultures were grown for 3 days in the dark at 25°C without shaking.

For the studies using albino mutants, a conidial suspension from one slant was added to 1000 ml of medium in a 2500 ml culture flask. The flask was shaken in the dark at 25°C for 3 days on a reciprocating shaker with a $2\frac{1}{4}$ inch stroke at 86 strokes/minute.

C. ILLUMINATION OF THE MYCELIUM AND HARVESTING

The action spectrum of the photoactivation process, as determined by Zalokar (92), has a broad maximum in the region of 450-488 mμ and drops rapidly above 490 mμ. For this reason, illumination during the harvesting of the mycelium was always limited to that of a 25 watt red

light bulb. Controls showed that this treatment did not initiate carotenoid synthesis.

After three days of aeration in the dark, the mycelium and medium of each carboy were poured over a double layer of cheese cloth. The mycelium was squeezed to remove excess medium, washed with 500 ml of water (precooled to 4°C), and squeezed again. The desired amount of mycelium, usually 135 g fresh weight, was added to a 2.5 gallon carboy, which contained a volume of medium equal to 9 times the fresh weight of mycelium.

The mycelium was illuminated with three 32 watt circular fluorescent lamps while the carboy was rotated at 37 revolutions/minute (see Figure 2). The carboy contained two indentations parallel to its longitudinal axis and spaced directly opposite each other. These served as baffles for mixing and allowed the mycelium to be uniformly illuminated and aerated (see Figure 3). In early experiments, a single 1¼ inch wide, lucite baffle, which was glued to the carboy along one edge with 3M Brand Weatherstrip Adhesive, was used. This baffle arrangement proved satisfactory for many experiments; however, more pigment was produced with the double baffle carboy, and so it was used in most cases.

Generally, the mycelium was illuminated continuously, but in some experiments a 15 minute light stimulus was

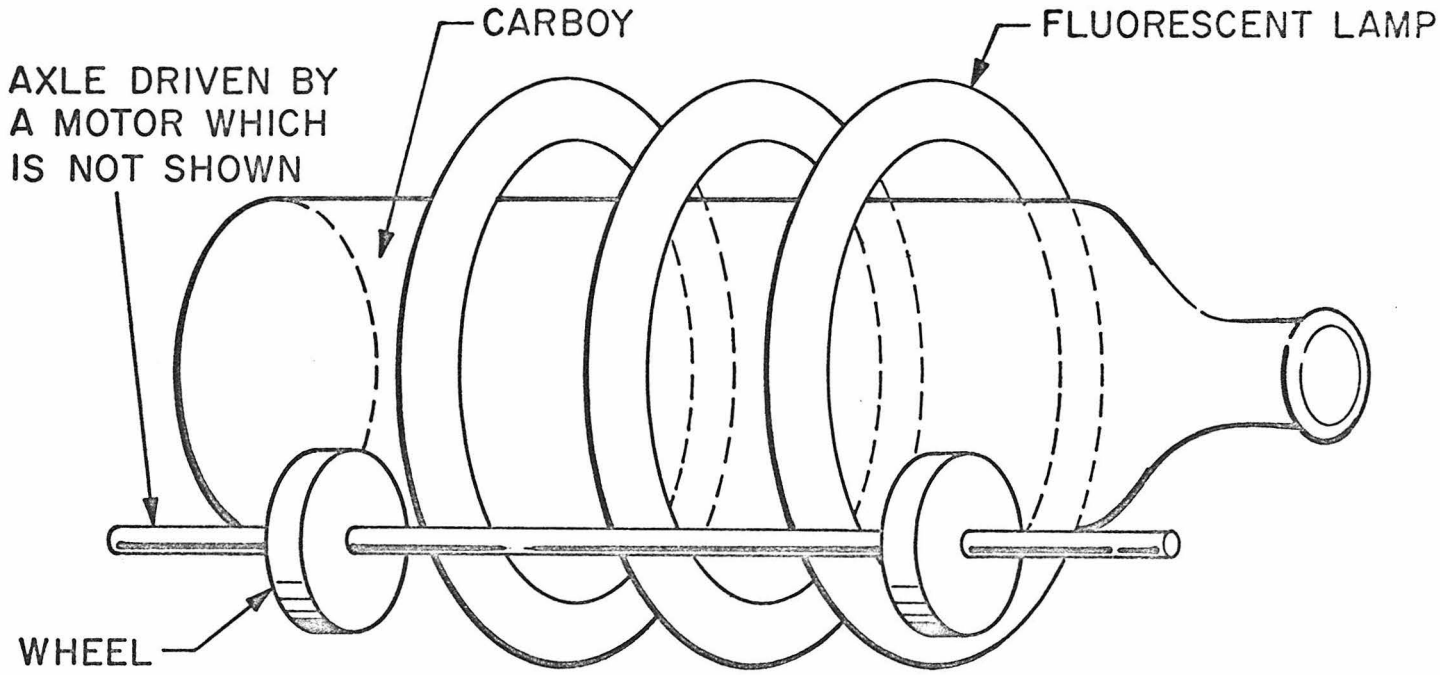

Fig. 2 Rotary apparatus used for illuminating and incubating mycelium. The carboy was supported by 2 pairs of wooden wheels. The pair shown in the figure is driven by a motor, and this in turn caused rotation of the carboy. Another pair of wheels, which is not shown, is located at the corresponding position on the opposite side of the carboy. The central lamp is an equal distance from the top and bottom of the carboy, and the center of each of the end lamps is 3 inches from the center of the middle lamp.

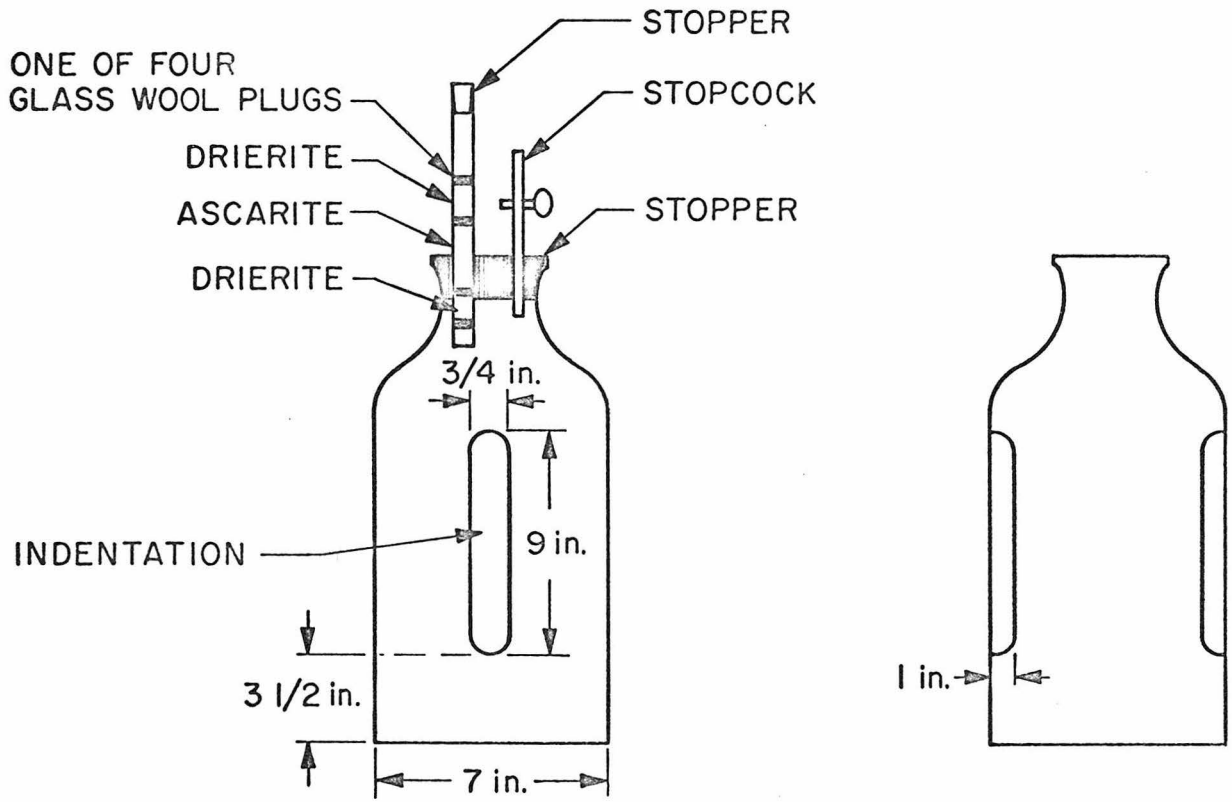

Fig. 3 Modified carboy used for the illumination of mycelium. The double glass baffle carboy is shown as seen from two directions. Two indentations, with the dimensions shown, were formed in the carboy. These served as baffles and caused the mycelium to be aerated during the incubation. The ascarite trap and stopcock which were used in experiments involving incubation under O_2 are shown in one drawing of the carboy.

used, and rotation was allowed to continue in the dark for the desired length of time. In other experiments, the entire incubation was carried out in the dark.

During rotation of the carboy, the rotator-illuminator was enclosed in a cardboard container, which was constructed for these experiments. The container, which was open on its top side during illumination, was covered with a large black cloth during dark incubations. Light was further excluded by attachment of the edge of the cloth with masking tape to the cardboard sides.

For illumination and incubation under O_2, the carboy was fitted with a stopcock and an ascarite (asbestos coated with sodium hydroxide) CO_2 trap (see Figure 3), evacuated with a water pump to a pressure of approximately 80 mm Hg, and filled with O_2 from an oxygen tank. The illumination and incubation were carried out at either 4°C or 25°C, and at 25°C the ascarite trap was replenished every 2-3 hours because of greater CO_2 evolution. When the illumination and incubation were carried out under air, the stopcock and CO_2 trap were replaced by a cotton plug.

Samples of mycelium were removed one of two ways. Either an aliquot of mycelium plus medium was removed and filtered on gauze, or the entire mixture was removed and filtered, the mycelium weighed and part of the

mycelium and filtrate placed back in the carboy for further incubation.

Mycelium samples were washed with approximately 100 ml of H_2O at 4°C per 45 g fresh weight of mycelium. The mycelium was either extracted immediately or wrapped in aluminium foil, dipped in liquid nitrogen for 30 seconds, and stored in the freezer at -15°C.

In order to obtain the dry weight of a fresh mycelial sample, several small pieces of mycelium (each less than 0.2 g) were randomly removed and weighed, dried in vacuo over drierite for approximately 12 hours, and reweighed.

The simultaneous illumination and incubation of several samples of a 3 day old dark-grown mycelium was carried out as follows. After harvesting the mycelium from a carboy, 22.5 g fresh weight samples were placed in 1000 ml flasks plus 90 ml of Vogel's minimal medium (precooled to 4°C), and the flasks were attached to a Burrell Wrist Action Shaker. Two 250 watt white light bulbs were used to illuminate the contents of the flasks at 4°C with the shaker operating at full speed, and a fan was used to prevent heating of the incubation solutions by the bulbs. After 15 minutes, the lights were turned off and shaking continued for 14 hours at 4°C at 7/10 full speed. The arrangement of the lamps is

shown in Figure 4. At the end of this period, the mycelium from each flask was harvested, washed, and extracted or frozen as described on page 16. In some experiments, the flasks were wrapped in aluminium foil and incubated as just described, but without any illumination.

The illumination of Em 5297a mycelial pads, which were grown in the dark in flasks for 3 days, was carried out by a procedure similar to that of Zalokar (92-94). Three pads and the medium in which they were grown were transferred to a petri dish 14 cm in diameter. The pads were spread out so that illumination would be uniform over their upper surfaces.

A linear, 40 watt fluorescent lamp was used to illuminate the pads, and different light intensities were obtained by varying the distance of the lamp above the petri dishes. The light intensity in foot-candles approximately at the mycelial surface was measured with a light meter.

After illumination, the pads were incubated in the dark for the desired length of time. At the end of this period, the three pads from each dish were removed and squeezed with a spatula to remove excess medium. The combined pad was washed with a small volume of water, squeezed again, pressed between several layers of paper towels, and extracted.

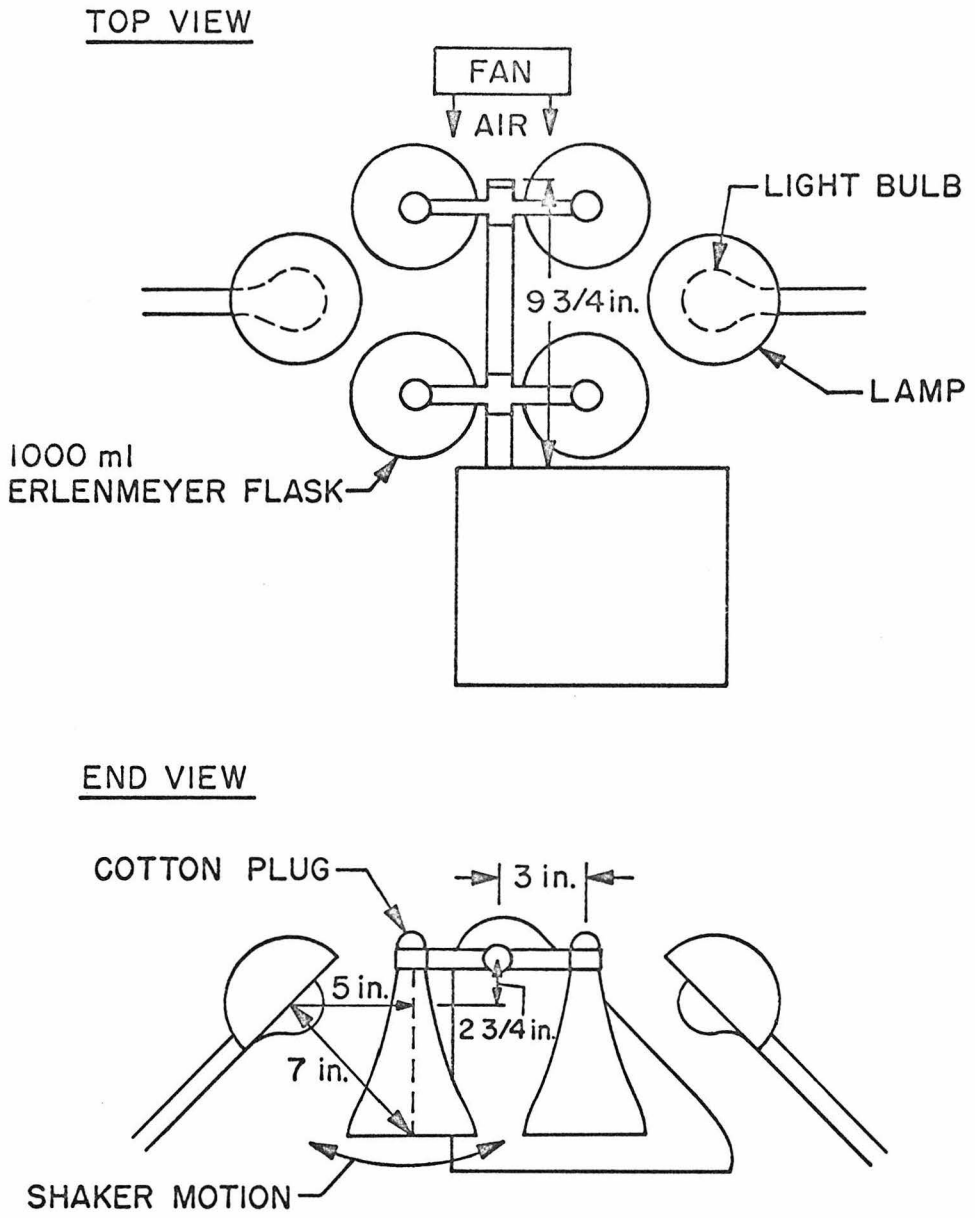

Fig. 4 Apparatus for the simultaneous illumination and incubation of 4 samples of mycelium. The approximate spatial relationship of the two light bulbs with respect to the incubation flasks is shown in the figure. The shaker can accommodate a total of 8 flasks, four as shown and four arranged in the same way on the opposite side of the shaker.

The albino cultures grown in the dark in 2500 ml culture flasks were harvested and illuminated as described for mycelium obtained from carboys.

D. INCUBATION AND ILLUMINATION OF MYCELIUM IN EXPERIMENTS USING RADIOACTIVE ISOTOPES

A carboy was inoculated, aerated for three days in the dark, and harvested as described on pages 11 and 12 In the labelling experiments with 1-C^{14}-β, β-dimethyl-acrylic acid, the total mycelium obtained from such a dark-grown culture was incubated in the dark at 25°C in a standing culture for one hour in 500 ml of water plus radioactive isotope. In early experiments with 2-C^{14}-mevalonate, a similar incubation in the dark was carried out for periods as long as 24 hours. In these experiments, 370 ml of water or Vogel's minimal medium per 135 g fresh weight of mycelium were used.

The procedure used in most cases for the dark incubation with label was to add 135 g fresh weight of mycelium obtained from a 3 day old carboy culture to 1200 ml of water plus the desired quantity of radioactive isotope. The incubation was carried out in a 2500 ml culture flask on a reciprocating shaker with a 2½ inch stroke at 67 strokes/minute for 24 hours in the dark.

At the end of this period, the incubation mixture was poured into the carboy with glass baffles (see Figure 3). A 50 ml solution consisting of 25 ml of water,

25 ml of Vogel's 50x concentrate (109) and 25 g of sucrose was also added to bring the final concentration of sucrose and salts to that of Vogel's minimal medium. The resulting slurry was illuminated using the apparatus already described and shown in Figure 2.

In other experiments, after the dark incubation period, the mycelium-water mixture was poured from the culture flask onto a double layer of cheese cloth. The mycelium was squeezed to remove excess liquid and washed with 300 ml of water (precooled to 4°C). The mycelium was again squeezed, and the washing was repeated with another 300 ml of water. The mycelium was placed in the carboy with double glass baffles plus a volume of medium equal to 9 times the fresh weight of the mycelium. The resulting slurry was illuminated as already described.

In some experiments it was desirable to synthesize a pool of unlabelled carotenoids before the incubation with radioactive isotope. At the end of 3 days of aeration in the dark, the mycelium was harvested and illuminated for the desired length of time as described on page 12. At the end of this illumination period, the mycelium-medium mixture was filtered on gauze, and the mycelium was washed, incubated with radioactive isotope in the dark, and illuminated a second time using the procedure already described in this section.

In labelling experiments with uniformly labelled C^{14}-leucine and 3-C^{14}-β-hydroxy-β-methyl glutaric acid, the mycelium obtained at the end of 3 days aeration in the dark was illuminated immediately in medium which contained the labelled compound.

In all labelling experiments ascarite was used to trap $C^{14}O_2$ emitted from the cultures.

E. SOLVENTS AND CHEMICALS

All chemicals and solvents were of analytical reagent grade, and for most experiments all solvents were redistilled. It was necessary in many cases to check the purity of the carotenoid pigments and colorless polyenes by both visible and uv absorption spectra. Petroleum ether, b.p. 60-71°C (predominently hexane), was found to be a suitable carotenoid solvent for the determination of visible absorption spectra, but it could not be used in the uv absorption region because it contains volatile uv absorbing impurities. Analytical reagent grade hexane also contains such impurities although to a much smaller extent than petroleum ether. For these reasons, most spectra determinations of carotenoid solutions were carried out by first removing the solvent by vacuum distillation at room temperature and redissolving the residue in spectroquality hexane (Fisher Scientific Company).

The solvents used in the chromatographic studies were dried over anhydrous $MgSO_4$ and redistilled from drierite (110).

F. EXTRACTION OF CAROTENOIDS

The extraction and purification procedures which are described in Materials and Methods-Sections F and G are summarized in the flow diagram of Figure 5. Extraction of the carotenoid pigments was carried out in a manner similar to that of Haxo (111) and Zalokar (92,94). Mycelium which had been frozen with liquid nitrogen and stored in the freezer was thawed in 4 parts methanol for 30 minutes at room temperature. The mycelium was broken apart with a spatula and the extraction continued for 15 minutes. With fresh mycelium a total extraction time of 15 minutes was used.

The resulting slurry was filtered with suction on a Büchner funnel through miracloth, the residue was washed with a small volume of methanol, and then pressed with a spatula to remove excess solvent. Using this same procedure, the residue was extracted once with 2 parts methanol for 15 minutes and 4 times with 2 parts acetone for approximately 5 minutes each time. The methanol and acetone extracts and the washes were combined for further treatment as described in Materials and Methods-Section G.

The mycelial pads which were illuminated and incubated in petri plates (see page 17) were extracted in a similar manner. The pad obtained from each petri plate was placed in 15 ml of methanol for 30 minutes, squeezed with a

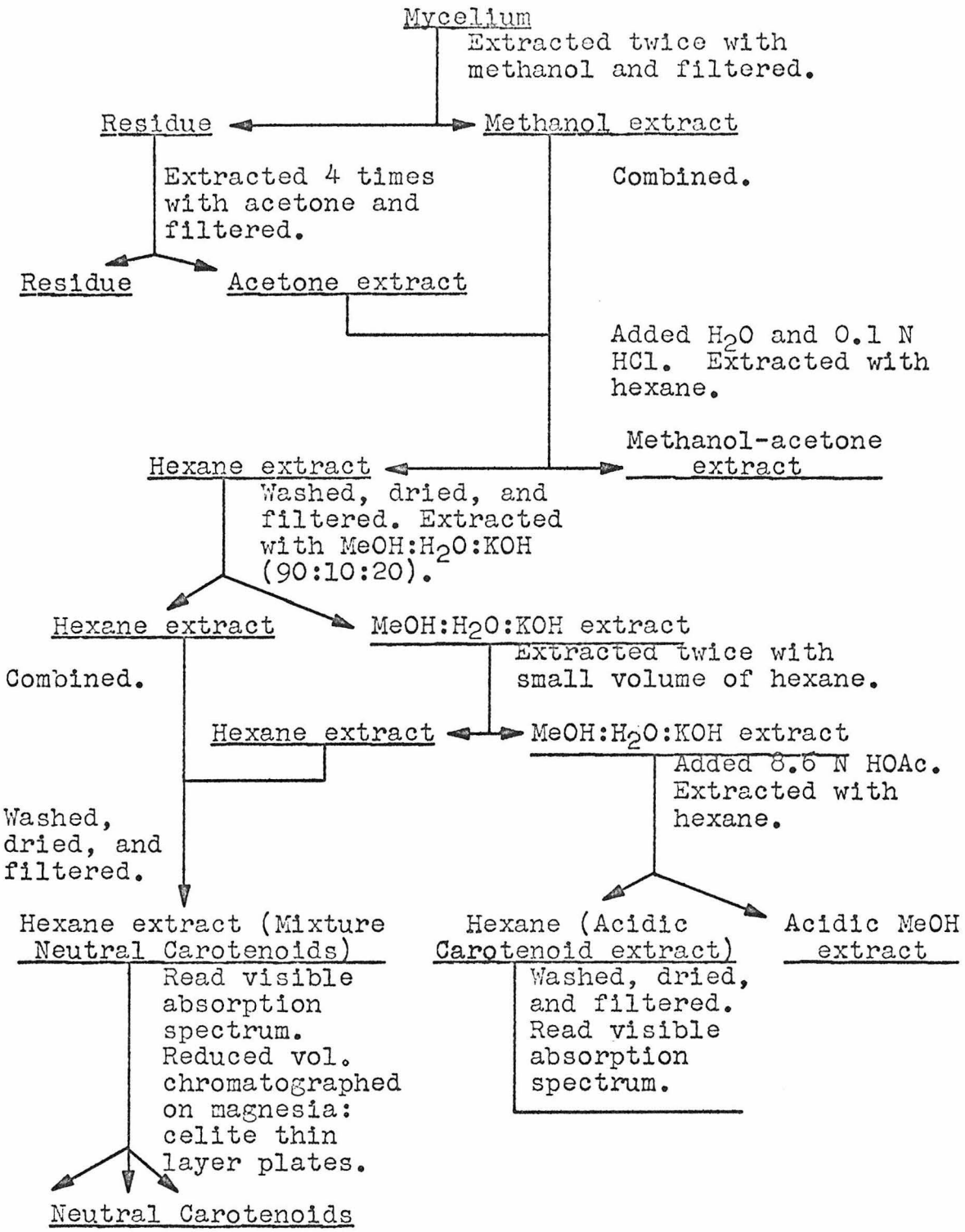

Fig.5 Flow diagram in which the extraction and purification procedures described in Materials and Methods-Sections F and G are summarized.

spatula, placed in acetone for two hours, and squeezed again. An estimate of the total quantity of carotenoid pigments originally present in the mycelium was obtained by measuring the spectrum of the methanol and acetone extracts with a Cary Recording Spectrophotometer, Model 11 M S or Model 15, in the range of 400-550 mμ .

G. IDENTIFICATION AND ABSOLUTE QUANTITIES OF THE CAROTENOID PIGMENTS

The procedure used to transfer the carotenoid pigments from a methanol-acetone extract to hexane is based on that of Haxo (111) and Zalokar (94,112). An equal volume of water plus 0.2 as much 0.1 N HCl were added to the extract. The pigments were then removed from the aqueous solution by repeated extraction in a separatory funnel with hexane or in some experiments with petroleum ether.

The hexane extract was washed 3 times, each time with an equal volume of water, dried over anhydrous sodium sulfate, and filtered through Whatman No. 1 filter paper. The filter paper was washed with a small volume of ethanol to remove any adsorbed pigment. The filtrate was evaporated to dryness by vacuum distillation with a flash evaporator at room temperature and the residue dissolved in hexane.

The acidic carotenoid pigment, which was first described in Neurospora by Haxo (111), partially purified by Zalokar (112), and identified by Aasen et al. (113), was separated from the neutral pigments by extracting the hexane extract with 3 equal portions of methanol: water:KOH (90:10:20). The basic methanol solution was extracted with two 20 ml portions of hexane, which were combined with the crude neutral carotenoid extract. The combined hexane solution was washed with water, dried over anhydrous Na_2SO_4, and filtered. In order to estimate the total quantity of pigment present in the mixture, the spectrum of an aliquot of the filtrate was recorded in the spectral range of 375-575 $m\mu$.

The method used to purify partially the acidic pigment is based on that of Zalokar (112). The basic methanol solution which was used to separate the acidic pigment from the neutral carotenoids was acidified by the addition of an equal volume of 8.6 N acetic acid. The resulting solution was extracted in a separatory funnel with four 50 ml portions of hexane. The combined hexane solution was washed with 3 equal portions of water, dried over anhydrous sodium sulfate, and filtered on Whatman No. 1 filter paper. An aliquot of the filtrate was removed, evaporated to dryness with a flash evaporator, and the residue redissolved in spectroquality hexane.

The visible spectrum of this solution was recorded in the range 375-575 mμ, and the OD at 477 mμ was used to estimate the quantity of acidic pigment present.

The mixture of neutral carotenoids was analyzed by thin layer chromatography, a technique which has been used with many types of compounds and in recent years with carotenoids (114,115). The thin layer plates used in this study were prepared by shaking together 50 g of Seasorb Magnesia (Fisher Scientific Company) and 50 g of celite plus binder (Research Specialties). To this mixture were added 100 ml of water. The solution was stirred rapidly for 2 minutes with a spatula and poured into an applicator specifically designed for preparing thin layer plates and commercially available from DESAGA (U.S. representative: C. A. Brinkmann).

The applicator was moved horizontally at a steady rate and the slurry allowed to flow onto clean glass plates with the dimensions 10 X 20 cm or 20 X 20 cm (116). The initial thin layer thickness could be varied by changing a setting on the applicator, and in all of these studies where the primary purpose was preparative, a thickness of 0.4 mm was used. For analytical work, 0.25 mm thick layers are recommended (116). As the thin layer dries, it will contract. For example, a 0.275 mm thick Silica Gel G layer will contract to approximately 0.150 mm upon drying (116).

The plates were allowed to air dry for 24 hours and stored over drierite in a lucite container. When ready for use, each plate was placed in an oven at 115°C for one hour, removed, and allowed to cool to room temperature. A mark was made in the thin layer with a needle at a distance of 1.5 cm from the bottom edge of the plate and 2.0 cm from the side. This was repeated at the corresponding position on the other side of the plate and at intervals of 2-3 cm between the two marks.

The solution of carotenoids to be analyzed was reduced to dryness with a flash evaporator and the residue redissolved in 5-10 ml of hexane. Using a syringe with a No. 20 needle and working under dim red light in order to minimize photo-oxidation of the pigments (116), a 0.5 to 1.0 ml aliquot was applied as a narrow streak to the adsorbent. This was accomplished by holding the needle just above the plate and moving it along the imaginary line formed by the needle marks while forcing the solution out of the syringe one drop at a time. The solvent evaporated rapidly so that it was possible to go back over the streak several times and add more solution. Although special equipment is available from DESAGA (U.S. representative: C. A. Brinkmann) for the application of sample to plates (116), and there have been reports in the literature of specially constructed

equipment for this purpose (117-120), it was found that with practice, the simplified method described above was suitable for these experiments.

Still working under red light, the thin layer plate was placed in a glass chromatographic jar which contained 100 ml of solvent. Several different solvent systems were used for development of the plates, and these included hexane, 2, 5, 10, 20, and 33% acetone in hexane, and 2, 5, and 10% ethanol in hexane. A glass cover was placed on the container, which had been coated lightly along its top edge with silicone grease, the entire chamber was covered with a black cloth, and ascending chromatography allowed to take place for 30 minutes (see Figure 6).

Under red light, the plate was removed from the chromatographic container. Since oxidation of the pigments is minimized by keeping the adsorbent wet (116), the plate was quickly covered with Saran Wrap in order to prevent evaporation of the solvent. The room lights were turned on, and using a narrow spatula, lines were lightly drawn across the plate in the adsorbent at the front and back of each carotenoid band. The Saran Wrap was removed under red light, and the powder in each zone was quickly scraped off the plates and put into a 15 ml clinical centrifuge tube which contained 10 ml of 10% ethanol in hexane.

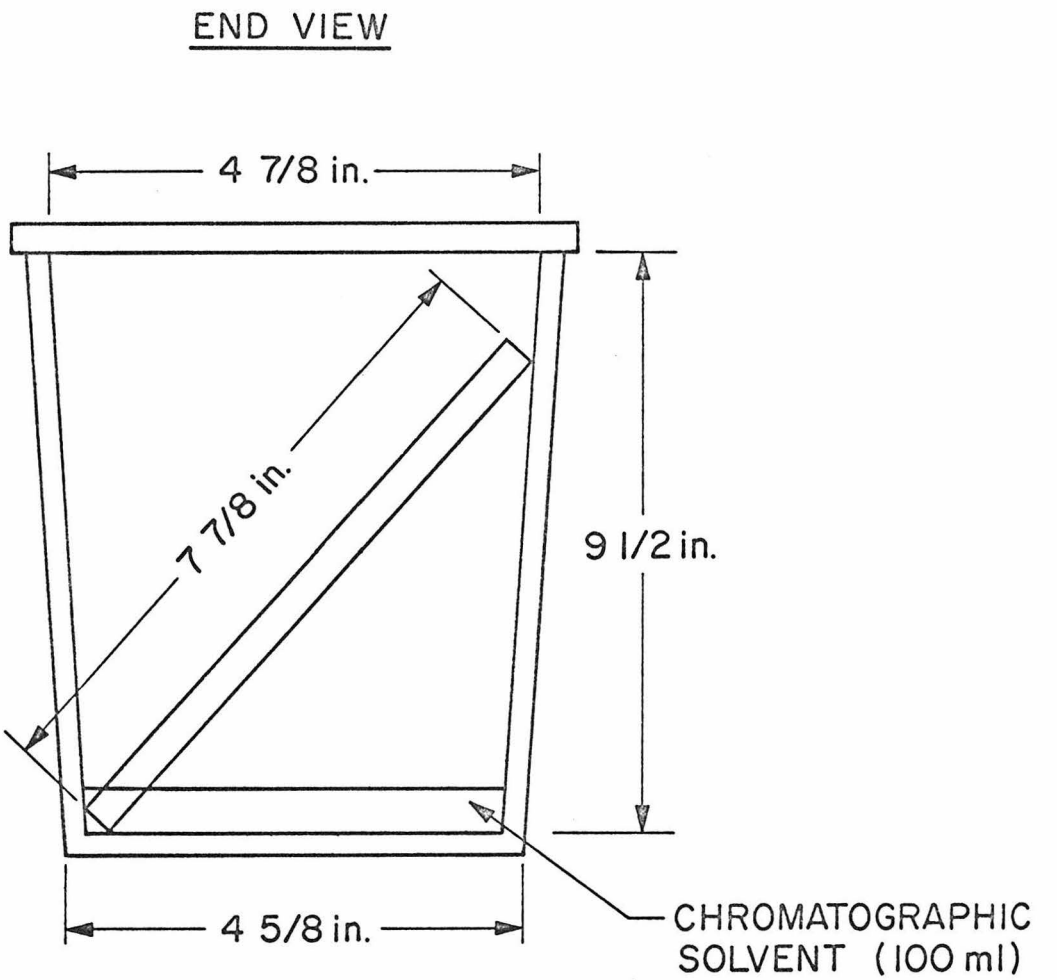

Fig. 6 Arrangement of a thin layer plate in a chromatographic jar during ascending chromatography. The jar is 9 inches long. Its other dimensions are shown in the figure.

The ethanolic hexane solution of each carotenoid fraction was stirred, centrifuged at full speed for one minute in a clinical centrifuge, and the supernatant decanted. The residue was washed 3 to 4 times, each time with 10 ml of 10% ethanol in hexane, and all supernatants were combined. The combined solution was dried over a small amount of anhydrous sodium sulfate and filtered on Whatman No. 1 filter paper. Each fraction was evaporated to dryness on a flash evaporator and the residue redissolved in spectroquality hexane. The visible spectrum of each of the resulting solutions was determined as already described. This procedure could be used to determine the absolute amount of each carotenoid since further purification steps (Materials and Methods-Section H) did not reveal the presence of any contaminants which absorbed in the visible region.

H. PURIFICATION OF RADIOACTIVE CAROTENOIDS

Several procedures were found to be successful in the purification of the labelled carotenoid pigments to a constant specific activity (disintegrations per minute per weight carotenoid), but one which could be used to purify all of the carotenoid pigments and phytoene was the following (see Figure 7 for a flow diagram). As described on page 22, the carotenoids were extracted, transferred to hexane, and the acidic carotenoid separated

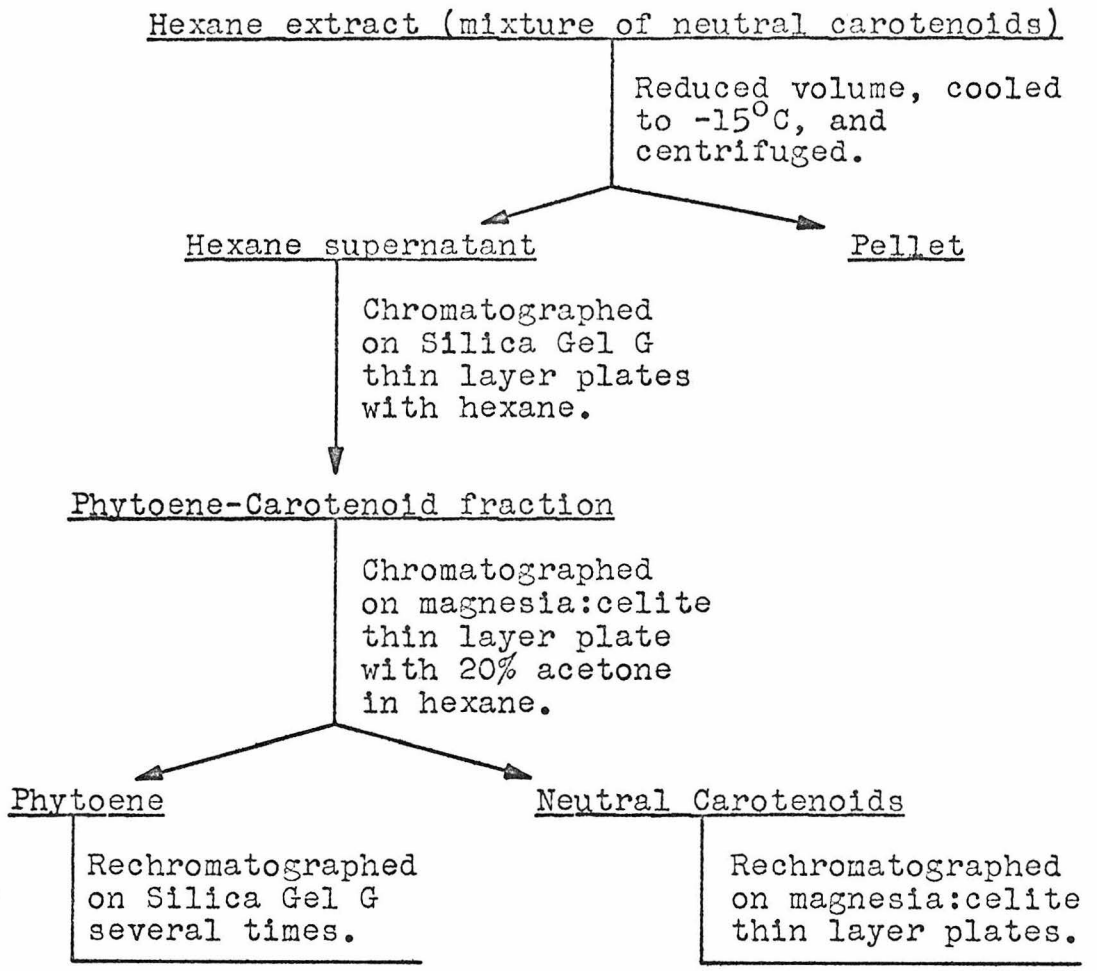

Fig. 7 Flow diagram in which the purification of the carotenoids and phytoene described by the procedure on pages 30-34 and pages 37-39 is summarized.

from the neutral ones. The hexane solution of neutral carotenoids was evaporated to dryness on a flash evaporator, and the residue was redissolved in 4 ml of spectroquality hexane. Usually a precipitate was observed at this stage, and further precipitation was allowed to take place by cooling the solution in a freezer at -15°C. The solution and precipitate were transferred by means of a Pasteur pipette to a clinical centrifuge tube. The residual pigment and precipitate were washed from the round bottom flask used in the flash evaporation, and the wash was pipetted to another centrifuge tube. The tubes were spun at full speed for one minute in a clinical centrifuge, and the supernatants decanted. The pellets were washed twice, each time with one ml of spectroquality hexane which had been precooled in the freezer, and the washes were combined with the first supernatants.

It was usually possible to precipitate some more material from the resulting solution by reducing it to a volume of one to two ml and repeating the above procedure once or twice. It was shown that the precipitation procedure just described removed a significant amount of radioactive ergosterol as compared with the loss of carotenoid pigments. This agrees with the observation that most sterols will precipitate under these conditions when they are present in large amounts (114).

The carotenoid mixture was further purified by thin layer chromatography on plates coated with Silica Gel G. This adsorbent has been used in the purification of a wide variety of compounds including colorless 40-carbon polyenes and carotenoid pigments (25, 114). The mixture to be chromatographed was reduced to dryness with a flash evaporator, and the residue was redissolved in 2 ml of spectroquality hexane. Using the procedure described on page 27, the solution was applied as a streak to the adsorbent on each of two 20 X 20 cm thin layer plates, one ml per plate, and ascending chromatography was carried out for 30 minutes using hexane as the developing solvent.

Most of the applied pigment moved up each plate as a wide band leaving a large quantity of radioactive impurities at the origin. The pigment band was scraped off each plate and elution from the adsorbent carried out as described on page 28. The eluates from the two bands were combined, evaporated to dryness with a flash evaporator and redissolved in 20 ml of spectroquality hexane. An aliquot of this solution was removed for spectral and radioactivity determinations.

The remainder of the carotenoid mixture was evaporated to dryness and the residue redissolved in one ml of spectroquality hexane. This was applied as a streak to

a 20 X 20 cm magnesia:celite thin layer plate and ascending chromatography carried out for 30 minutes with 20% acetone in hexane. The individual carotenoid bands obtained in this way were scraped off the plate and the carotenoid pigments eluted from the adsorbent with 10% ethanol in hexane as described on page 28. Each fraction was reduced to dryness and each residue redissolved in 10 ml of spectroquality hexane. An aliquot of each fraction was removed for spectral and radioactivity determinations.

Each carotenoid was rechromatographed several times on magnesia:celite thin layer plates to a constant specific activity using the procedures and solvent systems described on page 28.

When it was of interest to purify only the carotenoid pigments lycopene and 3,4-dehydrolycopene, chromatography with Silica Gel G could be omitted from the procedure just described.

Other purification techniques, which gave useful information, were saponification of carotenoid solutions and precipitation of sterols with digitonin (see Figure 8 for a flow diagram).

Saponification was carried out by a procedure similar to that of Davies (114). The hexane solution to be saponified was evaporated to dryness on a flash evaporator

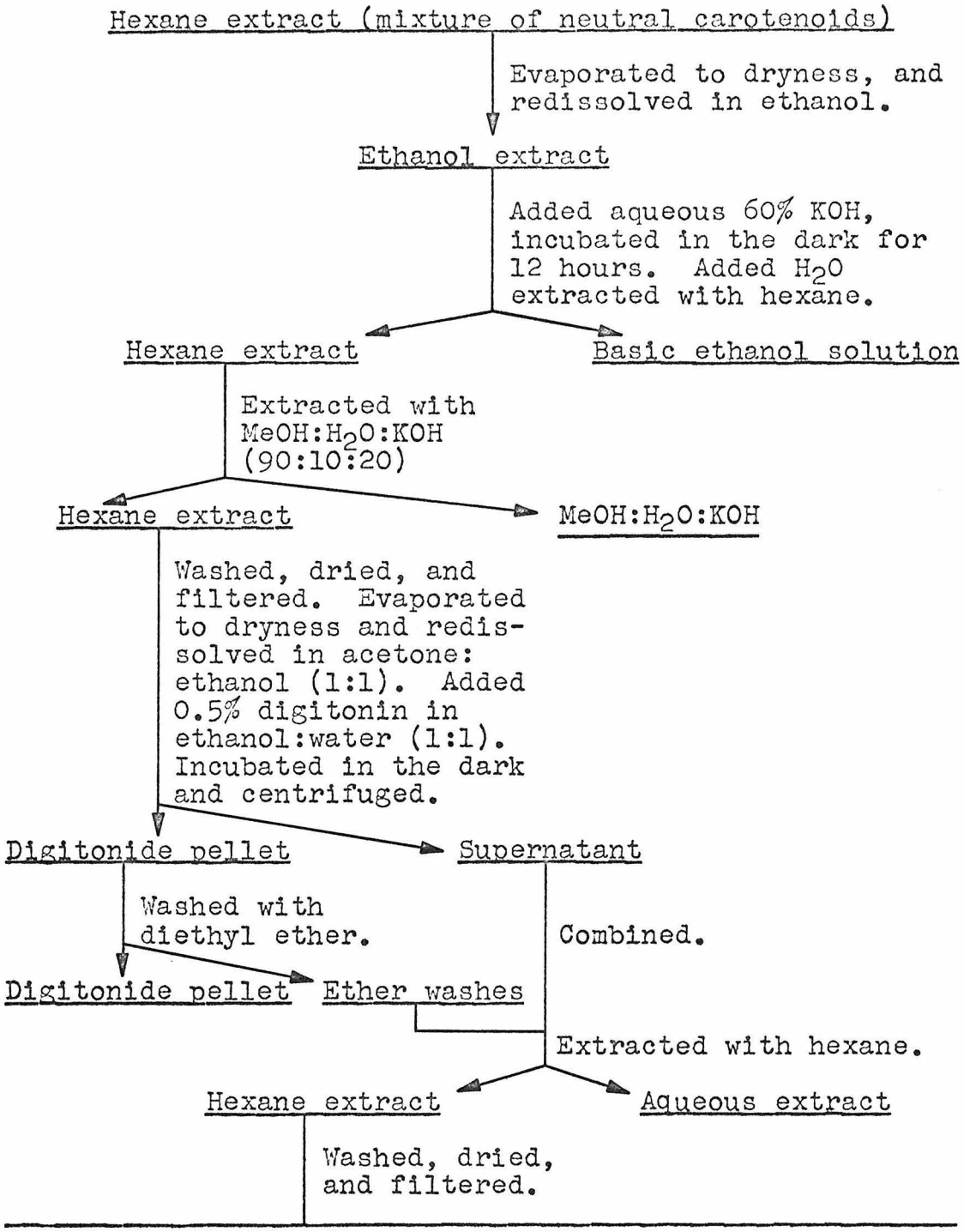

Fig. 8 Flow diagram in which the saponification and digitonin precipitation procedures described on pages 34-37 are summarized.

and redissolved in ethanol. A volume of 60 ml of solvent was used for an extract of 135 g fresh weight of mycelium. To the ethanol were added 6 ml of aqueous 60% KOH solution, and the mixture was incubated in the dark under N_2 at room temperature for 12 hours. The incubation solution was diluted with 3 volumes of water and extracted with four 150 ml portions of hexane. Small portions of ethanol were added to the mixture as needed to break up emulsions. The combined hexane extract was extracted with three 150 ml portions of methanol:water:KOH (90:10:20), washed with 3 equal portions of water, dried with Na_2SO_4, and filtered on Whatman No. 1 filter paper.

Digitonin, a compound which has been shown to precipitate 3β-hydroxy sterols as their digitonides (121), was used in some purification studies to remove most of the ergosterol from crude extracts. The procedure used was similar to that of Rinehart *et al.* (122). The hexane extract was evaporated to dryness with a flash evaporator, and the residue was redissolved in acetone: ethanol (1:1) using 60 ml of solvent for an extract of 135 g fresh weight of mycelium. The resulting solution was distributed to 12 clinical centrifuge tubes, 5 ml per tube, and to each was added 1 ml of a 0.5% digitonin solution in ethanol:water (1:1). The resulting solutions were stored in the dark at room temperature

for one hour and centrifuged at full speed for one minute in a clinical centrifuge.

Without decanting, an additional 5 ml of acetone: ethanol and 1 ml of 0.5% digitonin solution were added to each supernatant, and the tubes were stored in the dark at room temperature for one hour. Centrifugation was carried out as before, the supernatants decanted, and each pellet washed 3 times with 0.5 ml of diethyl ether, precooled to 4°C. The washes were combined with the original supernatants, and the combined solution was extracted 3 times with hexane. The hexane extracts were combined, washed with 3 equal portions of water, dried over Na_2SO_4, and filtered on Whatman No. 1 filter paper.

I. PURIFICATION OF RADIOACTIVE PHYTOENE

The initial steps in the purification of phytoene were carried out as described for the radioactive carotenoid pigments (see Materials and Methods-Section H and the flow diagram in Figure 7). After chromatography on magnesia:celite thin layer plates, the adsorbent between zeta-carotene and the solvent front was scraped off the plate and the phytoene eluted with 10% ethanol in hexane. The eluate was evaporated to dryness with a flash evaporator, and the residue was redissolved in 0.5 ml of spectroquality hexane.

This solution was applied to a 20 X 20 cm Silica Gel G thin layer plate and ascending chromatography carried out in the dark for 30 minutes with spectroquality hexane. Under red light, the plate was removed from the chromatograhic chamber, covered with Saran Wrap, and held under a mercury vapor long wavelength uv lamp. A narrow fluorescent band, which was shown to be phytofluene, was observed and its location was marked lightly in the adsorbent with a spatula. The Saran Wrap was removed, the desired fractions scraped off the plate, and each was eluted with 10% ethanol in spectroquality hexane. The fluorescent phytofluene acts as a marker, since phytoene moves just slightly ahead of it. Thus, the usual procedure was to scrape off fractions, each about 0.4 cm wide, starting at the back of the fluorescent band to a distance of 2-3 cm in front of it. A 0.4 cm wide band ahead of the solvent front was also removed and treated in the same way as the other fractions for use in background determinations.

The eluate of each fraction was evaporated to dryness with a flash evaporator, each residue redissolved in 10 ml of spectroquality hexane, and a 2 ml aliquot was removed from each of the resulting solutions for radioactivity determinations. The uv spectrum of each fraction was recorded in the range 240-375 mμ.

Using the same procedure, the phytoene fraction was rechromatographed on a 10 X 20 cm Silica Gel G plate once or twice to a constant specific activity. The purity of the phytoene was checked further in some experiments by rechromatographing on Silica Gel G an additional time with the solvent system carbon tetrachloride:hexane (40:60, both spectroquality solvents). No change in the specific activity of the phytoene was observed.

Another technique which was used in some experiments in the purification of phytoene was column chromatography with alumina. A similar procedure has been used by other investigators (25).

One hundred grams of neutral alumina, activity grade I, 100-200 mesh (available from Bio-Rad Laboratories) was mixed with 150 ml of redistilled analytical reagent grade hexane. The mixture was stirred into a slurry and quickly poured into a column which had an inside diameter of 2.5 cm. After the alumina had settled, the length of the column was 53 cm. A glass wool plug at the base of the column supported the adsorbent, and another at the top prevented disruption of the alumina by solvent.

The column was washed with 200 ml of hexane, and the solution to be chromatographed was applied to the adsorbent in a small volume of hexane with a Pasteur

pipette. Increasing concentrations of diethyl ether in hexane were used for elution of compounds off the column. It was found that 40% diethyl ether in hexane was required to elute phytoene in a reasonable length of time.

J. RADIOACTIVE ISOTOPES AND COUNTING PROCEDURES

Most of the radioactive isotopes used in these studies were obtained from New England Nuclear Corporation. These included uniformly labelled C^{14}-leucine (240 millicuries per millimole), 3-C^{14}-β-hydroxy-β-methyl glutaric acid (2.37 millicuries per millimole), and 2-C^{14}-mevalonate (N,N-dibenzylethylenediamine salt; 3.28-3.78 millicuries per millimole).

The radioactive compound 1-C^{14}-β, β-dimethyl-acrylic acid (1.42 millicuries per millimore) was obtained from Tracerlab Inc.

Radioactive isotopes were counted in a Nuclear Chicago 720 Series liquid scintillation counter by the channels ratio method (123, 124). Aliquots of solutions to be counted were added to liquid scintillation vials, each of which contained 10 ml of scintillation solvent. Two types of scintillation solvents were used (see Table 1 for compositions). These were Bray's solvent (125), which was used for counting radioactive isotopes dissolved in aqueous solutions, and toluene plus

TABLE 1

Composition of Liquid Scintillation Solvents

Bray's Scintillation Solvent

Compound	Quantity
Naphthalene	60 g per liter of scintillation solvent
2,5-diphenyloxazole (PPO)	4
p-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP)	0.2
Methanol	100 ml per liter of scintillation solvent
Ethylene glycol	20
p-dioxane	Add to make a total volume of 1000 ml of scintillation solvent

Toluene Plus Fluors

Compound	Quantity
Liquifluor* 25 x concentrate (Nuclear Chicago Corp.)	42 ml
Toluene	1000 ml

* 50 g PPO+0.625 g POPOP in 500 ml of toluene.

liquifluor (available from Nuclear Chicago Corporation), which was used for radioactive isotopes dissolved in nonpolar solutions. The quantity of radioactive aliquot added to each vial was generally 0.5 or 1.0 ml.

In order to count the total label extracted by methanol and acetone (see Materials and Methods-Section F), one ml aliquots of the combined methanol-acetone extract were added to empty scintillation vials. Nitrogen gas, emitted through a Pasteur pipette was used to evaporate the organic solvents from each aliquot, before the addition of 10 ml of toluene plus fluors. Whenever necessary, a minimum volume of methanol was added to each vial to make the solutions homogeneous before counting.

The digitonide pellet, formed by the addition of digitonin to the crude carotenoid extract (see page 36), was dissolved in a few ml of methanol and aliquots of this solution were added to vials of toluene plus fluors and counted. The combined supernatant and washes of the digitonin precipitation was counted by adding aliquots to vials containing Bray's solution.

As mentioned on page 21, ascarite was used to trap the CO_2 given off from cultures incubated with labelled compounds. In some experiments, the quantity of emitted $C^{14}O_2$ was measured. The ascarite was extracted with

water, and a procedure similar to that of Snyder and Godfrey (126) was used to determine the quantity of label in the aqueous solution. For this purpose, a 1000 ml Warburg Flask (see Figure 9) was constructed. Five ml of the labelled solution were placed in the bottom of the flask, 8 ml of 9 N H_2SO_4 were added to the side arm, and a scintillation vial which contained 2 ml of hyamine hydroxide (available from Nuclear Chicago Corporation) was placed in the center well. The flask was stoppered, the acid cautiously mixed with the basic aqueous solution, and the flask incubated at 25°C for 8 hours. At the end of this time, 10 ml of toluene plus fluors were added to the scintillation vial and the resulting solution counted.

In order to determine the counting efficiency of each radioactive sample by the channels ratio method, a calibration curve for carbon-14 was determined (see Figure 10). A series of scintillation vials containing samples of known activity and quenched to varying amounts is available from Nuclear Chicago Corporation. These were counted and the channels ratio of each determined (123, 124). A plot was made of the per cent efficiency $\left[\frac{(\text{counts per minutes})\ (100)}{(\text{disintegrations per minute})}\right]$ versus R, the channels ratio.

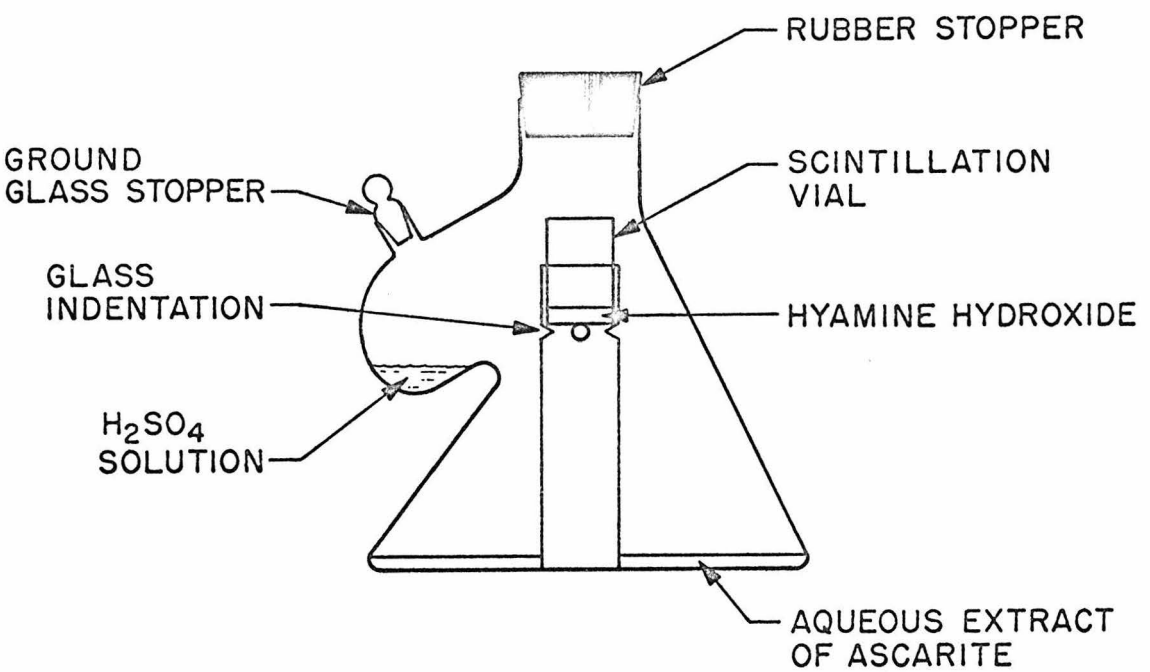

Fig. 9 Warburg flask for measuring the total $C^{14}O_2$ trapped by ascarite. The apparatus shown in the figure was prepared from a 1000 ml Erlenmeyer flask. A glass tube was fused to the bottom of the flask, and each scintillation vial was supported within it by 3 indentations.

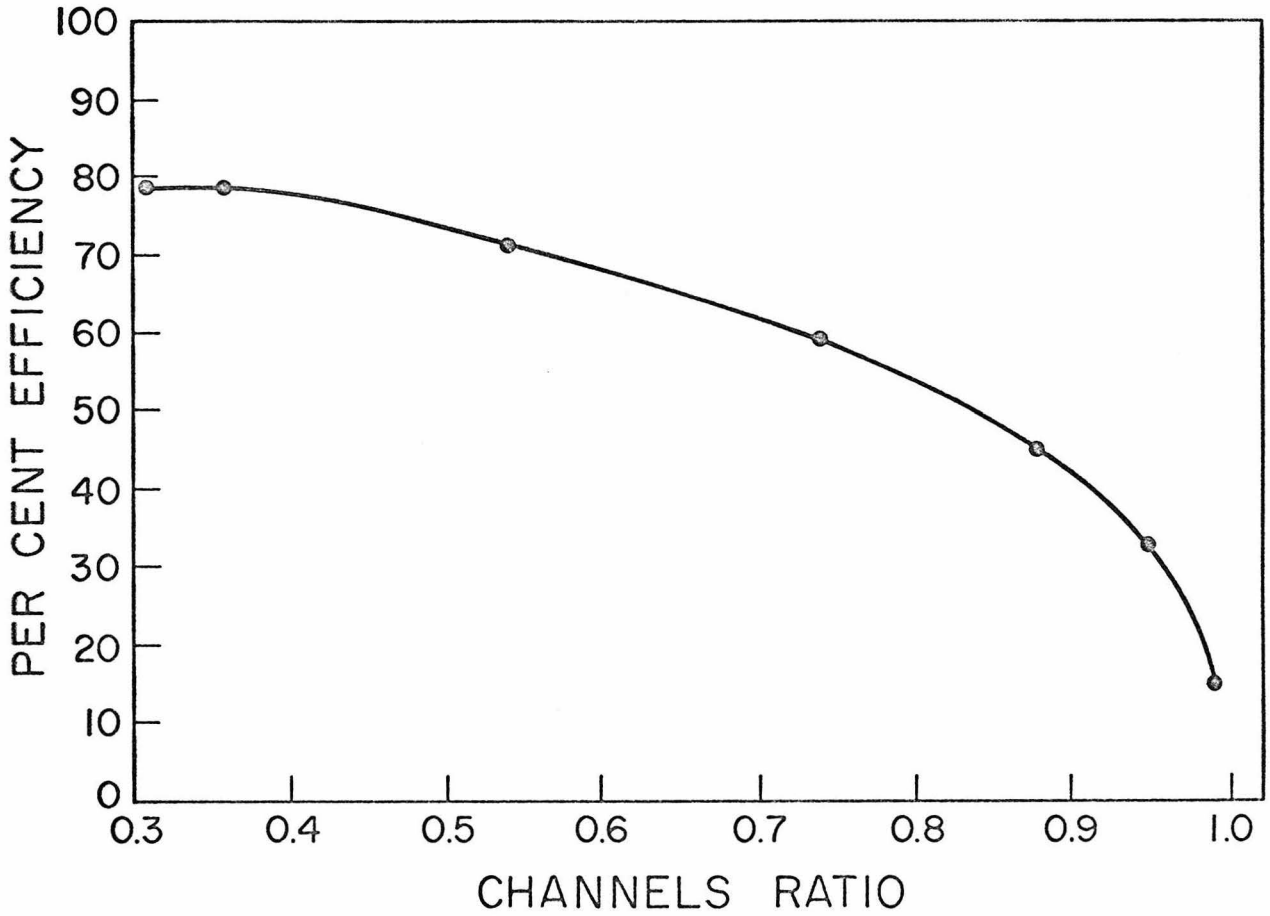

Fig. 10 Correction for quenching by the channels ratio method. Standard samples which contain a known amount of radioactivity were counted, and the channels ratio of each was determined. The calibration curve which is shown was prepared by plotting per cent counting efficiency versus this ratio.

For a sample of unknown efficiency, R can be measured and the corresponding efficiency read off the calibration curve.

The counting efficiency of samples was also determined by internal standarization. Standard C^{14}-benzoic acid (8260 ± 5% dpm/mg, New England Nuclear) was dissolved in toluene to a concentration of $\frac{826 \text{ dpm}}{10\lambda}$. After samples were counted, 10λ of this solution were added to each vial and the samples recounted. The per cent efficiency was calculated as $\left[\frac{(\text{increase in cpm})\ (100)}{826}\right]$. The two methods of determining efficiency gave comparable values except for highly quenched samples (less than 30% efficiency) in which the channels ratio method gave values that were too high.

Determination of the background to be subtracted from measured activities was obtained by counting vials which contained only scintillation solvent. This will involve some error because the background in vials containing sample are not quenched to exactly the same extent as in vials without sample (124, 127); however, except for highly quenched samples of low activity, such an effect can be neglected.

RESULTS

A. THE CAROTENOID BIOSYNTHETIC PATHWAY

1. Carotenoid Pigments and Colorless Polyenes of *Neurospora crassa*

Examples of the absorption spectra of the carotenoid pigments and 40-carbon colorless polyenes of *Neurospora crassa* are presented in Figures 11-20. These spectra were obtained by the purification procedures summarized in Figures 5 and 7, and in each case the wavelengths of the absorption maxima are shown to agree closely with published values. References to published spectral curves are also presented, and these spectra closely coincide with those shown in the figures. On this basis, it is concluded that the carotenoids and colorless 40-carbon polyenes are spectroscopically pure in the wavelength range of each spectrum. This criterion of purity is satisfactory for studies in which the absolute quantity of each of these compounds is determined; however, for radiochemical work, additional criteria are necessary as will be shown later in the Results section (see page 86).

Beta-zeacarotene is the only one of these 10 compounds which has not previously been identified and shown to be present in *Neurospora*. In an earlier study (96), Haxo found a carotenoid which had absorption maxima

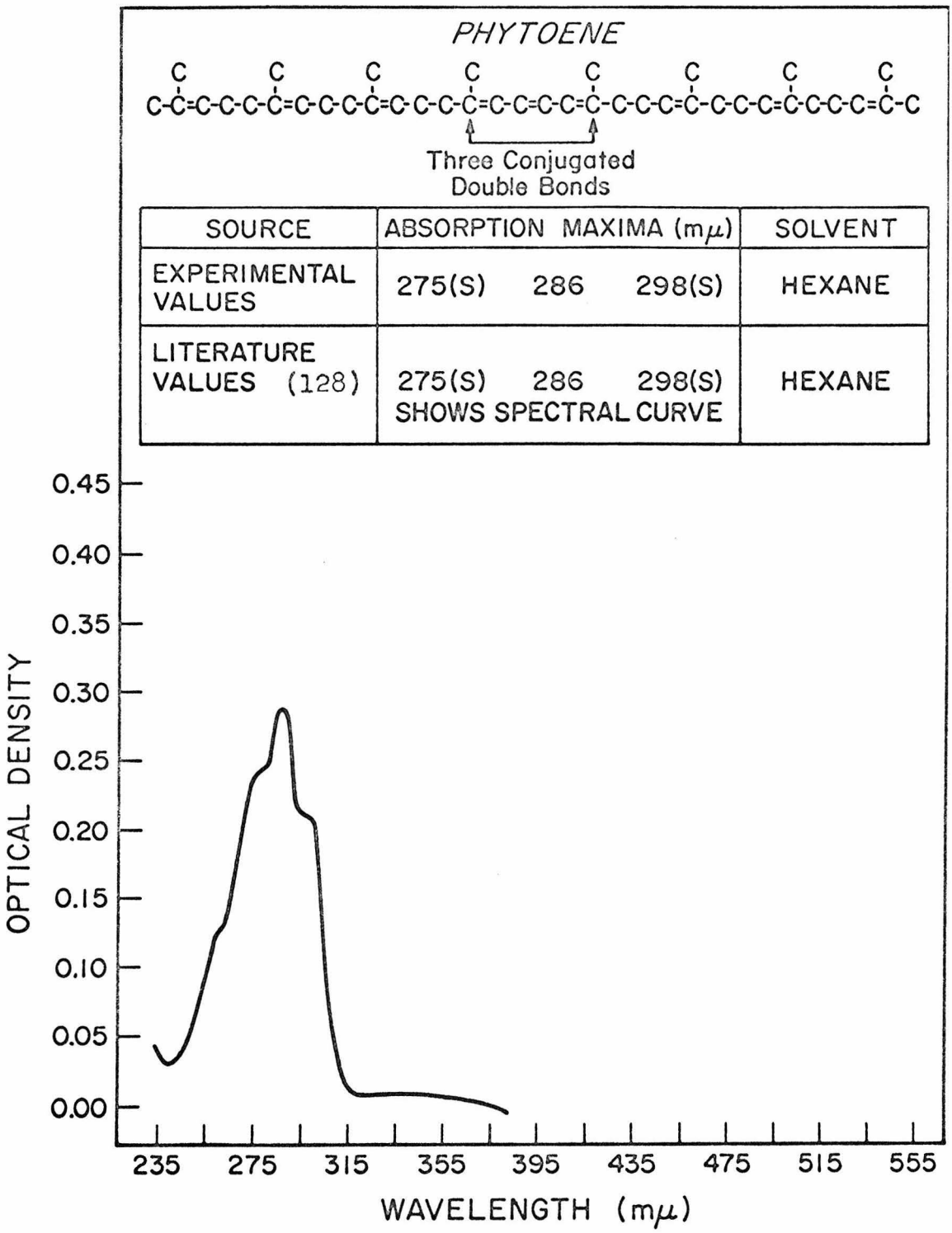

Fig. 11 The structure and ultraviolet absorption spectrum of phytoene. The spectrum was obtained by use of the purification procedure summarized in Figure 7. Concentration--2.2 μg/ml.

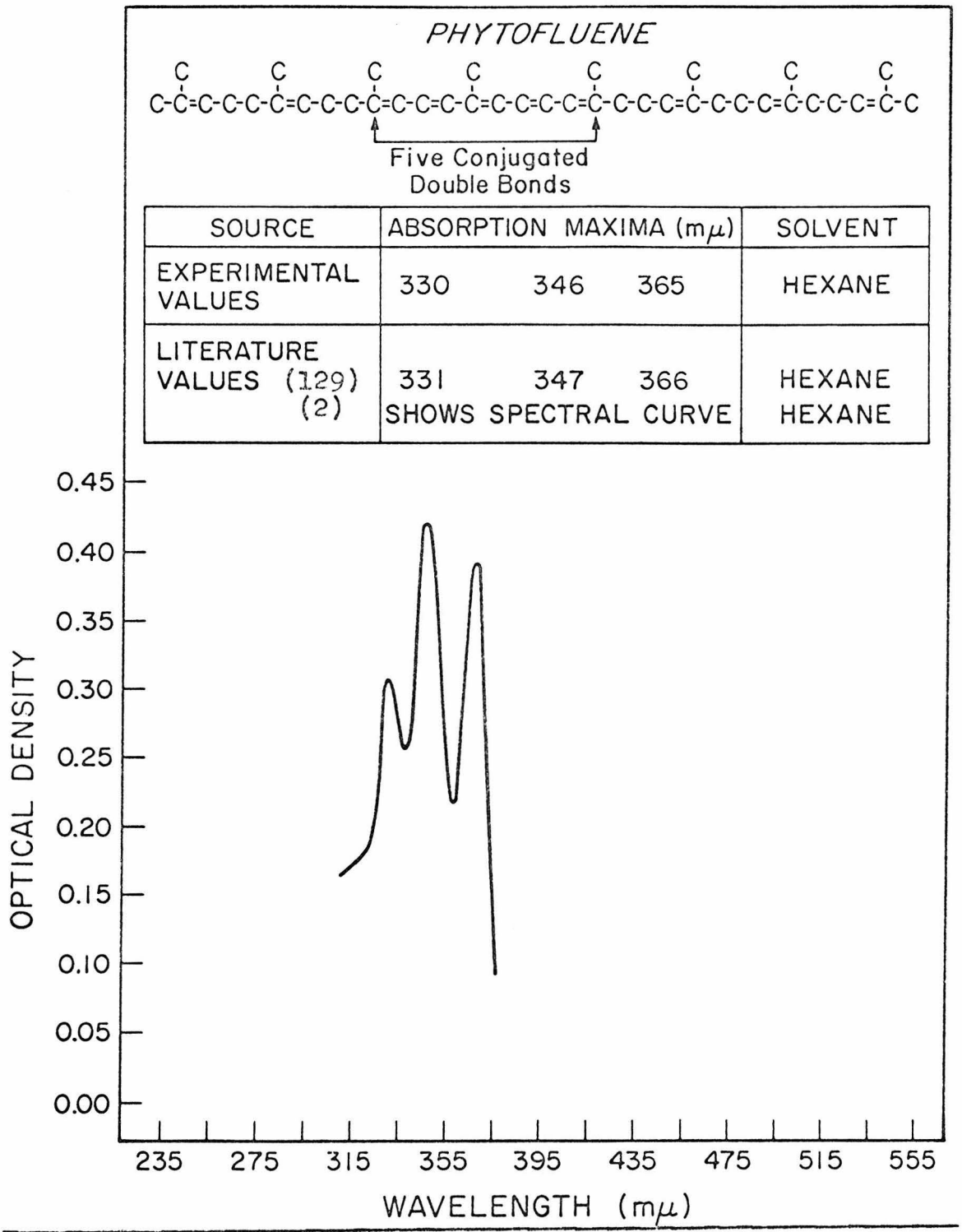

Fig. 12 The structure and ultraviolet absorption spectrum of phytofluene. The spectrum was obtained by alumina column chromatography (see p. 39). Concentration--3.0 μ g/ml.

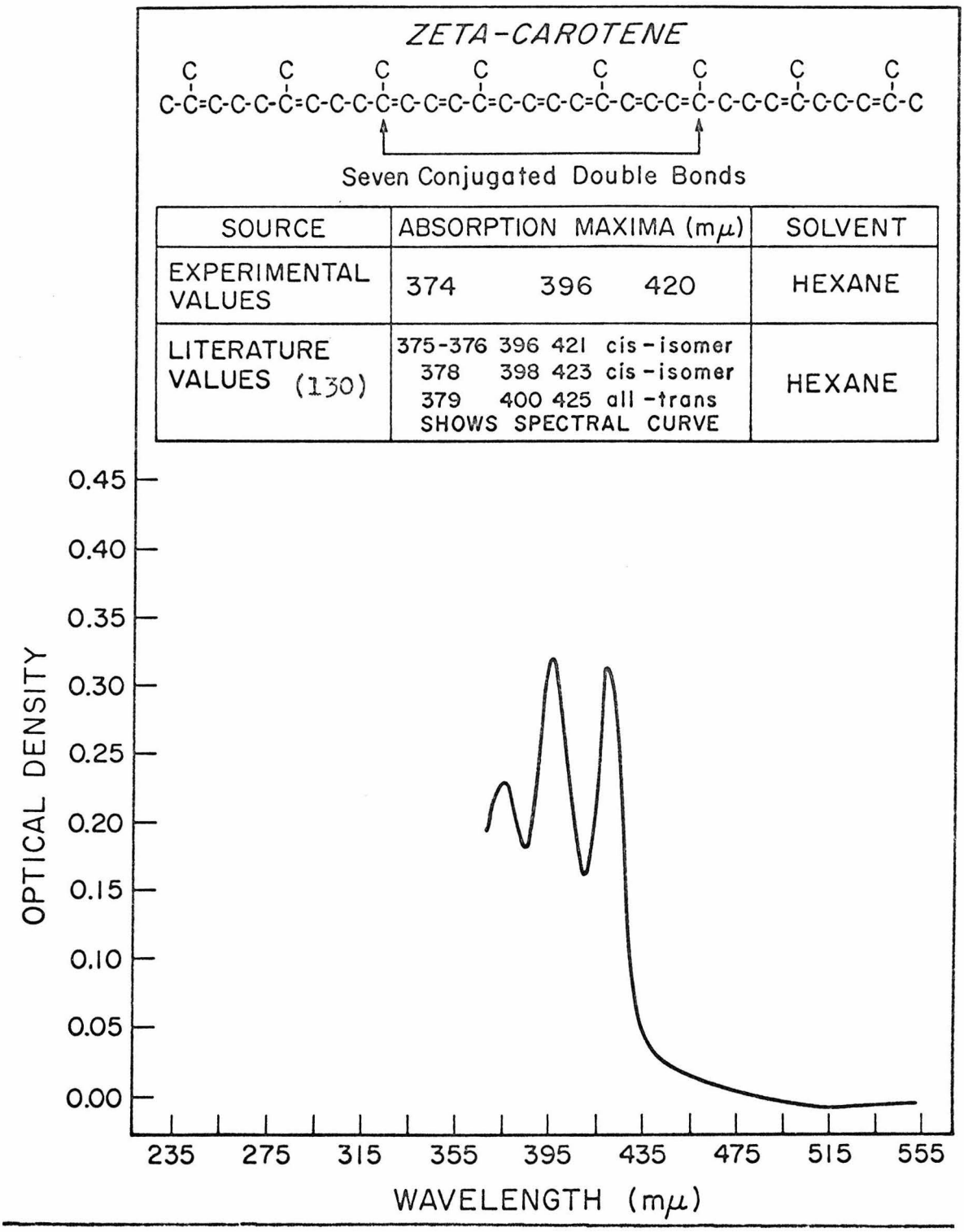

Fig. 13 The structure and visible absorption spectrum of zeta-carotene. The spectrum was obtained by use of the purification procedure summarized in Figure 5. Concentration--1.4 μg/ml.

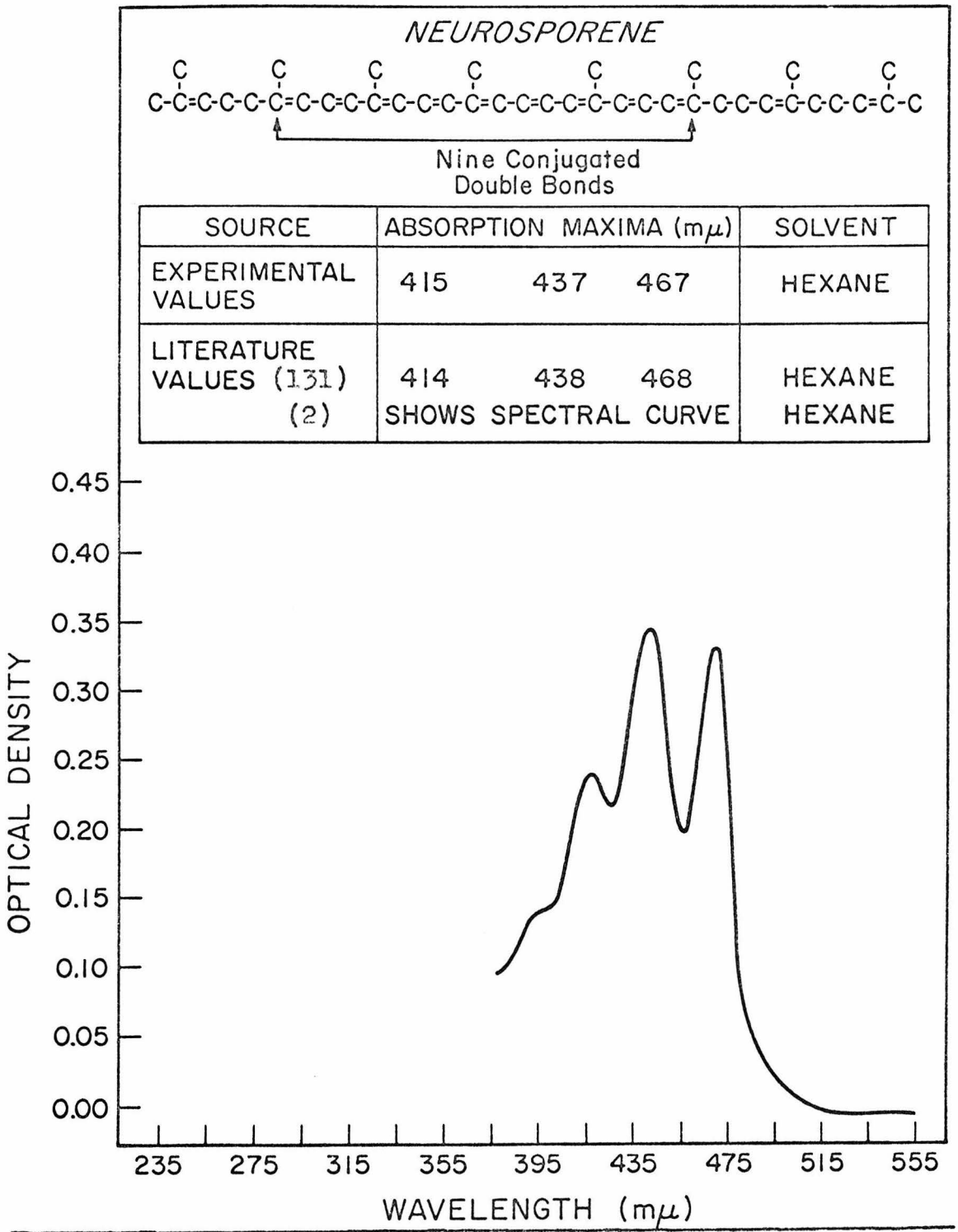

Fig. 14 The structure and visible absorption spectrum of neurosporene. The spectrum was obtained by use of the purification procedure summarized in Figure 5. Concentration--1.2μ g/ml.

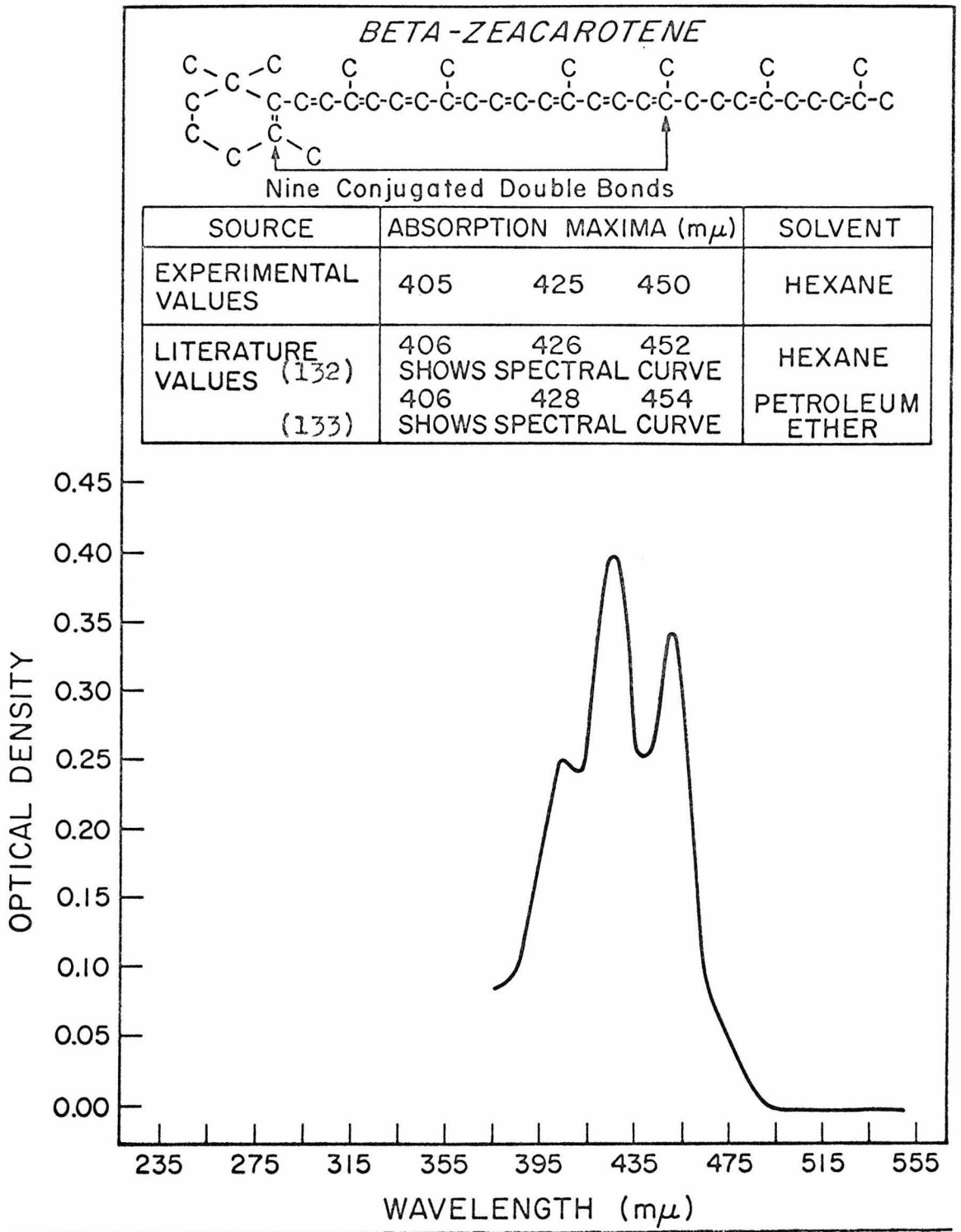

Fig. 15 The structure and visible absorption spectrum of beta-zeacarotene. The spectrum was obtained by use of the purification procedure summarized in Figure 7. Concentration--1.6 μg/ml.

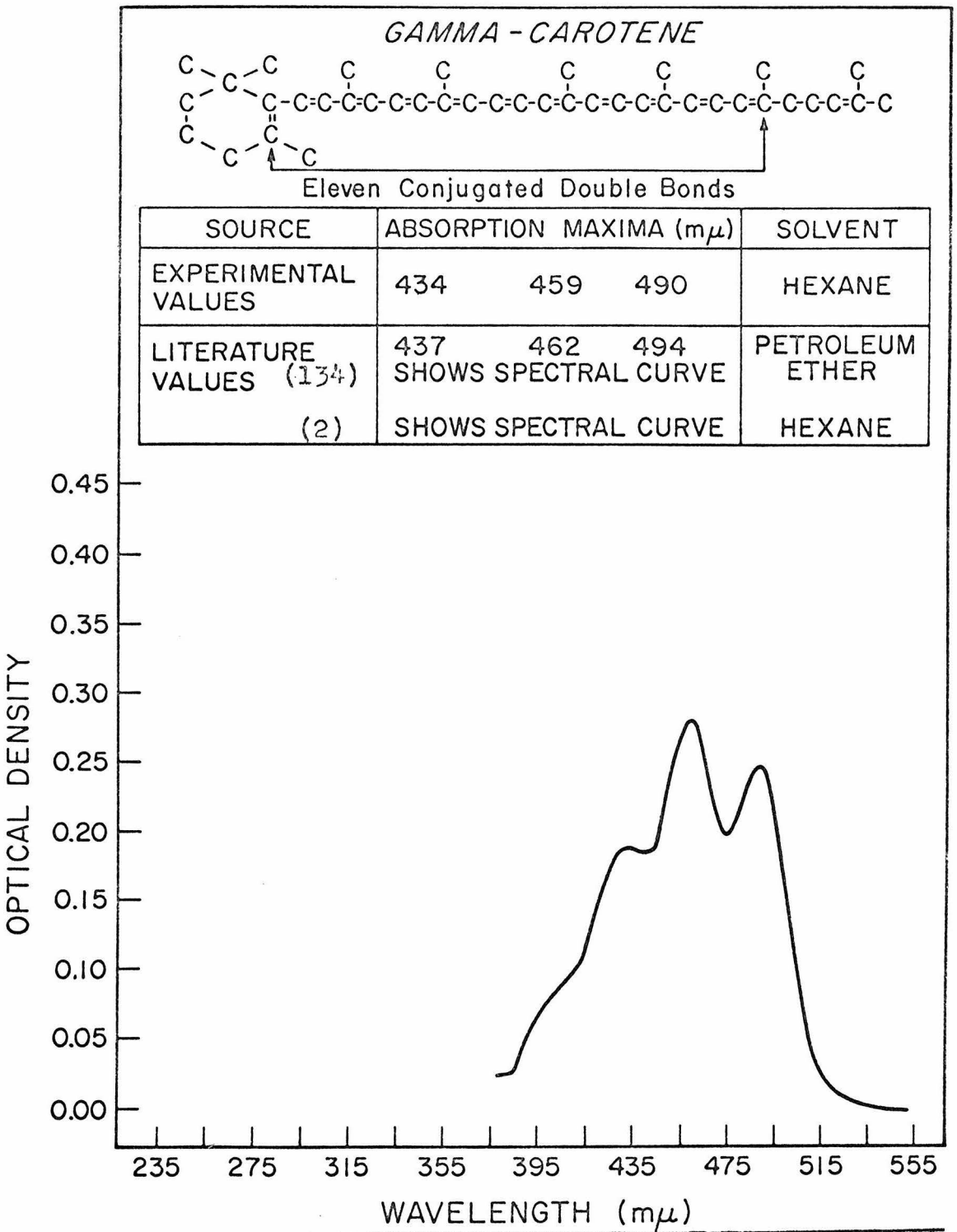

Fig. 16 The structure and visible absorption spectrum of gamma-carotene. The spectrum was obtained by use of the purification procedure summarized in Figure 5. Concentration--0.90 μ g/ml.

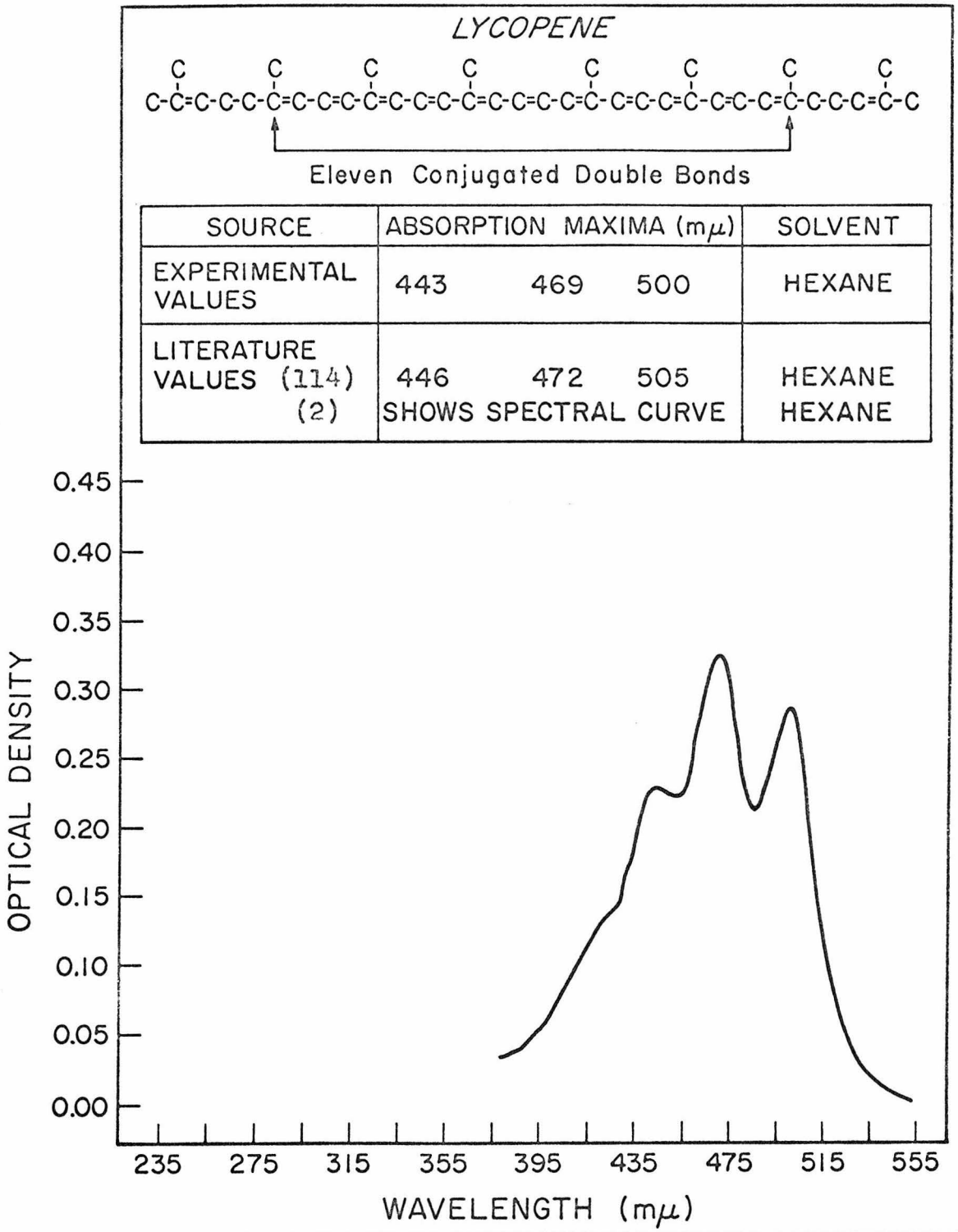

Fig. 17 The structure and visible absorption spectrum of lycopene. The spectrum was obtained by use of the purification procedure summarized in Figure 5. Concentration--0.94μ g/ml.

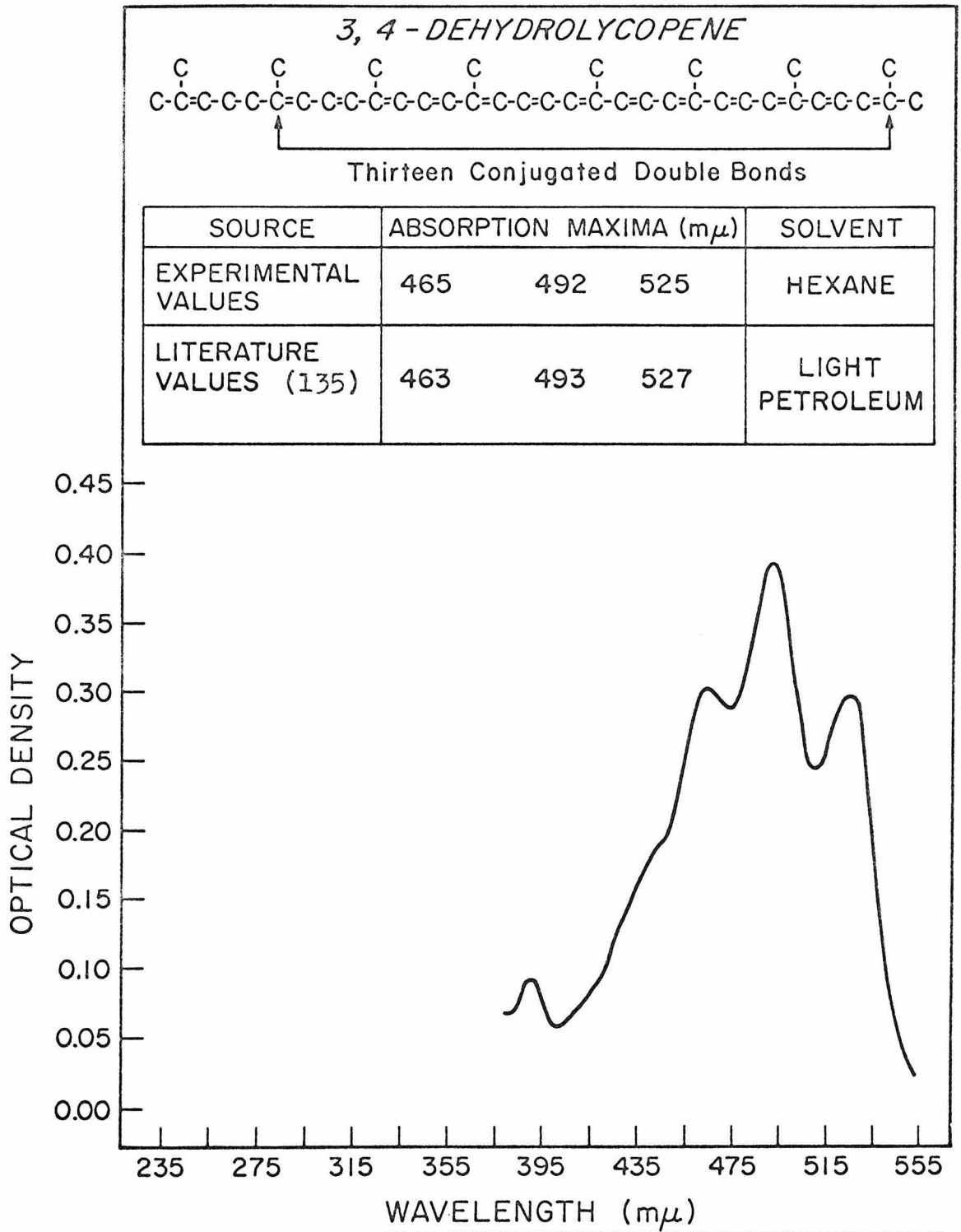

Fig. 18 The structure and visible absorption spectrum of 3,4-dehydrolycopene. The spectrum was obtained by use of the purification procedure summarized in Figure 5. Concentration--1.3 μ g/ml.

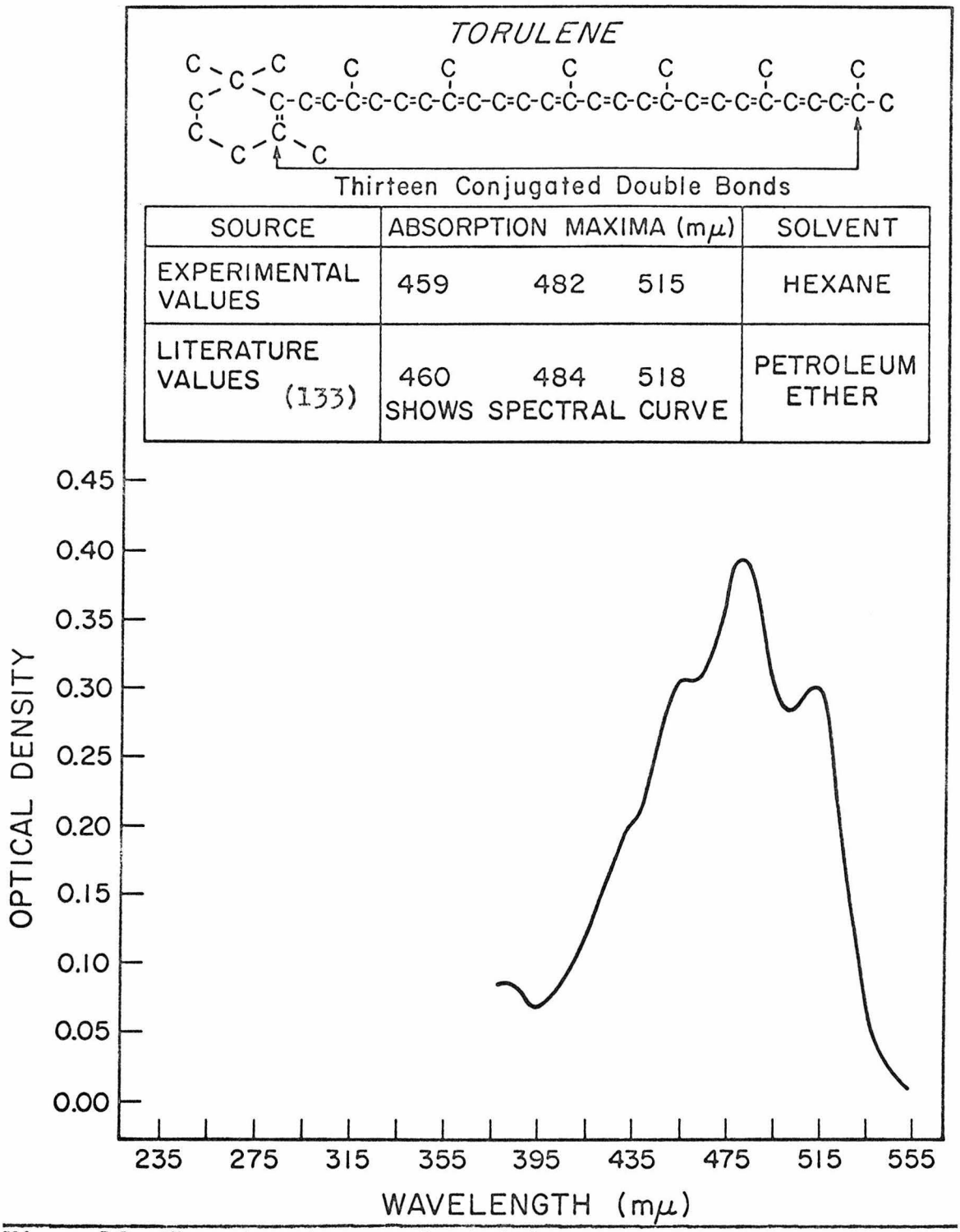

Fig. 19 The structure and visible absorption spectrum of torulene. The spectrum was obtained by use of the purification procedure summarized in Figure 5. Concentration--1.2 μ g/ml.

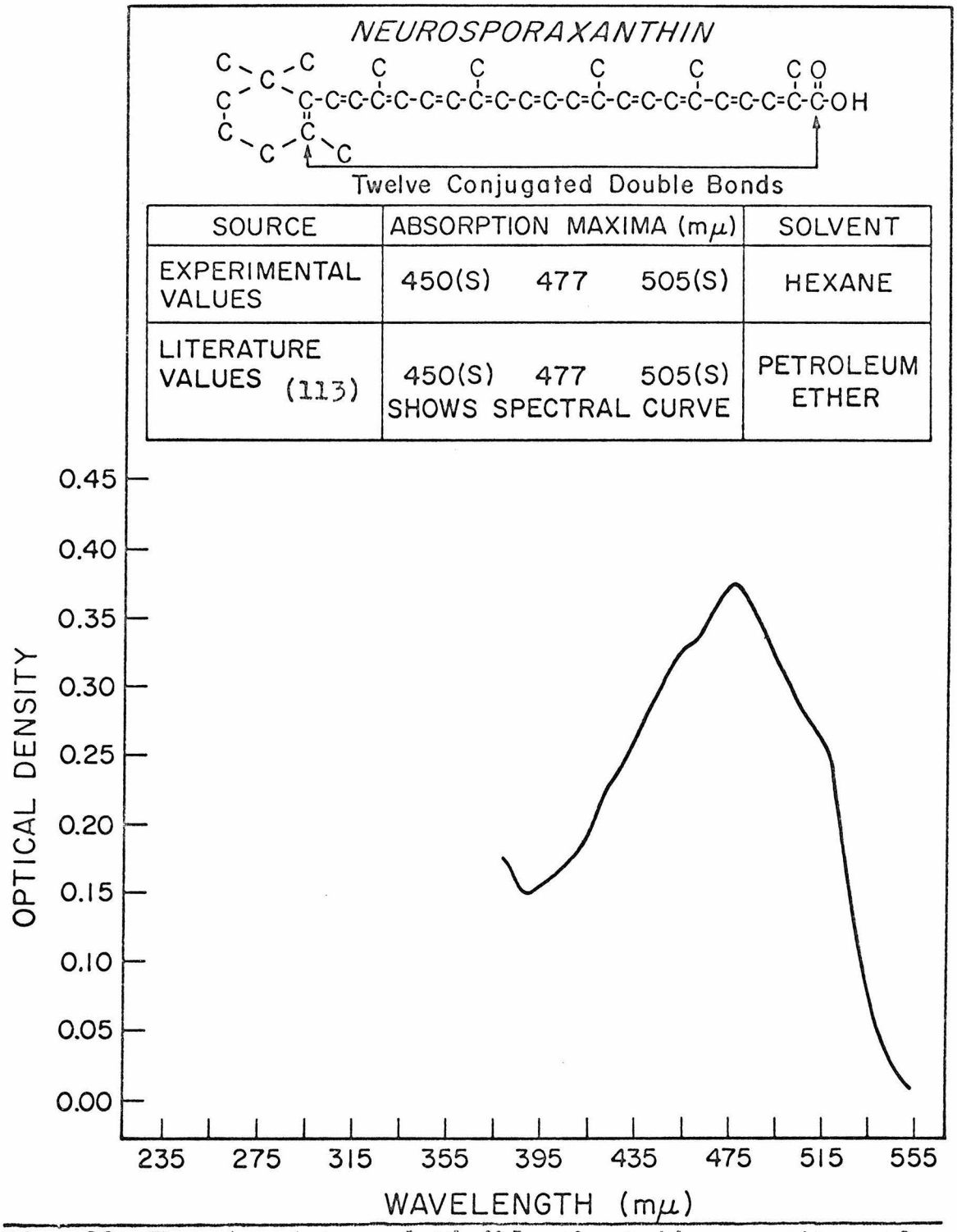

Fig. 20 The structure and visible absorption spectrum of neurosporaxanthin. The spectrum was obtained by use of the purification procedure summarized in Figure 5. Concentration--2.2 μg/ml.

at 454, 427, and 406 mμ, but since beta-zeacarotene was unknown at the time, he was unable to identify the compound.

As shown in Figure 13, the wavelengths of the absorption maxima in the spectrum of zeta-carotene are closer to those of one of the cis isomers reported by Zechmeister (130) than to the all trans compound. Since a stereochemical study of the carotenoids was not undertaken, the possible cis configuration of the purified zeta-carotene was not confirmed. In this connection, it should be mentioned that in the process of purifying the carotenoids, a certain amount of unavoidable isomerization will take place. This subject has been extensively discussed by Zechmeister (2). In general, each cis isomer has lower extinction values than the corresponding all trans isomer, and the wavelengths of the absorption maxima are shifted to lower values by a few mμ (2). For the purposes of this investigation, any possible changes in the extinction values of these 10 compounds due to isomerization would be small enough to be neglected.

The wavelengths of the absorption maxima in the carotenoid pigments and colorless polyenes are dependent on the number of conjugated double bonds which they contain. A consideration of the spectra in Figures

11-20 shows that the absorption maxima are found at longer wavelengths as this number increases. Absorption maxima occur at shorter wavelengths if one of the conjugated double bonds is included within the cyclic part of the molecule. This is illustrated by a comparison of the spectra of neurosporene and beta-zeacarotene (Figures 14 and 15) and of gamma-carotene and lycopene (Figures 16 and 17). The effect of chemical structure on carotenoid absorption spectra is considered in detail by Zechmeister (2) and Davies (114).

2. The Proposed Carotenoid Biosynthetic Pathway

In Figure 21, the proposed pathway of carotenoid biosynthesis in Neurospora crassa is presented. Haxo (96) originally suggested that the carotenoid pigments in Neurospora are synthesized by a series of sequential dehydrogenations from phytoene, even though the exact chemical structures of several of the carotenoids were unknown at the time. Other investigators have proposed that the pigments are synthesized in parallel without interconversion (23, 25, 94) and from precursors which branch off the pathway from mevalonic acid to phytoene (25).

3. The Relative Rates of Synthesis of the Carotenoids

Time course studies of the accumulation of each of the carotenoid pigments were found to give some

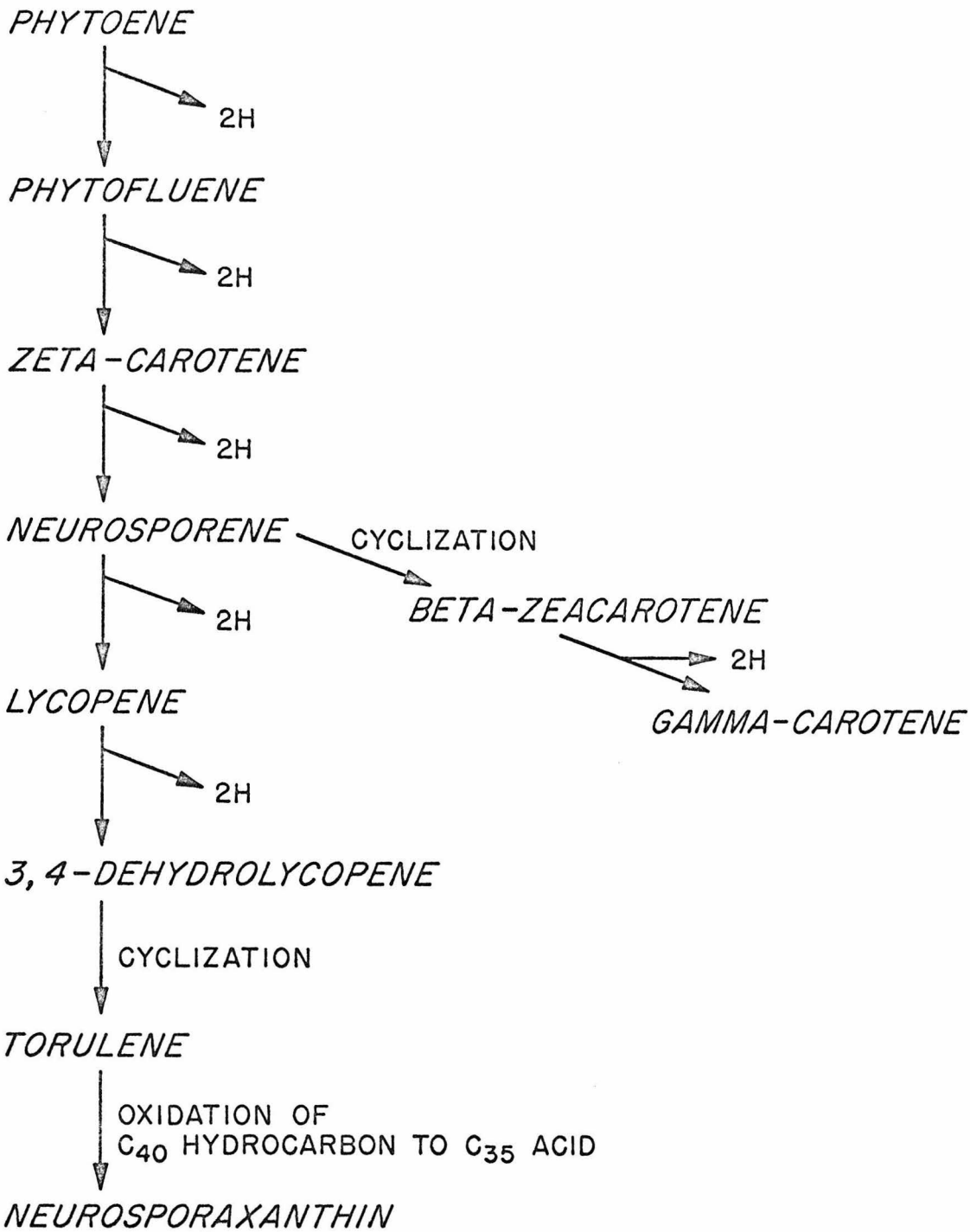

Fig. 21 Proposed pathway of carotenoid biosynthesis in Neurospora crassa.

useful information concerning the question of interconversion of the carotenoids. Dark-grown mycelium obtained from carboys was illuminated under various conditions, and the spectra of the acidic and neutral pigment extracts were determined at different illumination times. In Figure 22, such a time series of spectra of the neutral carotenoid extracts are presented.

These spectra show that there is a large increase in the total quantity of neutral pigments during the interval of 3 to 6 hours. From 6 to 12 hours, there is a significant decrease in the absorption of the maxima at 397 and 421 mμ and an increase at 494 and 525 mμ. There is also a shift of the peak at 467 mμ to 464 mμ.

In order to evaluate these changes in the spectra, the quantity of each of the neutral carotenoids was determined as a function of time. This was accomplished by using the purification procedure summarized in Figure 5.

In Table 2, some R_f values of the carotenoids are shown using two solvent systems and magnesia:celite thin layer chromatography. The pigments move farther when 2% ethanol is used because it is a more polar solution than 20% acetone. The choice of solvent system in adsorption thin layer chromatography is discussed by Stahl (136). R_f values can be quite variable, and are

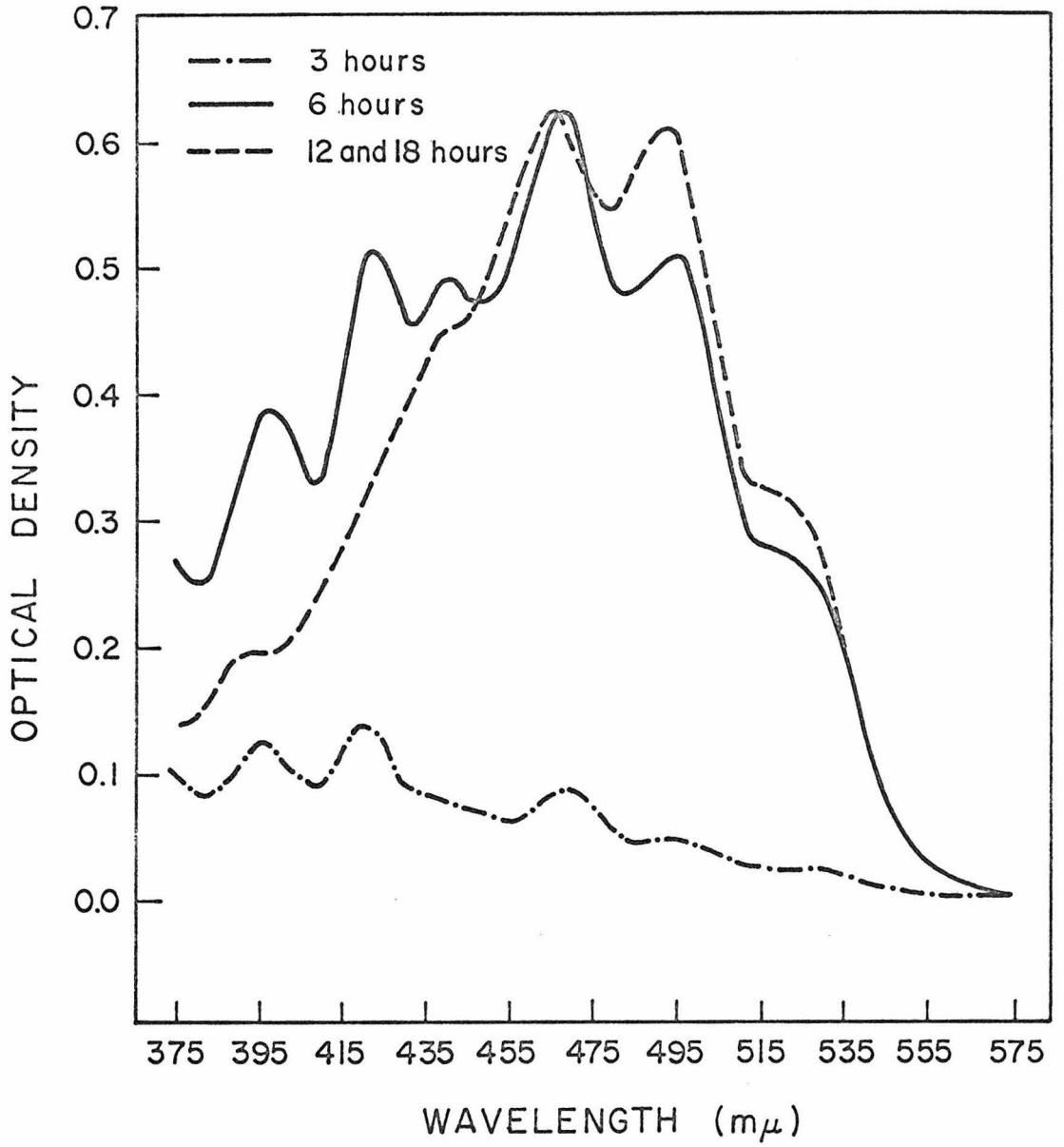

Fig. 22 The absorption spectra of the neutral carotenoid extracts obtained from mycelium illuminated for different lengths of time. Three day old dark-grown mycelium was harvested and then illuminated in Vogel's minimal medium in air at 4°C as described on page 12. Samples of mycelium were removed, and the neutral carotenoid extracts were prepared as summarized in Figure 5.

TABLE 2

Examples of R_f Values of the Carotenoids Chromatographed with Magnesia:celite Thin Layer Chromatography

Carotenoid	R_f
20% Acetone in Hexane	
Zeta-carotene	1.00
Gamma-carotene	.78
Neurosporene	.44
Lycopene-Torulene	.17
3,4-dehydrolycopene	0
2% Ethanol in Hexane	
Zeta-carotene, gamma-carotene, and neurosporene	1.00
Torulene	.67
Lycopene	.40
3,4-dehydrolycopene	.15

R_f values are defined as:

$$\left[\frac{\text{distance to compound from origin}}{\text{distance to solvent front from origin}}\right]$$

Chromatography was carried out as previously described (see page 27).

generally not used to identify unknown compounds (136). For this reason the numbers presented in Table 2 should be considered only as qualitative values.

Generally 75-90 per cent of the applied pigment was recovered from each thin layer plate when the precautions against photo-oxidation described in the Materials and Methods section were taken. There was also no indication that any of the carotenoids was more favorably recovered than the others. The extinction coefficients which were used to calculate the quantity of all the neutral pigments except 3,4-dehydrolycopene and torulene were taken from the list compiled by Davies (114) for absorption in hexane or light petroleum. The values for 3,4-dehydrolycopene and torulene were taken from the paper by Liaaen Jensen (135).

The results of chromatographic analysis of the neutral carotenoid extracts of Figure 22 are presented in Figure 23 by plotting the quantity of each carotenoid versus illumination time. After 3 hours, zeta-carotene is the predominent pigment. It increases in amount during the next 3 hours and decreases during the final 12 hours of illumination. This accounts for the significant decrease in absorption of the 397 and 421 mμ maxima in the spectra of the neutral carotenoid extracts during the interval of 6 to 12 hours.

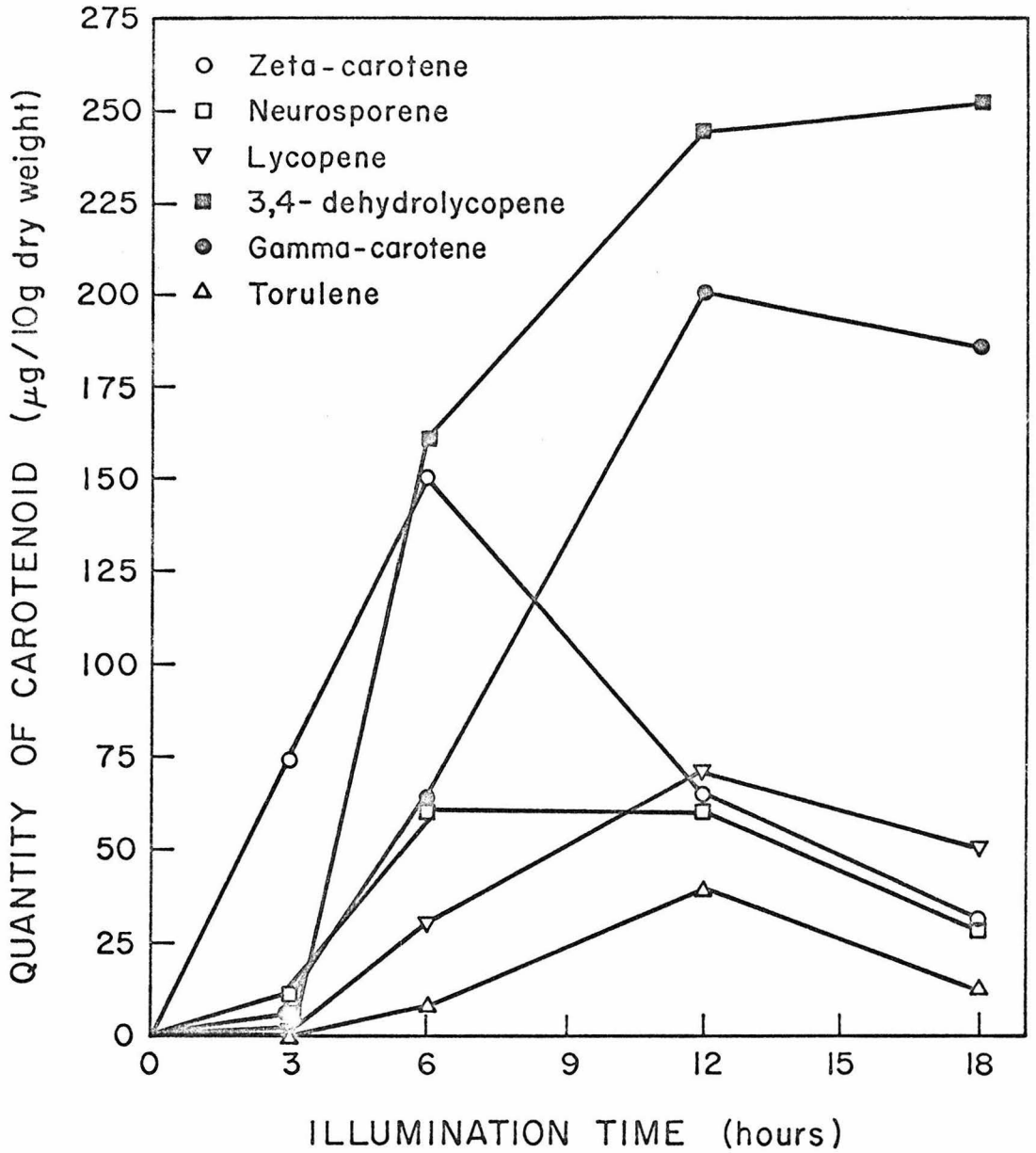

Fig. 23 Accumulation of each neutral carotenoid as a function of illumination time. Three day old dark-grown mycelium was harvested and then illuminated in Vogel's minimal medium in air at 4°C as described on page 12. Samples of mycelium were removed, and the carotenoids were extracted and purified as summarized in Figure 5.

The accumulation of the other pigments occurs after a lag period of at least 3 hours. As with zeta-carotene, the quantity of neurosporene attains a maximum value after a total illumination time of about 6 hours, while each of the other pigments accumulates for an additional 6 hours. The accumulation of 3,4-dehydrolycopene in the 6-12 hour interval accounts for the increase in absorption of the 494 and 525 mμ maxima in Figure 22. The increase in gamma-carotene during this interval accounts for the shift of the maxima at 467 to 464 mμ .

As shown in Figure 24, there is about a 6 hour lag in the accumulation of the acidic pigment; however, during the remaining 12 hour period, accumulation takes place at a linear rate. A comparison of Figures 23 and 24 shows that the amount of neurosporaxanthin which accumulates is large compared with that of any of the individual neutral carotenoids. This is also apparent from the data of Table 3 where the total quantity of neutral carotenoids and acidic pigment are compared at different illumination times. As shown there, after 12 hours the total quantity of neutral carotenoids remains constant or perhaps decreases. On the other hand, the accumulation of the acidic carotenoid proceeds at a constant rate after 6 hours of illumination, and by 18

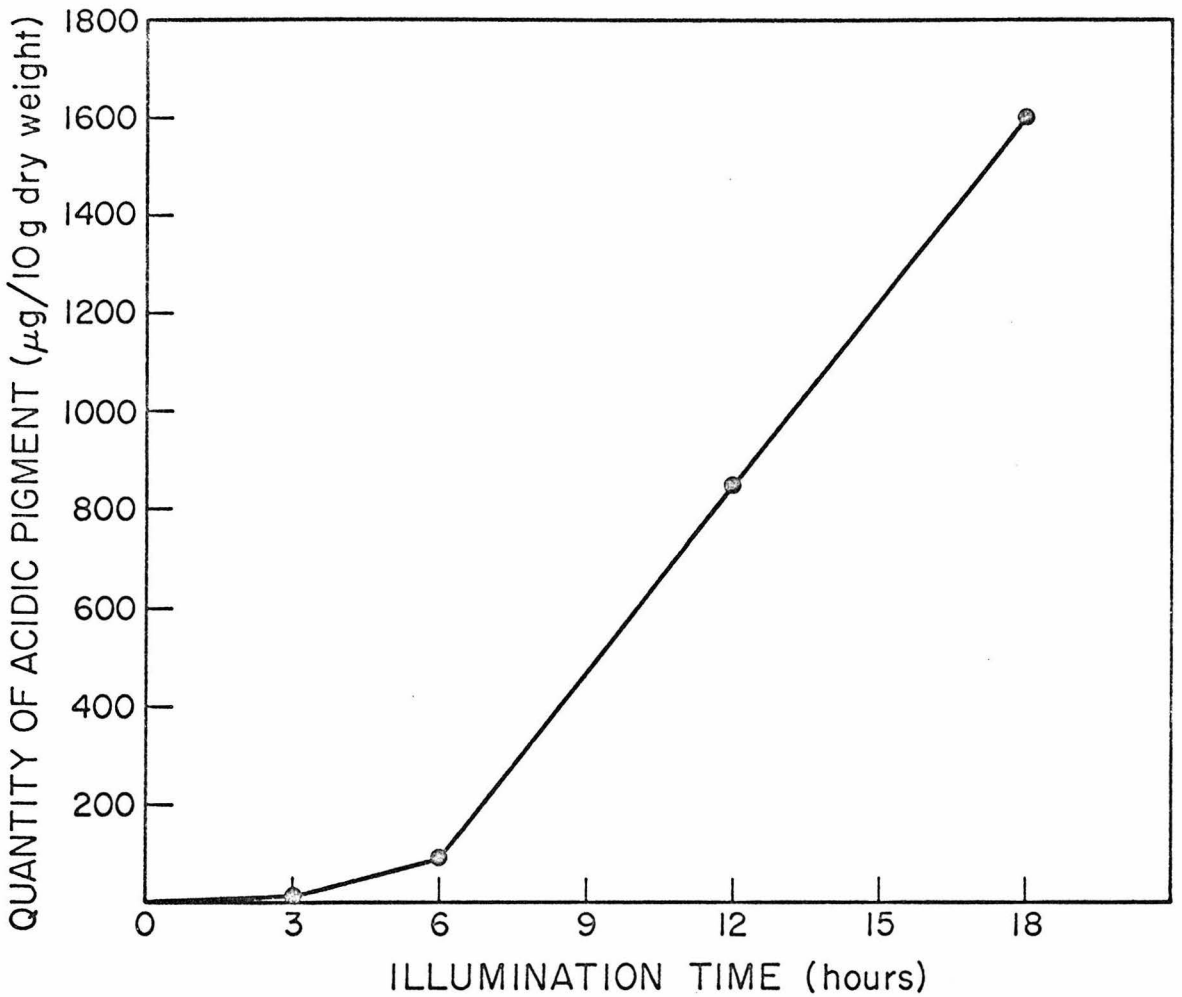

Fig. 24 Accumulation of acidic pigment as a function of illumination time. Three day old dark-grown mycelium was harvested and then illuminated in Vogel's minimal medium in air at 4°C as described on page 12. Samples of mycelium were removed and the acidic carotenoid extracts were prepared as summarized in Figure 5.

TABLE 3

Comparison of the Total Quantity of Neutral Carotenoids and the Acidic Carotenoid, Neurosporaxanthin

Hours of Illumination	Total Neutral Carotenoids (μg/10g dry weight)	Total Acidic Carotenoid (μg/10g dry weight)
3	102	10
6	471	87
12	677	844
18	563	1600

Three day old dark-grown mycelium was harvested and then illuminated in Vogel's minimal medium at 4°C in air for a total of 18 hours using the procedure described on page 12. Samples were removed and extracted after 3, 6, 12, and 18 hours of illumination. The extraction and purification of the carotenoids were carried out as summarized in Figure 5.

hours there is almost 3 times as much neurosporaxanthin as total neutral pigment.

It was previously shown by Zalokar (94) that the total synthesis of the carotenoid pigments is greatly reduced if the incubation of illuminated mycelium is carried out in nitrogen and greatly enhanced if carried out in oxygen. In Table 4, a comparison is made between the quantities of each of the carotenoid pigments extracted from mycelium illuminated for 12 hours in air and 12 hours in oxygen. As shown, each of the neutral pigments, except zeta-carotene, accumulates to a lesser extent when the incubation is in oxygen. On the other hand, the quantity of acidic pigment as well as the total of acidic plus neutral pigment is greater in oxygen than in air.

Mycelium which has been illuminated and incubated in oxygen for 12 hours and then incubated in the dark in water, under poorly aerated conditions, accumulates the carotenoids beta-zeacarotene and torulene. This is apparent from the data of Table 5. During the dark incubation, zeta-carotene and 3,4-dehydrolycopene decrease in amount.

4. Color Mutants of Neurospora crassa

In order to obtain additional information about the carotenoid biosynthetic pathway, a preliminary

TABLE 4

Comparison of Pigment Accumulation by Mycelium Illuminated in Air and in Oxygen

Carotenoid	12 hours in air (µg/10g dry weight)	12 hours in O_2 (µg/10g dry weight)
Zeta-carotene	64	52
Neurosporene	60	20
Gamma-carotene	200	36
Lycopene	71	31
3,4-dehydrolycopene	244	48
Torulene	38	10
Total Neutral Carotenoids	677	197
Neurosporaxanthin	844	2440
Total Neutral and Acidic Carotenoids	1521	2637

Three day old dark-grown mycelium was harvested and then illuminated in Vogel's minimal medium at 4°C in oxygen for a total of 12 hours as described on page 12. At the end of this time, the mycelium was harvested, and a sample was extracted and the carotenoids purified as summarized in Figure 5. The quantities of the carotenoids obtained were compared with those from dark-grown mycelium which was illuminated in air at 4°C for 12 hours.

TABLE 5

The Effect of a Dark Incubation Under Poorly Aerated Conditions on the Quantity of Each Carotenoid Pigment

Carotenoid	12 hours in light and O_2 (μg/10g dry weight)	12 hours in light and O_2 plus 24 hours in darkness (μg/10g dry weight)
Zeta-carotene	52	21
Neurosporene	20	17
Beta-zeacarotene	--	31
Gamma-carotene	36	80
Lycopene	31	24
3,4-dehydrolycopene	48	21
Torulene	10	61
Neurosporaxanthin	2440	2300

Three day old dark-grown mycelium was harvested and then illuminated in Vogel's minimal medium at 4°C in oxygen for a total of 12 hours as described on page 12. At the end of this time, the mycelium was harvested and a sample extracted. The remaining mycelium was incubated in water on a shaker at 25°C for 24 hours (1200 ml of water per 135 g fresh weight of mycelium). At the end of this period, the mycelium was harvested and a sample was extracted. Using the procedure summarized in Figure 5, the quantities of the carotenoids obtained at the two extraction times were compared.

investigation of several color mutants was made. These included yellow-1 (Y30539yA), peach (L-pe-4a), and golden (70007gA).

The conidia of the yellow mutant are initially yellow, but in 3-4 day old slants, they turn light orange. Illumination and incubation of the yellow-1 mycelium causes it to turn a deep orange color, which is indistinguishable from that of colonial-4 (70007A) mycelium.

In Table 6, the quantity of each carotenoid in the yellow mutant and colonial-4 are compared for two different illumination times. The yellow mutant was found to synthesize little or no acidic pigment in agreement with a previous observation (108). The maximum quantity of neurosporaxanthin which the mutant synthesized at each illumination time is shown in the table. The values are actually lower, as indicated there, because the acidic extracts are contaminated with a small amount of neutral pigment.

Other differences between the yellow mutant and colonial-4 are that the former accumulates much more lycopene and less neurosporene, gamma-carotene and 3,4-dehydrolycopene than the latter. Also the total quantity of pigment produced by yellow-1 is less than that of colonial-4 at each of the two illumination times.

TABLE 6

Comparison of the Quantities of the Carotenoid Pigments Obtained from Yellow-1 and Colonial-4

Carotenoid	Quantity (μg/10g dry weight)			
	Illumination Time (hours)			
	6		12	
	Yellow-1 (Y30539yA)	Colonial-4 (70007A)	Yellow-1 (Y30539yA)	Colonial-4 (70007A)
Zeta-carotene	48	150	60	64
Neurosporene	20	61	24	60
Beta-zeacarotene	--	--	--	--
Gamma-carotene	20	62	103	200
Lycopene	133	29	362	71
3,4-dehydrolycopene	44	162	138	244
Torulene	8	7	73	38
Neurosporaxanthin	11 max.	87	27 max.	844
Gamma-carotene derivative	--	--	73	--
Lycopene derivative	--	--	53	--
TOTAL	284	558	913	1521

Dark-grown mycelium of yellow-1 and colonial-4 was harvested and illuminated in Vogel's minimal medium in air at 4°C as described on page 12. At the times indicated, mycelial samples were extracted and purified as summarized in Figure 5.

On the other hand, yellow-1 was found to accumulate two unknown carotenoids which were not observed in colonial-4 under these conditions. One of these has a visible absorption spectrum similar to that of lycopene and is more strongly adsorbed in magnesia:celite thin layer chromatography than 3,4-dehydrolycopene. The other has a visible absorption spectrum which closely matches that of gamma-carotene and is found between lycopene and 3,4-dehydrolycopene on thin layer plates.

The conidia of the peach mutant appear to be pale orange at all ages, while peach mycelium turns a deep orange during illumination and incubation. In Table 7, a comparison of the quantity of each carotenoid accumulated in peach and colonial-4 is made. The total synthesis of carotenoid pigment in the former is about one third that of the latter. The peach mutant accumulates smaller quantities of lycopene, 3,4-dehydrolycopene, neurosporaxanthin and gamma-carotene, and larger quantities of zeta-carotene, neurosporene, and beta-zeacarotene.

The conidia of the golden mutant remain yellow in 4 day old slants; however, after an additional 3-4 days, they turn orange. The mycelium of the mutant becomes a deep orange during illumination and incubation. In Table 8, the quantity of each carotenoid accumulated in golden and colonial-4 are compared. In golden, the total

TABLE 7

Comparison of the Quantities of the Carotenoid Pigments Obtained from Peach and Colonial-4

Carotenoid	Quantity (μg/10g dry weight)	
	Illumination Time (12 hours)	
	Peach (L-pe-4a)	Colonial-4 (70007A)
Zeta-carotene	84	64
Neurosporene	89	60
Beta-zeacarotene	18	--
Gamma-carotene	147	200
Lycopene	16	71
3,4-dehydrolycopene	22	244
Torulene	38	38
Neurosporaxanthin	107	844
Total	521	1521

Dark-grown mycelium of peach and colonial-4 was harvested and illuminated in Vogel's minimal medium in air at 4°C as described on page 12. After 12 hours, mycelial samples were extracted and purified as summarized in Figure 5.

TABLE 8

Comparison of the Quantities of the Carotenoid Pigments Obtained from Golden and Colonial-4

Carotenoid	Quantity (μ g/10g dry weight)	
	Illumination Time (12 hours)	
	Golden (70007gA)	Colonial-4 (70007A)
Zeta-carotene	34	64
Neurosporene	28	60
Beta-zeacarotene	--	--
Gamma-carotene	122	200
Lycopene	20	71
3,4-dehydrolycopene	96	244
Torulene	52	38
Neurosporaxanthin	343	844
Total	695	1521

Dark-grown mycelium of golden and colonial 4 was harvested and illuminated in Vogel's minimal medium in air at 4°C as described on page 12. After 12 hours, mycelial samples were removed, and the carotenoids were extracted and purified as summarized in Figure 5.

synthesis of carotenoid pigment is about half that of colonial-4, and except for torulene, the quantity of each pigment is smaller by about the same amount.

5. Relative Specific Radioactivities of the Carotenoids and Phytoene

In the study of carotenoid biosynthesis in Neurospora crassa by Krzeminski and Quackenbush (23), several different radioactive compounds were tested as possible precursors of the carotenoids. Evidence was obtained that 2-C^{14}-mevalonate gave the maximum amount of incorporation.

As will be shown later (see Figure 26), Neurospora crassa does not appear to have an active transport system for the uptake of large quantities of mevalonic acid from the medium. For this reason, several radioactive compounds which had not yet been tested as carotenoid precursors in Neurospora were tried. These included uniformly labelled C^{14}-leucine, 1-C^{14}-β, β-dimethyl-acrylic acid, and 3-C^{14}-β-hydroxy-β-methyl-glutarate. These compounds have all been shown to be incorporated into carotenoids in other organisms by the pathway presented in Figure 25 (60).

The intermediates in the carotenoid pathway in Neurospora beyond mevalonic acid are likely to be pyrophosphate compounds (see Figure 1), and since

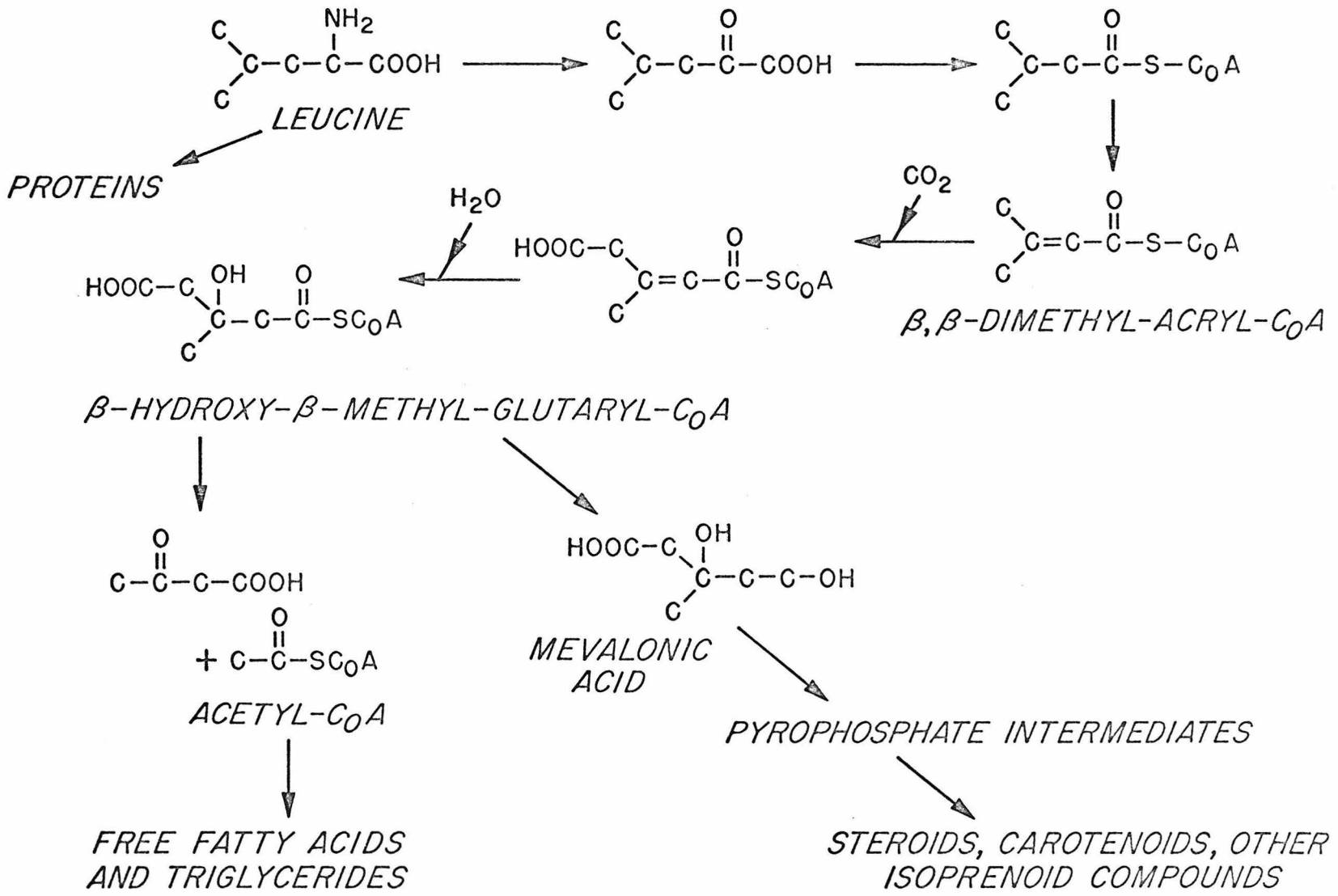

Fig. 25 Pathway of degradation of leucine.

phosphate compounds are not taken up from the medium by Neurospora (137), no attempt was made to demonstrate the incorporation of any of these proposed intermediates into the carotenoids.

In Table 9, some of the labelling data obtained using 3 of the intermediates of the pathway of Figure 25 are presented. The conditions of each experiment are given at the bottom of the table.

After incubation with uniformly labelled-C^{14}-leucine, a small amount of label was found in the total carotenoid extract; however, most of the radioactivity taken up from the medium was extractable from the mycelium with hyamine hydroxide. Most of the label in this extract is probably incorporated into proteins, although this was not confirmed.

During incubation with 3-C^{14}-β-hydroxy-β-methyl-glutaric acid, there was little or no uptake of label from the medium and no incorporation of radioactivity into compounds found in the total carotenoid extract. In contrast to this, with 1-C^{14}-β, β-dimethyl-acrylic acid, 73 per cent of the label in the medium was taken up by the mycelium. A significant quantity of this label was incorporated into the neutral and acidic carotenoid extracts, but after extensive purification of the neutral carotenoid pigments by magnesia:celite

TABLE 9

Partial Summary of Labelling Experiments Using Compounds Previously Shown to be Precursors of the Carotenoids in Other Organisms besides *Neurospora*

Solution Counted	Disintegrations Per Minute
Uniformly labelled-C^{14}-leucine[1]	
Medium-initially	2,900,000
Uptake	1,500,000
Total carotenoid extract	7,900
Hyamine hydroxide	1,400,000
3-C^{14}-β-hydroxy-β-methyl-glutaric acid[1]	
Medium-initially	640,000
Uptake	60,000[2]
Total carotenoid extract	Background
1-C^{14}-β,β-dimethyl-acrylic acid[3]	
Medium-initially	770,000
Uptake	560,000
Neutral carotenoid extract	82,000
Acidic extract	11,000

(1) The incubation of mycelium with uniformly labelled-C^{14}-leucine and 3-C^{14}-β-hydroxy-β-methyl glutaric acid is described on page 21. Illumination was for 6 hours at 4°C as described on page 12. In the case of the leucine experiment, after extraction of the carotenoids, an aliquot of the mycelial residue was refluxed with hyamine hydroxide for 1.5 hours.

(2) Since this value was obtained by a measurement of a small difference between two large numbers, it should be considered as only an estimate of the uptake.

(3) The incubation of mycelium with 1-C^{14}-β,β-dimethyl-acrylic acid is described on page 19. Illumination was for 6 hours at 4°C as described on page 12.

All data are based on 27 g (dry weight) of mycelium.

thin layer chromatography, it was shown that they contained little or no radioactivity. In another experiment, carried out in the same way, except that a larger quantity of label (12.4×10^6 dpm) was initially added to the medium, the purified carotenoid pigments were also shown to contain essentially no radioactivity. It was found in this experiment that saponification of the neutral carotenoid extract and subsequent removal of acidic compounds (see Figure 8 for summary of procedure) removed 85% of the radioactivity. From this information, it was apparent that a large quantity of the label was incorporated into an acid (or acids) which has been esterified. One explanation for this is that under these conditions much of the label is converted to acetyl-CoA instead of to mevalonic acid (see Figure 25) and consequently ends up in fatty acids which become esterified to glycerol to form triglycerides.

Since none of these labelled compounds appeared to be incorporated into the carotenoids to a significant extent, further investigations were carried out using 2-C^{14}-mevalonate, even though it does not appear to be actively taken up from the medium by the mycelium. A procedure which was found to improve the uptake was incubation of 3 day old dark-grown mycelium in water instead of medium. This is shown in Figure 26 where the uptake of label in water and medium during incubation of

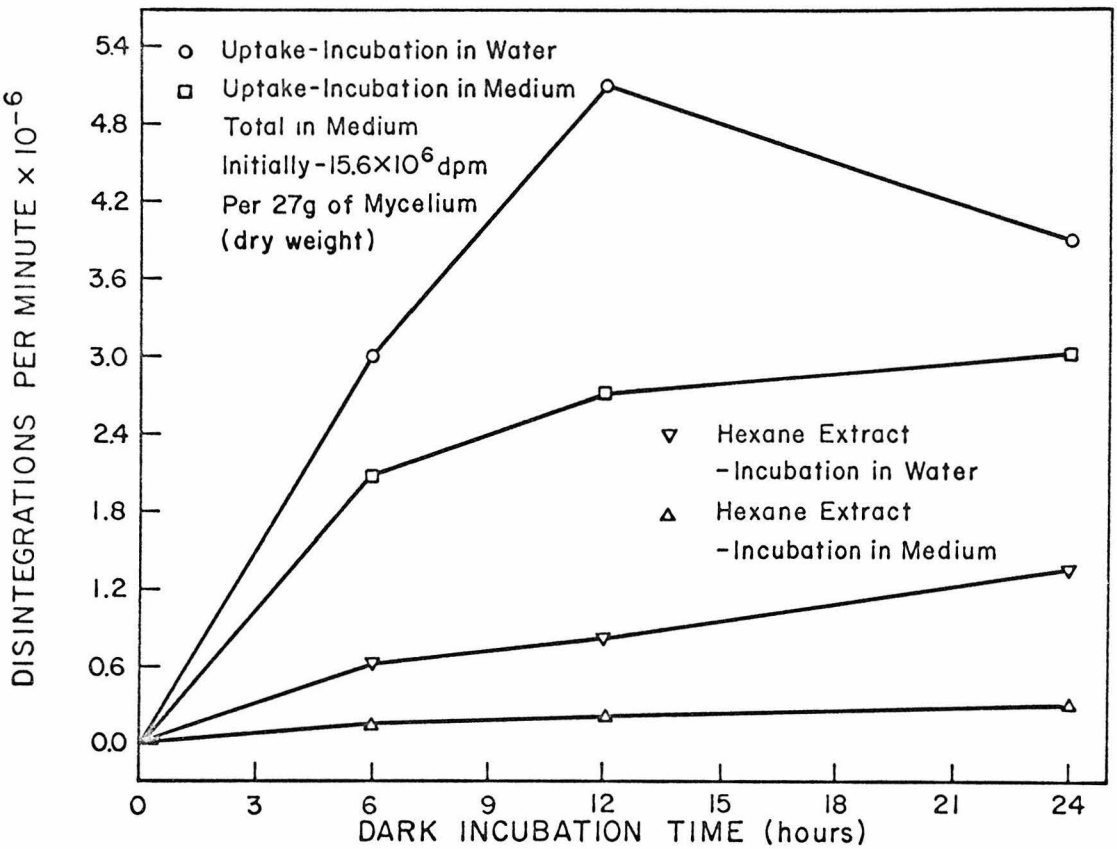

Fig. 26 Uptake of 2-C^{14}-mevalonate and incorporation of label into hexane soluble compounds. Three day old dark-grown mycelium was harvested and incubated with 2-C^{14}-mevalonate in standing cultures as described on page 19 for 6, 12, or 24 hours in the dark. The mycelium from each incubation time was harvested, and extraction and preparation of the hexane extracts were carried out as described in Figure 5. The quantity of label in each extract is based on 27 g of mycelium (dry weight).

mycelium in the dark is compared in the two upper curves. The total label in the medium initially was 15.6 X 10^6 dpm so that in water the uptake after 12 hours is 33% of the total. The two lower curves show the incorporation of label into the total hexane extract as a function of incubation time. It is apparent that incorporation is greater for mycelium incubated in water. In both cases, the incorporation takes place at an approximately constant rate throughout the 24 hour incubation period.

In order to increase the incorporation of label further, the quantity of label used was increased and the incubation was allowed to take place on a shaker. At the end of 24 hours, the contents of the culture flask were poured into the carboy with double baffles and a sucrose and salt solution added as described on page 19. The mycelium was then illuminated for the desired length of time under O_2 at 25°C using the apparatus shown in Figure 2. At the end of the illumination period, the mycelium was harvested and extracted and the carotenoids purified.

The specific radioactivities of the carotenoids and phytoene determined in several experiments in which this incubation procedure was used are summarized in Table 10. As shown in the upper portion of the table, the specific radioactivities of the carotenoids are essentially equal

TABLE 10

Comparison of Relative Specific Radioactivities of the Carotenoids and Phytoene

Compound	Specific Radioactivity $\left[\frac{dpm}{\mu g}\right]$			
	Illumination Time (hours)			
	0	3	6	9
Incubation with 28 μ Curies of Label				
Phytoene	186	---	---	---
Zeta-carotene	---	---	139	---
Neurosporene	---	---	118	---
Gamma-carotene	---	---	136	---
Lycopene	---	124	109	129
3,4-dehydrolycopene	---	99	112	116
Incubation with 14μ Curies of Label				
Phytoene	73		48	
Lycopene	---		43	
3,4-dehydrolycopene	---		43	

Three day old dark-grown mycelium was incubated with 2-C^{14}-mevalonate in water on a shaker at 25°C for 24 hours as described on page 19. The mycelium was either harvested and extracted (0 hours of illumination) or the incubation mixture was transferred to the baffled carboy, salts and sugar added (see page 19), and illumination carried out at 25°C in O_2 for the times indicated. The specific activities of phytoene were determined by the procedure summarized in Figure 7 plus alumina column chromatography (see page 39). The specific activities of the carotenoids were either determined by the methods shown in Figures 5 and 8, or for lycopene and 3,4-dehydrolycopene by the procedure of Figure 7 (without the Silica Gel G chromatographic step). Specific radioactivity measurements of the carotenoids at zero hours of illumination cannot be made because little or no pigment is present.

after 6 hours of illumination. Such measurements were also made for lycopene and 3,4-dehydrolycopene after 3 and 9 hours of illumination, and the values obtained for these carotenoids were all equal to each other and to those of the other carotenoids after 6 hours. The specific radioactivity of phytoene at zero hours of illumination was found to be slightly higher than that attained by the carotenoids.

In the lower part of the table, some similar data are presented for incubation with 14μ Curies of label. The use of one-half as much label in these experiments was inadvertent and due to an incorrect designation by the manufacturer of the quantity of label sent. These data are still of interest, because they show that the specific radioactivities of phytoene at zero time, and lycopene and 3,4-dehydrolycopene after 6 hours, are all smaller than the corresponding values obtained in the incubation with 28μ Curies by about a factor of 2. They also show that the specific radioactivity of phytoene during 6 hours of illumination decreases slightly and becomes equal to that of lycopene and 3,4-dehydrolycopene.

In another experiment (data not shown) the mycelium was incubated in the dark in 28μ Curies of label as already described and then washed twice, each time with 300 ml of water. The washed mycelium was placed in

medium which did not contain label and illuminated, and samples of mycelium were removed after 3, 6, and 9 hours. The specific radioactivities of lycopene and 3,4-dehydrolycopene were measured at each illumination time, and again the values obtained were all about equal; however, their magnitude was approximately half of those presented in the upper portion of Table 10 in the experiment where no attempt was made to wash the label out of the mycelium.

An important consideration in the experiments summarized in Table 10 is whether these compounds are pure. As already mentioned (see page 47), it is relatively easy to prepare carotenoid pigments which are pure from the standpoint of visible absorption spectra. In general, without further purification the specific radioactivities of these compounds are much too high, since they are contaminated with radioactive compounds which do not have a visible absorption spectrum. The most significant of these is ergosterol which absorbs in the uv region at 294, 282, and 271 mμ in hexane. Thus, two criteria of radiochemical purity which were used are purification to a constant specific radioactivity and removal of uv absorbing compounds. In addition, the adsorbent immediately in front and behind each carotenoid fraction in thin layer chromatography was shown to be free of radioactivity.

The following control was also carried out as a check on the radiochemical purity of the carotenoids. Mycelium was grown, incubated with label, and rotated under O_2 for 6 hours exactly as in the experiment used to compile the data shown in the upper portion of Table 10, except no illumination was used. The mycelium was extracted and the carotenoid free hexane extract treated as before in the purification procedure up to thin layer chromatography. At this point, a mixture of unlabelled carotenoids was added and chromatography was carried out. The specific radioactivities of all the carotenoids except zeta-carotene were 10-20 times lower than those of Table 10 after a single chromatographic run. Further chromatography of zeta-carotene reduced its specific activity to one-fourth that shown in the table. These data support the supposition that most of the radioactivity found in each pigment fraction of Table 10 is due to incorporation of label into the carotenoid and not to radioactive contaminants which have exactly the same chromatographic properties.

The criteria used to support the conclusion that the phytoene fraction is radiochemically pure are purification to a constant specific activity, no absorption in the visible range, an ultraviolet spectrum which coincides

with those in the literature, and coincidence of label and phytoene in thin layer chromatography.

In Table 11, the data obtained from a different type of labelling experiment are presented. In this case, carotenoid synthesis was allowed to take place, and then the mycelium was incubated in label in such a way that significant incorporation of radioactivity into phytoene, but not the carotenoids, occurred. At the end of this period, a sample of mycelium was removed for extraction, and the remaining mycelium was illuminated and incubated for a second time under conditions in which carotenoid production took place. At the end of this incubation, another sample of mycelium was removed and extracted. In Table 11, the specific radioactivities of several of the carotenoids and phytoene and the quantities of the carotenoids at the two extraction times are presented. As shown there, the quantity of zeta-carotene was constant or decreased slightly during the second illumination, while that of each of the other carotenoids increased. During this same period, the specific radioactivity of phytoene decreased slightly, while those of zeta-carotene and neurosporene have increased significantly. The specific radioactivities of zeta-carotene, neurosporene, and lycopene wereabout equal at the end of the illumination and higher than those of beta-zeacarotene and gamma-carotene.

TABLE 11

Comparison of the Relative Specific Radioactivities of the Carotenoids and Phytoene

Compound	Specific Activity $\frac{dpm}{\mu g}$		Quantity (μg/10g dry weight)	
	Second Illumination Time (hours)		Second Illumination Time (hours)	
	0	6	0	6
Phytoene	157	112	---	---
Zeta-carotene	14	72	148	101
Neurosporene	25	76	21	104
Beta-zeacarotene	11	29	62	117
Gamma-carotene	19	19	30	48
Lycopene	---	67	---	44
Neurosporaxanthin	---	---	42	282

Dark-grown mycelium was harvested and illuminated in Vogel's minimal medium in air at 4°C for 6 hours. At the end of this time, the mycelium was again harvested and incubated on a shaker with 2-C^{14}-mevalonate for 24 hours in the dark as described on page 19. At the end of this period, the culture flask and its contents were allowed to cool for 6 hours in the dark in the 4°C room under stationary conditions. Under red light, the mycelium was filtered and half was frozen with liquid nitrogen. The other half was added to one-half of the filtrate in addition to sugar and salts at the final concentrations found in Vogel's minimal medium. The mixture was illuminated at 4°C under O_2 for 6 hours as described on page 12. At the end of this period, the mycelium was harvested and frozen. The two mycelial samples were extracted, and the carotenoids and phytoene were purified as summarized in Figure 7.

B. INITIATION OF CAROTENOID BIOSYNTHESIS

1. Effect of Length of Light Stimulus on the Induction of Carotenoid Synthesis

Zalokar (94) has pointed out that if light of sufficient intensity is used to illuminate *Neurospora* mycelium, then short illumination times (1 minute or more) are as effective in initiating carotenoid synthesis as continuous illumination. The results of a further study of the induction of carotenoid synthesis using short illumination times is shown in Figure 27.

Mycelial pads were illuminated, as described on page 17, with three different light intensities for various lengths of time and then incubated in the dark for a total of 24 hours. The pads were extracted (see page 22), and the absorption spectrum of each acetone extract was used to estimate the total quantity of pigment synthesized. As shown in Figure 27, illumination at 175 foot-candles for 30 seconds is as effective in triggering the synthesis of carotenoids as illumination for 30 minutes. At an intensity of 35 foot-candles, 30 seconds of illumination did not significantly stimulate carotenoid production, while illumination for 2-4 minutes at this intensity was as effective as 30 minutes. At an intensity of 5 foot-candles, carotenoid synthesis does not reach the level attained by illumination at the

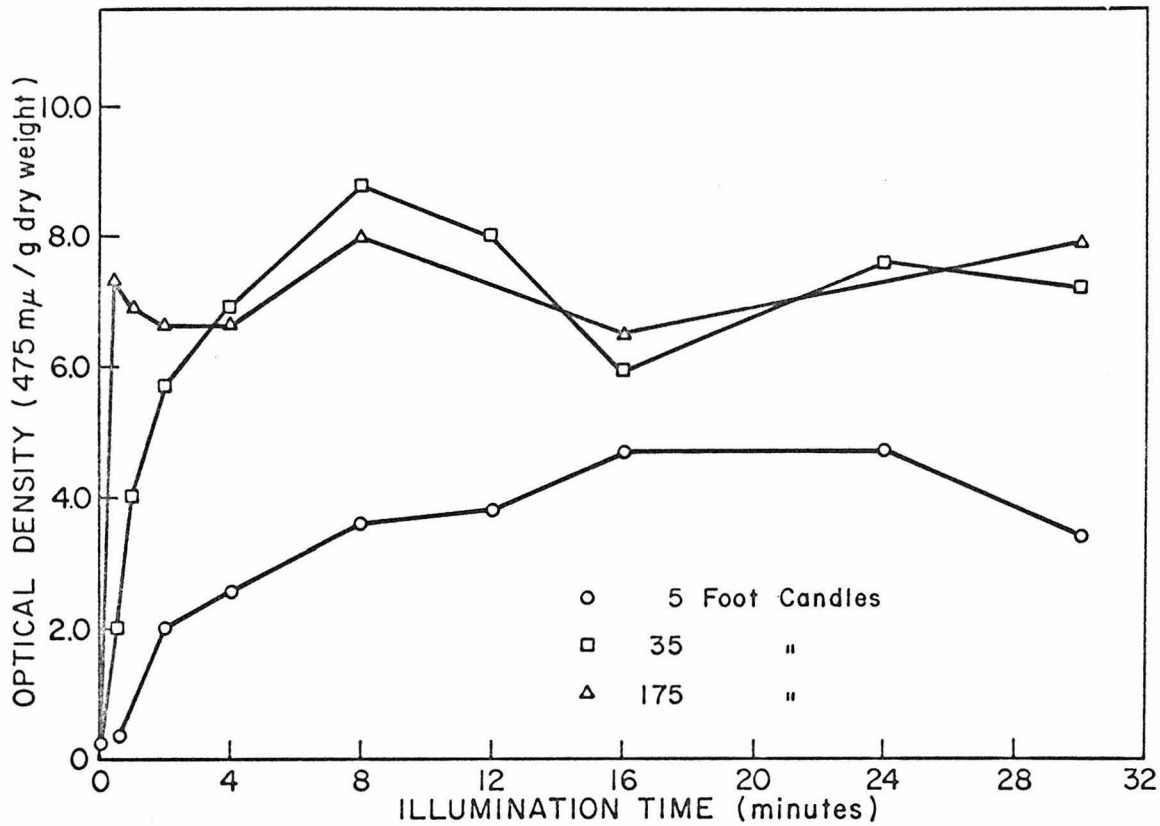

Fig. 27 The effect of variation of illumination time and light intensity on carotenoid synthesis. Dark-grown mycelial pads (Em5297a) were illuminated as described on page 17. After illumination, the pads were incubated in the dark for a period of 24 hours measured from the start of illumination. At the end of this time, each sample was extracted as described on page 22 and the spectrum of the acetone extracts recorded. The optical density at 475 mμ in these extracts was used to estimate the total quantity of pigment synthesized.

higher intensities even after 30 minutes of illumination. A longer illumination time of 60 minutes at 5 foot-candles (not shown in Figure 27) was not effective in increasing the level of carotenoid synthesis further.

In most experiments, it was convenient to use the rotator-illuminator diagramed in Figure 2, because large quantities of mycelium could be illuminated. Generally, dark-grown mycelium was harvested and then illuminated for a continuous period of time, but in some experiments a 15 minute light stimulus was used and the subsequent incubation carried out in the dark.

A comparison of the optical densities at the maxima in the spectra of the carotenoid extracts obtained from mycelium illuminated in these two ways is shown in Table 12. In this experiment, the illumination and incubation was under oxygen. It is apparent that 15 minutes of illumination plus 5.75 hours of dark incubation is as effective in promoting carotenoid synthesis as 6 hours of continuous illumination.

In Table 13, the results of a similar experiment are shown in which the illumination and incubation were carried out in air. It was found that under these conditions the 15 minute induction time was not as effective as continuous illumination, although a significant amount of carotenoid synthesis was initiated.

TABLE 12

Comparison of Effect of Short Versus Continuous Illumination on Carotenoid Synthesis for a 6 Hour Incubation in Oxygen

Wavelength of Maxima (mμ)	Optical Density (Per Cent of Control)	
	Illumination Time	
	15 minutes	6 hours (control)
	Neutral Carotenoid Extract	
397	122	100
421	110	100
440	109	100
467	110	100
494	104	100
525	108	100
	Acidic Carotenoid Extract	
477	70	100

Dark-grown mycelium was harvested and then illuminated as shown in the table. Illumination and any subsequent dark incubations were carried out in Vogel's minimal medium at 4°C in oxygen as described on page 12. The carotenoid extracts were prepared as summarized in Figure 5.

TABLE 13

Comparison of Effect of Short Versus Continuous Illumination on Carotenoid Synthesis for a 6 Hour Incubation in Air

Wavelength of Maxima (mμ)	Optical Density (Per Cent of Control)	
	Illumination Time	
	15 minutes	6 hours (control)
	Neutral Carotenoid Extract	
397	65	100
421	63	100
440	57	100
467	53	100
494	37	100
525	30	100
	Acidic Carotenoid Extract	
477	43	100

Dark-grown mycelium was harvested and then illuminated as shown in the table. Illumination and any subsequent dark incubations were carried out in Vogel's minimal medium at 4°C in air as described on page 12. The carotenoid extracts were prepared as summarized in Figure 5.

The data of Tables 12 and 13 imply that with these illumination and incubation conditions the light reaction goes almost to completion in the first 15 minutes of illumination. This is particularly true if the illumination and incubation are carried out in oxygen. These results are consistent with those of Zalokar (94) where he showed that both the initial light reaction and subsequent dark reactions in carotenoid synthesis required oxygen.

2. Inhibition of Carotenoid Synthesis by Cycloheximide

There are many plausible mechanisms which can be proposed to explain how light induces carotenoid synthesis in Neurospora. Most of these involve either the activation of an inactive enzyme, or induction of the de novo synthesis of an enzyme missing in dark-grown cultures. Thus, it is of interest to know whether protein synthesis is a necessary requirement for carotenoid production. In order to investigate this question, use was made of cycloheximide, an effective inhibitor of protein synthesis in Neurospora (138, 139).

As shown in Table 14, there is a potent inhibitory effect of this compound on carotenoid synthesis. In the experiments summarized, dark-grown mycelium was placed in medium which contained cycloheximide at a concentration of either 2.0 or 0.5 μg/ml. Eight minutes later incubation and illumination were started and allowed to continue

TABLE 14

Effect of Concentration of Cycloheximide on Carotenoid Synthesis

Wavelength of Maxima (mμ)	Optical Density (Per Cent of Control)		
	Concentration of Cycloheximide (μg/ml of medium)		
	2.0	0.5	None (control)
Neutral Carotenoid Extract			
397	1	7	100
421	1	5	100
440	0	3	100
467	0	2	100
494	0	1	100
525	0	2	100
Acidic Carotenoid Extract			
477	0	0	100

Dark-grown mycelium was harvested and then illuminated and treated with the concentrations of cycloheximide shown in the table. Illumination was carried out at 4°C in Vogel's minimal medium in air for 6 hours as described on page 12, and the carotenoid extracts were prepared as summarized in Figure 5.

for 6 hours. At the end of this time, the mycelium was extracted and the absorption spectra of the carotenoid extracts determined. In Table 14, the optical densities of the maxima in these extracts are compared with those of the control (no cycloheximide in the medium). At a concentration of 2.0 μg/ml, the inhibition of carotenoid production is essentially complete, while at 0.5 μg/ml a small amount of synthesis takes place.

If the cycloheximide is added to the medium after illumination has begun, the inhibition of carotenoid production is reduced. This is shown in Table 15 where the effect of adding the inhibitor at 3 different times is presented. It is apparent that when cycloheximide is added after 3 hours of illumination, a significant quantity of pigment production takes place in the subsequent 3 hour period. The quantity of pigment synthesized in the total 6 hour incubation when the inhibitor was added just before or 30 minutes after the start of illumination is much smaller.

In Table 16, the actual quantity of each pigment which accumulates in 6 hours, when cycloheximide was added after 3 hours, is shown. For comparison, the quantities of the carotenoids after 3 and 6 hours of illumination in the absence of the inhibitor are also included in the table.

TABLE 15

Effect of Varying the Time of Addition of Cycloheximide on the Synthesis of Carotenoids

Wavelength of Maxima (mμ)	Optical Density (Per Cent of Control)				
	Illumination Time at which Cycloheximide was Added (hours)			No Cycloheximide--Illumination and Incubation Time (hours)	
	0*	0.5	3	3	6 (control)
	Neutral Carotenoid Extract				
397	7	12	50	27	100
421	5	9	46	22	100
440	3	5	42	14	100
467	2	4	38	11	100
494	1	2	28	6	100
525	2	2	22	7	100
	Acidic Carotenoid Extract				
477	0	0	43	2	100

Dark-grown mycelium was harvested and then illuminated for 6 hours, and at various times, the mycelium was treated with cycloheximide as shown in the table. Illumination was carried out at 4°C in Vogel's minimal medium under air as described on page 12, and the carotenoid extracts were prepared as summarized in Figure 5. The concentration of inhibitor used was 0.5 μg/ml.

* Cycloheximide was added 8 minutes before illumination.

TABLE 16

The Quantity of Each of the Carotenoids Accumulated in 6 Hours when Cycloheximide was Added after 3 Hours of Illumination

Carotenoid	Quantity (µg/10g dry weight)		
	3 Hours of Illumination-- No Cycloheximide	6 Hours of Illumination-- Cycloheximide Added after 3 Hours	6 Hours of Illumination-- No Cycloheximide
Zeta-carotene	74	67	150
Neurosporene	12	26	61
Gamma-carotene	8	11	62
Lycopene	2	13	29
3,4-dehydrolycopene	6	17	162
Torulene	--	--	7
Neurosporaxanthin	10	48	87
Total	112	182	558

Dark-grown mycelium was harvested and then illuminated and treated with cycloheximide as shown in the table. Illumination was carried out at 4°C in Vogel's minimal medium under air as described on page 12, and the carotenoids were extracted and purified as summarized in Figure 5. The concentration of cycloheximide used was 0.5 µg/ml.

These data reveal that in the first 3 hours in the absence of cycloheximide, zeta-carotene is the major pigment. Small quantities of several of the other pigments accumulate, and thus according to the biosynthetic scheme proposed in Figure 21, all the reactions in the pathway are functioning. In the interval of 3-6 hours in the presence of cycloheximide, there is a further increase in the total quantity of carotenoids and an increase in the accumulation of each pigment except for zeta-carotene and torulene. Again according to the pathway of Figure 21, this means that all the biosynthetic reactions are still taking place even in the presence of the inhibitor.

One plausible interpretation of the data of Tables 15 and 16 is that a certain amount of critical protein synthesis takes place in the first 3 hours of illumination so that addition of cycloheximide after this time cannot completely inhibit carotenoid production. On the other hand, addition of the inhibitor at earlier times prevents most of this protein synthesis. The fact that addition of cycloheximide at the 3 hour point still causes a significant inhibition of carotenoid production indicates that further critical protein synthesis is being prevented.

Taken together, the results of Tables 12, 13, 15, and 16, as well as those of Figures 23 and 24, suggest that carotenoid production in Neurospora under these conditions can be divided into three phases: (1) an initial light reaction (or reactions) which goes to completion within about 15 minutes; (2) a period of protein synthesis which occurs during the first 3 hours and longer; and (3) significant accumulation of the carotenoid pigments, which except for zeta-carotene, occurs after 3 hours.

In Table 17, data are presented which show that the inhibitory effect of cycloheximide is not due to irreversible damage to the carotenoid biosynthetic system. After 6 hours of illumination and incubation with cycloheximide, the mycelium was washed twice with water. The mycelium was then illuminated and incubated for 6 hours, and a significant (50-60% of control) synthesis of carotenoids was found to occur.

Zalokar (92) has carried out experiments in which he illuminated and kept mycelium in the dark at low temperatures (0°C) without subsequent carotenoid production. Twenty-four hours later, the mycelium was warmed to 25°C and carotenoid synthesis took place in the dark. This suggested that the initial light effect was stable. The use of cycloheximide has provided support for this

TABLE 17

Partial Reversal of Cycloheximide Inhibition by Washing the Mycelium and Incubating and Illuminating a Second Time

Wavelength of Maxima (mμ)	Optical Density (Per Cent of Control)		
	Illumination for 6 hours with cycloheximide in the medium	Illumination for 6 hours with cycloheximide in the medium; then mycelium washed and illuminated for 6 hours without inhibitor	Illumination for 6 hours without cycloheximide in the medium (control)
		Neutral Carotenoid Extract	
397	7	56	100
421	5	56	100
440	3	59	100
467	2	56	100
494	1	51	100
525	2	47	100
		Acidic Carotenoid Extract	
477	0	74	100

Dark-grown mycelium was harvested and then illuminated and treated with cycloheximide as shown in the table. Illumination was carried out at 4°C in Vogel's minimal medium under air as described on page 12, and the carotenoid extracts were prepared as summarized in Figure 5. The concentration of cycloheximide used was $\left[\frac{0.5\mu g}{ml}\right]$. The mycelium was washed with two 600 ml portions of water per 135 g of mycelium (fresh weight) to remove the inhibitor.

conclusion as shown by the data of Table 18. Mycelium was illuminated for 15 minutes in medium which contained cycloheximide and then incubated for 5.75 hours in the dark. At the end of this period, only a small amount of carotenoid synthesis had taken place. Working under red light, the mycelium was washed twice with water and the incubation continued in the dark for 6 hours. It was found that at the end of this period, a significant amount of carotenoid production had occurred.

3. Attempt to Induce Carotenoid Biosynthesis Chemically in Dark-Grown Cultures

As already mentioned, some characteristics of the primary light reaction which is involved in the initiation of carotenoid biosynthesis in Neurospora crassa have been described by Zalokar (94). The actual chemical reaction, however, is still unknown.

Several experiments were carried out to determine whether the product of this reaction is a diffusible inducer, but no evidence to support this suggestion was obtained. In addition, dark-grown mycelium was incubated in medium supplemented with specific compounds, and in each case there was no initiation of carotenoid synthesis in the dark. Besides incubation with compounds which might have been inducers, samples of dark-grown mycelium were also incubated with metabolic inhibitors in the

TABLE 18

Partial Reversal of Cycloheximide Inhibition by Washing the Mycelium and Incubating in the Dark

Wavelength of Maxima (mμ)	Optical Density (Per Cent of Control)		
	Incubation and Illumination Conditions		
	Addition of cycloheximide, 15 minutes light, 5.75 hours dark	Addition of cycloheximide, 15 minutes light, 5.75 hours dark; mycelium washed, 6 hours dark without inhibitor	No cycloheximide added; 15 minutes light, 5.75 hours dark (control)
	Neutral Carotenoid Extract		
397	15	42	100
421	12	38	100
440	8	32	100
467	6	28	100
494	4	21	100
525	3	18	100
	Acidic Carotenoid Extract		
477	0	27	100

Dark-grown mycelium was harvested and then illuminated and treated with cycloheximide as shown in the table. Illumination and subsequent dark incubations were carried out at 4°C in Vogel's minimal medium under air as described on page 12, and the carotenoid extracts were prepared as summarized in Figure 5. The concentration of cycloheximide used was $\left[\frac{0.5\ \mu g}{ml}\right]$. The mycelium was washed with two 600 ml portions of water per 135 g of mycelium (fresh weight) to remove the inhibitor.

hope that they would prevent further synthesis of a labile repressor, or prevent it from functioning, but addition of these compounds also failed to initiate carotenoid synthesis.

These experiments do not rule out the possibility that the product of the light reaction is an inducer of enzyme synthesis. Negative results would be expected if any of the following conditions exist:

1. The inducer is present in extremely small quantities in illuminated mycelium.
2. The inducer is a very unstable compound.
3. The chemical structure of the inducer is such that it cannot be taken up from the medium by mycelium.
4. The inducer is tightly bound to some macromolecular component.

Another possibility is that the primary light reaction actually involves the inactivation of a repressor.

Further investigations along these lines may yet reveal information about the mechanism of induction by light. For this reason, a description of the experiments which have been tried is presented below.

In all experiments described in this section, mycelium was obtained by growth under well aerated conditions for 3 days in the dark in carboys (see page 11), and harvesting of mycelium was always carried out under red light (see page 11).

a. Use of Extracts of Illuminated *Neurospora crassa* Mycelium

(1) Aqueous extract of illuminated mycelium

A 135g (fresh weight) aliquot of dark-grown mycelium was placed in the carboy with double baffles (see Figure 3). Nine parts of medium precooled to 4°C were added, and the carboy was evacuated with a water pump, filled with O_2, and then illuminated for 15 minutes at 4°C as described on page 12. At the end of this time, the mycelium was harvested and then ground in 2.5 parts of Vogel's minimal medium and 0.5 parts of sand (washed and ignited) for 10 minutes. The resulting slurry was filtered through miracloth, and the filtrate was added to a 34 g (fresh weight) aliquot of dark-grown mycelum. The mixture was incubated in the dark for 7 hours, under air at 4°C, using the rotator-illuminator shown in Figure 2. At the end of this time, the mycelium was harvested and then extracted as described on page 22. The resulting methanol and acetone extracts were combined and extracted with hexane (see page 24), and the visible spectrum of the hexane extract was read in the range of 375 to 575 mμ.

(2) Methanol-acetone extract of illuminated mycelium

A 127 g aliquot of dark-grown mycelium was placed in the carboy with double baffles (see Figure 3),

9 parts of medium at 4°C were added, and illumination at 4°C was carried out for 15 minutes (see page 12). The mycelium was harvested and extracted with methanol and acetone using the same procedure as in the extraction of the carotenoids (see page 22). The combined extract was reduced to a volume of 19 ml on a flash evaporator at room temperature. Eighteen ml of this solution were added to 90 ml of Vogel's minimal medium precooled to 4°C. As one control, the remaining 1 ml of extract was later diluted by a factor of 5 with medium, and the resulting solution was shown to support growth of Neurospora crassa mycelium. To the 90 ml of solution were added 22.5 g of dark-grown mycelium. The mixture was then incubated in the dark for 14 hours at 4°C using the shaker apparatus shown in Figure 4 at 7/10 maximum speed. At the end of this period, the mycelium was harvested and then extracted and analyzed for the presence of carotenoids as already described above.

b. Dark Incubation of Illuminated and Dark-Grown Mycelium in Common Medium

This experiment was designed to show whether there was any diffusion of an inducer of carotenoid synthesis between mycelium by way of the medium. As already mentioned no evidence for such a compound was obtained. A rectangular piece of nylon with a fine mesh was used to divide the inside of a 6 X 18 inch

chromatographic jar in half. Three edges of nylon were glued to the inside of the container. Masking tape was used to increase the outside diameter of the jar from 6 inches to approximately 7 inches at the positions where it came into contact with the wheels of the apparatus shown in Figure 2. In one-half of the jar were placed 22.5 g (fresh weight) of mycelium, which had been illuminated for 15 minutes under O_2, in 9 parts of medium precooled to 4°C, using the apparatus shown in Figure 2. In the other half of the jar were placed 22.5 g of dark-grown mycelium. Nine parts of Vogel's minimal medium were added, and the top of the container was covered with a piece of cotton. A rubber band was used to hold the cotton in place. The container plus its contents was then rotated in the dark on the apparatus shown in Figure 2 for 6 hours. At the end of this period, a piece of cheese cloth was clamped to the nylon and used to cover the opening to one-half of the jar. The mycelium was poured out of the other half onto a piece of cheese cloth. Using the same procedure, the remaining mycelium in the jar was poured onto another piece of cheese cloth. Excess medium was removed from each sample of mycelium, and each was washed with 50 ml of water (precooled to 4°C) and then extracted and analyzed for the presence of carotenoids as already described.

In the subsequent experiments described in this section, the following experimental procedure was used. Dark-grown mycelium was obtained as already described and 22.5 g aliquots (fresh weight) were each added to 90 ml of Vogel's minimal medium (precooled to 4°C) plus the compound to be tested in a 1000 ml Erlenmeyer flask. The flask was wrapped in aluminum foil and incubation in the dark for 14 hours was carried out using the shaker apparatus shown in Figure 4, at 7/10 maximum speed.

c. Addition of Possible Inducers

(1) Hydrocortisone

Neidleman has reported that hydrocortisone-21-acetate initiated some carotenoid synthesis in Neurospora crassa dark-grown mycelium (140). With hydrocortisone-21-succinate (6.2×10^{-4}M) and hydrocortisone-21-acetate (10.8×10^{-4}M), no such induction was observed.

(2) Indole derivatives

Each of the following compounds was tested at a concentration of 10^{-2}M: indole-3-acetic acid, indole-3-pyruvic acid, indole-3-aldehyde, indole-3-carbinol, and indole-3-carboxylic acid.

(3) Terpene compounds

It has been shown in Phycomyces blakesleeanus that beta-ionone stimulates carotenoid synthesis (141). The following terpene compounds were tested with

Neurospora crassa: beta-ionone, alpha-ionone, nerolidol, nerol, and citronellol (all at concentrations of 1%), and citral at 0.5%. With citral the mycelium turned bright yellow in the dark, but the compound (or compounds) produced could not be identified as a carotenoid.

d. Addition of Metabolic Inhibitors

(1) Inhibitors of RNA or protein synthesis

Each of the following compounds was tested: DL-ethionine (0.5 mg/ml and 6.25 mg/ml) and actinomycin-D (100 μg/ml).

(2) SH group inhibitors

It has been shown that p-hydroxymercuri-benzoate and p-chloromercuribenzoate will initiate carotenoid synthesis in dark-grown Fusarium aquaeductuum (101, 102). The inhibitor, p-chloromercuribenzoate, was tested with Neurospora crassa mycelium at concentrations of 10^{-2}M, 10^{-5}M, 10^{-7}M, or 10^{-8}M. No initiation of carotenoid synthesis was observed.

DISCUSSION

A. THE CAROTENOID BIOSYNTHETIC PATHWAY

A knowledge of the chemical structures of the carotenoids and the colorless polyenes which are present in Neurospora crassa allows one to postulate a finite number of reasonable pathways by which they can be synthesized. Information of this nature served as one basis for formulation of the biosynthetic scheme presented in Figure 21, page 60.

The first study designed to identify these polyene compounds in Neurospora crassa was undertaken by Haxo (111). Some of his conclusions were modified as a result of subsequent investigations. For example, Zalokar reported that zeta-carotene is synthesized by Neurospora crassa mycelium (94), and the carotenoid previously identified as spirilloxanthin was shown to be 3,4-dehydrolycopene by Liaaen Jensen (135). In addition, the presence of torulene in Neurospora crassa was reported for the first time (135). In another investigation (113), the chemical structure of the acidic pigment, neurosporaxanthin, was elucidated, and based on this structural information, it was suggested that gamma-carotene or torulene is the precursor of this pigment (113). Evidence that an additional carotenoid, beta-zeacarotene,

is synthesized by Neurospora crassa mycelium has been obtained (see Results). Identification was based on the spectral information presented in Figure 15, page 52. Furthermore, the relative chromatographic mobility of this carotenoid, compared to the others in the carotenoid mixture, was found to be consistent with information on beta-zeacarotene in the literature (114).

Additional data which support the pathway presented in Figure 21 were obtained by time course measurements of the accumulation of each carotenoid pigment. Under the conditions used in these experiments, it could readily be shown that zeta-carotene is the only carotenoid which accumulates significantly during the first 3 hours of illumination (see Figure 23, page 65). The quantity of this pigment continues to increase for an additional 3 hours and then decreases during the next 12 hours. After a lag period of approximately 3 hours, neurosporene begins to accumulate and attains a maximum concentration after a total of 6 hours of illumination. The quantity of each of the more unsaturated neutral pigments continues to increase for an additional 6 hours. The acidic pigment, neurosporaxanthin, accumulates at a linear rate after a lag period of about 6 hours (see Figure 24, page 67), and the total acidic pigment present after 18 hours is almost 3 times as great as the total neutral pigment.

The fact that zeta-carotene is the first neutral pigment to accumulate significantly is consistent with the biosynthetic scheme presented in Figure 21. The same is true of the significant decrease in the quantity of zeta-carotene which takes place in the interval of 6-12 hours. Another result which is consistent with this proposal is that neurosporene attains a maximum accumulation before the more unsaturated neutral pigments and the acidic pigment.

Further support for the pathway presented in Figure 21 is indicated by the relatively large accumulation of the acidic pigment. It is shown as an end product in the pathway and thus would be expected to accumulate, while the intermediates would likely reach steady state levels and turn over. There is a relatively larger accumulation of the acidic pigment compared to gamma-carotene, another proposed end product of the pathway. According to Figure 21, this would occur if the rate of conversion of neurosporene to lycopene is much greater than that of neurosporene to beta-zeacarotene.

Zalokar has shown that molecular oxygen is required for carotenoid synthesis in Neurospora (94). The data of Table 4, page 70, are consistent with this observation. As shown, the mycelium accumulates almost twice as much pigment with illumination and incubation in oxygen than in air. This difference is due entirely to a larger

quantity of neurosporaxanthin, and there is actually about one-third as much neutral pigment in mycelium treated in oxygen. In addition, each of the neutral pigments, except zeta-carotene, is present in significantly smaller quantities.

In terms of the pathway shown in Figure 21, these data imply that in oxygen each of the steps from zeta-carotene to the acidic pigment is taking place at a faster rate. As a consequence, a larger quantity of the end product neurosporaxanthin is produced, and the steady state levels of the intermediates in the pathway are lower. The increased rate of pigment production is likely due to a requirement for oxygen in the dehydrogenation steps and in the oxidation of torulene to neurosporaxanthin.

As shown in Figure 21, neurosporene is a common substrate for two branches of the pathway. In oxygen, the relative rate of conversion of neurosporene to lycopene compared to that of neurosporene to beta-zeacarotene should be increased since the former reaction would likely require oxygen while the latter would not. Such an assumption would explain the decrease in accumulation of gamma-carotene for mycelium illuminated and incubated in oxygen compared to that in air.

In Table 5, page 71, the change in the quantity of each carotenoid as a result of incubation under poorly aerated conditions is presented. It is clear that there is an accumulation of beta-zeacarotene and torulene, the two carotenoids which are synthesized by cyclization according to the pathway proposed in Figure 21. The accumulation of beta-zeacarotene can be explained in terms of this pathway if it is assumed that under poorly aerated conditions this pigment is synthesized at a faster rate from neurosporene than it is converted to gamma-carotene. Such an assumption seems reasonable since the former reaction would probably not require molecular oxygen while the latter would. On this same basis, the explanation for the accumulation of torulene would be that under these conditions it is synthesized more rapidly from 3,4-dehydrolycopene than it is converted to neurosporaxanthin. It should be noted that there is a net decrease in the quantity of 3,4-dehydrolycopene which is consistent with its conversion to torulene.

The demonstration of the presence of beta-zeacarotene in Neurospora crassa adds support to the proposal that gamma-carotene is synthesized from neurosporene by cyclization and then dehydrogenation rather than from lycopene by cyclization. Radioisotope data, which will be discussed later, also support this contention.

The investigation of several color mutants of Neurospora crassa yielded additional pertinent information concerning the pathway of carotenoid biosynthesis. Unfortunately none of the mutants exhibited a complete block at any step in the pathway; however, it was possible to draw some conclusions from the data and these supported the proposal of Figure 21.

As shown in Table 6, page 73, the mycelium of the yellow mutant (Y30539yA) contains an increased accumulation of lycopene, little or no neurosporaxanthin, and a reduced level of 3,4-dehydrolycopene as compared to that of colonial-4 (the control). This relative distribution of pigment is best explained by the proposed pathway of Figure 21. The data are consistent with this proposal if it is assumed that the conversion of lycopene to 3,4-dehydrolycopene is partially blocked in the color mutant. It should also be noted that two unidentified carotenoids accumulate in the yellow mutant under these conditions. Their visible absorption spectra are similar to those of lycopene and gamma-carotene. It is possible that they are hydroxy derivatives of these carotenoids since this functional group would not cause a change in the absorption spectra but would give the compounds different chromatographic mobilities (114). Positive identification of

these compounds is necessary before the significance of their accumulation in this mutant can be evaluated further.

In the peach mutant (L-pe-4a), there is an increase in accumulation of zeta-carotene, neurosporene, and beta-zeacarotene, and a decrease in gamma-carotene, lycopene, 3,4-dehydrolycopene, and neurosporaxanthin as compared to colonial-4 (see Table 7, page 75). These results are also consistent with the pathway of Figure 21. In this case, it seems likely that the step from neurosporene to lycopene is partially blocked in the color mutant. The fact that there is some accumulation of beta-zeacarotene and a lower quantity of gamma-carotene in this mutant can be explained if the same enzyme which converts neurosporene to lycopene also converts beta-zeacarotene to gamma-carotene. This may very well be the case since in both reactions, the dehydrogenation occurs at the same carbon-carbon bond. Furthermore, the two substrates, neurosporene and beta-zeacarotene, are chemically very similar compounds.

Data obtained in a study using a golden mutant (70007gA) are presented in Table 8, page 76. As with the yellow and peach mutants, the total quantity of pigment synthesized is less than in colonial-4; however, in this case the relative distribution of pigment is the same,

and consequently no conclusions about the sequence of intermediates in the pathway can be made.

Results relevant to the carotenoid biosynthetic pathway shown in Figure 21 were obtained in radioisotope experiments in which 2-C^{14}-mevalonate was employed. Previous evidence that mevalonic acid is a precursor of the carotenoids and phytoene in Neurospora crassa (23) was confirmed, and the specific radioactivities of these 40-carbon compounds were measured and compared under various conditions. In order for these determinations to be meaningful, each of the carotenoids and phytoene must be spectroscopically (uv and visible spectra) and radiochemically pure. Criteria were established to insure that these requirements were met (see Results, page 86). For the carotenoids, these included:

1. Purification to a constant specific radioactivity.
2. Coincidence of the absorption spectrum in the visible range with those in the literature.
3. Removal of uv absorbing impurities.
4. Coincidence of label and pigment in thin layer chromatography.

In addition, it was concluded from data obtained in a control experiment (see Results, page 87) that the radioactivity associated with each of the purified

carotenoid pigments was not due to colorless contaminants which have the same R_f values in thin layer chromatography.

For phytoene the criteria of spectroscopic and radiochemical purity included:

1. Purification to a constant specific radioactivity.
2. Absence of absorption in the visible range.
3. Coincidence of the ultraviolet spectrum with those in the literature.
4. Coincidence of label and phytoene in thin layer chromatography.

The purification procedures which proved successful in meeting these criteria for the carotenoids and phytoene are described in the Materials and Methods section. Extensive use was made of thin layer chromatography, a technique which proved to be more convenient than column chromatography.

Two basic incubation procedures were used in these experiments (see Materials and Methods). In one of these, a dark-grown culture was incubated with 2-C^{14}-mevalonate, and then carotenoid synthesis was initiated by illumination. In the other procedure, a culture which contained a pool of unlabelled carotenoids was incubated with label, and then further carotenoid synthesis was allowed to take place.

The results obtained using the first procedure are presented in Table 10, page 84. The specific radioactivities of the carotenoids synthesized during the period following the incubation with label were observed to be all equal to each other and to the specific radioactivity of phytoene.

During the interval shown, phytofluene is present in such small quantities that its specific radioactivity could not be accurately measured. Since there are no significant pools of carotenoids at the beginning of illumination to dilute the label, these data are consistent with the proposal that phytoene is a precursor of the carotenoids.

This proposal is also supported by the data presented in Table 11, page 89. Further information about the sequence of the carotenoid intermediates in the pathway is also revealed by these results. In this experiment, phytoene was labelled in such a way that its specific radioactivity was higher than that of the carotenoid pigments; then carotenoid synthesis was initiated for a second time. It can be seen that there was a decrease in the quantity of zeta-carotene while the quantity of neurosporene increased 5 fold. At the end of the illumination period, the specific radioactivities of these two compounds were essentially equal. The fact

that the specific radioactivity of zeta-carotene increases significantly during this period shows that it is being synthesized and that its precursor has a higher specific radioactivity. Since there is only a small pool of neurosporene present at time zero, its specific radioactivity at the end of the incubation should be essentially equal to that of its proposed precursor zeta-carotene, and this is shown to be true. The specific radioactivity of lycopene at the end of this period is also essentially equal to that of zeta-carotene and to that of its proposed precursor neurosporene as would be predicted for the same reason. On the other hand, the specific radioactivities of beta-zeacarotene and gamma-carotene are lower. This is to be expected if the relatively large increase in neurosporaxanthin occurs by way of lycopene instead of gamma-carotene. Under the conditions of these experiments, the proposed intermediates between lycopene and the acidic pigment do not accumulate significantly. In another similar experiment, radiochemically pure neurosporaxanthin was prepared and its specific radioactivity was found to be higher than that of beta-zeacarotene and of gamma-carotene and about equal to that of lycopene as expected.

As shown in both Tables 10 and 11, there is a decrease in the specific radioactivity of phytoene during carotenoid

synthesis. This shows that it is being synthesized from a precursor which has a lower specific radioactivity. Even if this precursor is not labelled, there would still be about a 30 per cent increase in the quantity of phytoene if phytoene is a metabolic dead end. It is likely that the specific radioactivity of the precursor is greater than zero so this would mean that the increase would be more than 30 per cent. Estimates of the absolute quantity of phytoene (approximately 600 μg/10g dry weight mycelium) did not show such an increase. This is further evidence that phytoene is being converted to the carotenoids.

While this investigation was in progress, a labelling experiment analogous to the one summarized in Table 10 was reported (25). From the data which were obtained, it was concluded that phytoene could not be a precursor of the carotenoids in Neurospora crassa. This contradictory conclusion is probably due to a failure of the investigators to establish the purity of each of the pigments. In this experiment, carotenoid production was initiated by regulating the supply of medium to a rapidly growing culture. Apparently illumination was continuous thoughout the incubation. During the rapid growth period, radioactive mevalonate was added and phytoene was labelled to a specific radioactivity of about 10^5 cpm/mg, a value

which is remarkably close to the ones shown in Tables 10 and 11. After pigment production had begun, the mycelium was extracted and the carotenoid pigment mixture was chromatographed several times on columns. The radioactivity in the total carotenoid fraction was measured, and from the absorption spectrum of the mixture an estimate of the quantity of total pigment was made. An average specific radioactivity was then calculated and its value was found to be as much as 100 times that of phytoene; however, as reported (25) the pigment fraction contained traces of phytofluene, and based on the experience of the investigations described in this thesis, it seems likely that the fraction was contaminated with other radioactive, colorless impurities. This would explain why the estimated average specific radioactivity of the carotenoid pigments was so much higher than that of phytoene.

B. INITIATION OF CAROTENOID BIOSYNTHESIS

The biosynthesis of carotenoids in Neurospora crassa mycelium is almost completely blocked in the dark; however, upon illumination of dark-grown mycelium, these pigments are rapidly produced (92-96). The action spectrum for the initiation of carotenoid synthesis by light has been investigated by Zalokar (92). He found that the spectrum increases slowly from 400 to 449 mμ,

has a plateau between 449 and 488 mμ, and drops sharply between 490 and 510 mμ. Measurements were not made in the uv absorption range.

On the basis of the action spectrum and from an examination of the pigments present in dark-grown mycelium, Zalokar suggested that the photoreceptor is probably a flavoprotein (92). Such a proposal has also been made for several other organisms in which carotenoid synthesis is initiated by light. These include Fusarium aquaeductuum (100) and Mycobacterium sp. (105, 106).

Zalokar has shown that the initial light reaction goes to completion in one minute if light of sufficient intensity is used (94). This observation was confirmed as shown in Figure 27. At an intensity of 175 foot-candles, 30 seconds of illumination of mycelial pads is as effective in triggering carotenoid synthesis as 30 minutes. In contrast to this, illumination for as long as 60 minutes (see Results) at an intensity of only 5 foot-candles does not initiate as much pigment production as the 30 second stimulus at the higher light intensity.

Zalokar has observed that during the dark period following illumination, the rate of carotenoid synthesis is dependent on temperature and is essentially zero at 0°C. On the other hand, if the dark incubation is

carried out at 25°C, the rate of synthesis is found to be independent of whether the temperature during illumination was 0, 25, or 37°C (92). As shown in Figures 23 and 24, significant pigment production occurs during the 18 hour incubation at 4°C under the conditions of this experiment; however, in agreement with the observations of Zalokar, it was found that the quantity of pigment synthesized at 25°C is 2-3 times larger than that at 4°C when the incubation is under well aerated conditions. In addition, the 3-6 hour lag in pigment production shown in the two figures is reduced to less than 2 hours at 25°C.

As already mentioned (see Results), it was shown by Zalokar that molecular oxygen is required during both the illumination period and subsequent dark incubation in order for significant pigment production to take place (94).

In Mycobacterium sp., it was demonstrated that carotenoid synthesis is initiated by a mechanism analogous to that of Neurospora crassa (103, 105, 106). As already mentioned, the action spectra for initiation of carotenoid synthesis in these organisms are similar (92, 105, 106). In addition, the initial light reaction (or reactions) in Mycobacterium sp. is rapid, temperature independent, and requires oxygen; the dark reaction (or reactions) which

occurs after illumination is temperature dependent and also requires oxygen (103, 105, 106).

Another organism which appears to have a similar regulatory system is Fusarium aquaeductuum (97-102). Its action spectrum for the initiation of carotenoid synthesis is similar to those of Neurospora crassa and Mycobacterium sp. (92, 100, 105, 106). Furthermore, short illumination times will stimulate carotenoid synthesis (99), the primary light reaction (or reactions) is temperature independent (98), and the subsequent dark reaction (or reactions) is temperature dependent (98).

Carotenoid biosynthesis is blocked by the addition of chloramphenicol to Mycobacterium sp. cultures immediately after illumination (103, 105) or cycloheximide to Fusarium aquaeductuum cultures (101). In both cases, if addition of the inhibitors is delayed several hours, significant carotenoid production takes place (101, 103, 105). Chloramphenicol (142) and cycloheximide (143) are known to be inhibitors of protein synthesis. Thus, these data strongly suggest that an enzyme (or enzymes) which is missing in dark-grown cultures is synthesized during a period of time following illumination.

Cycloheximide has been previously demonstrated to be an inhibitor of protein synthesis in Neurospora crassa (138, 139). As shown in Table 14, page 96, carotenoid

synthesis is also blocked by this compound. At a concentration of 2.0 μg/ml, inhibition of pigment production is essentially complete, while at 0.5 μg/ml a small amount of synthesis takes place.

In Tables 15 and 16, pages 98 and 99, the effect of adding cycloheximide at various times is presented. Inhibition of pigment production is almost complete if the inhibitor is added to the medium 8 minutes before or 30 minutes after illumination has begun. On the other hand, the inhibition of carotenoid synthesis by this compound is significantly reduced if it is added 3 hours after the start of illumination. As shown in Table 13, page 94, 15 minutes of illumination is sufficient to initiate significant carotenoid synthesis. Futhermore, the data of Figures 23 and 24 show that there is a lag in the accumulation of the pigments. This varies from less than 3 hours for zeta-carotene to 6 hours for neurosporaxanthin. These facts all taken together demonstrate that there are at least three phases of carotenoid synthesis in Neurospora crassa. These include:

1. An initial light reaction (or reactions).
2. A subsequent period of protein synthesis.
3. Accumulation of the carotenoid pigments.

Such a 3-phase system was originally demonstrated with Mycobacterium sp. by Rilling (103) and later for Fusarium aquaeductuum by Rau (101). It has been proposed that the photoreceptor, most likely a flavoprotein, is activated by light and then catalyzes the photo-oxidation of a compound to form an inducer. The inducer then initiates the functioning of the appropriate gene, and an enzyme (or enzymes) required in the carotenoid biosynthetic pathway and absent in cultures grown in the dark is synthesized (101, 105).

Reactions which are catalyzed by riboflavin, or flavoproteins, plus oxygen and light are known. For example, indoleacetic acid is converted to indolealdehyde in vitro under these conditions (144). It is believed that light converts riboflavin to a metastable compound (possibly in a triplet state) which then oxidizes indoleacetic acid to indolealdehyde. Riboflavin is then recovered by auto-oxidation. It also has been suggested that a similar mechanism is operative in phototropism by Avena coleoptiles (145). According to this proposal, a flavoprotein absorbs light and catalyzes the inactivation of indoleacetic acid on the illuminated side of the coleoptile. As a consequence, the dark side grows faster due to the presence of a relatively larger quantity of the growth promoter and bending toward the light occurs.

Additional information which is pertinent to the mechanism of initiation of carotenoid synthesis by light has been reported recently by Rau (101) and by Rau *et al.* (102). It was shown that p-chloro- or p-hydroxymercuribenzoate could replace light in inducing carotenoid synthesis in *Fusarium aquaeductuum*. Furthermore, cycloheximide was found to block this effect as in the case when light is used as a stimulus (101). Since these compounds are relatively specific for sulfhydryl groups, it was suggested that the photoreceptor, activated by light, catalyzes the photo-oxidation of the SH group (or groups) of a compound and that this results in the formation of an active inducer. Alternatively the photo-oxidation may involve the inactivation of a repressor which contains sulfhydryl groups. In either case, as a result of the oxidation, the pertinent enzyme would then be synthesized (101). It was reported that these compounds were found to be ineffective in initiating carotenoid synthesis in *Neurospora crassa* (101). This result has been confirmed (see Results-Section B 3). This experimental difference between *Neurospora* and *Fusarium aquaeductuum* could mean that different functional groups are involved in the initial photo-oxidation step in the two organisms. Another possibility is that in *Neurospora* the mercury compounds not only affect the SH

groups involved in the light induction but also inactivate a required enzyme which contains SH groups. A third possibility is that in Fusarium aquaeductuum addition of these compounds causes initiation of carotenoid synthesis by a different mechanism from that of light, and such a mechanism is absent in Neurospora. Hopefully, further investigation along these lines will clear up this point.

One case of chemical induction of the synthesis of an enzyme involved in carotenoid production by a compound normally found in nature is known. In the (-) strain of Blakeslea trispora, carotenoid synthesis is stimulated by a series of terpene compounds known as trisporic acids (146, 147). These are excreted into the medium by mated cultures, but they do not stimulate pigment production in the (+) strain. It was found that the effect of one of these compounds, trisporic acid C, can be blocked by the addition of cycloheximide to the medium. The biosynthetic step in the pathway affected by trisporic acid C is unknown, but it is thought to be before the production of phytoene.

As described in Results-Section B 3, a specific compound or crude extract which would replace light in initiating carotenoid synthesis in Neurospora crassa dark-grown mycelium was not found. Further studies of this nature may eventually provide useful information; however, identification of the

inducer by this method will not be successful if its chemical structure is such that it cannot be taken up from the medium by the mycelium. Furthermore, extracts of illuminated mycelium would be unable to induce carotenoid synthesis in dark-grown cultures if the light reaction involves the inactivation of a repressor.

The investigation of the pathway of carotenoid synthesis in Neurospora crassa reveals information concerning its regulation by light since the initial biosynthetic step which is blocked in the dark can be determined. The data presented in Tables 10 and 11 are consistent with the proposal that phytoene is a precursor of the carotenoid pigments in Neurospora crassa. Since phytoene accumulates in dark-grown mycelium, the block in the pathway is likely between phytoene and phytofluene. Thus, the addition of cycloheximide may prevent carotenoid production by inhibiting the de novo synthesis of phytoene dehydrogenase; however, confirmation of this proposal depends on the future development of a workable cell-free system. Such a system presumably could also be used to determine whether subsequent steps in the pathway are blocked in the dark-grown cultures as well.

A study of the albino mutants may reveal further information about initiation of carotenoid synthesis by

light. Hungate (78) showed that the albino mutants are located in two recombination units which are closely linked and that heterokaryons formed between mutants from the two units produce pigment. This investigation was extended later by Huang (77). He observed that albinos from each of the two classes of mutants apparently contain phytoene.

A preliminary investigation of two albinos which complement to form pigment, aur (34508A) and al-2 (15300A), was made. One of these, aur (34508A), is "leaky" and produces small quantities of the pigments when illuminated. It was found that a large quantity of phytofluene does not accumulate in the dark or after illumination in either of these mutants. In terms of the pathway of Figure 21, this information plus the observation of Huang mentioned above implies that the genetic defect in both of the albino mutants affects the conversion of phytoene to phytofluene. If this is the case, then possibly one mutant can carry out the initial light reaction but then induces the formation of a defective enzyme, while the other mutant cannot carry out the light reaction but has the potential to make an active enzyme. Since the two genetic units are closely linked, such a regulatory mechanism may have many of the properties of the operon system found in bacteria. Further studies are necessary to confirm or disprove such a suggestion.

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