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# GERANYLGERANYL PYROPHOSPHATE AS THE CONDENSING UNIT FOR ENZYMATIC SYNTHESIS OF CAROTENES

TUNG-CHING LEE and C. O. CHICHESTER

Department of Food Science and Technology, University of California, Davis, California 95616

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**Abstract**—A cell-free enzyme system capable of converting 4,8,12,16-*trans*-$^{14}$C-geranylgeranyl pyrophosphate into phytoene, phytofluene, ζ-carotene, neurosporene, and lycopene was obtained from a *Phycomyces blakesleeanus* $R_1$ mutant. The conditions of incubation and cofactor requirements for the conversion were defined. The incorporation of radioactive substrate into carotenes was maximal at pH 7·8 with Tris-HCl buffer and in the presence of a sulfhydryl reagent (dithiothreitol) at 25° for 24 hr. Geranylgeranyl pyrophosphate was a much more effective precursor than mevalonic acid in this system and it was demonstrated that geranylgeranyl pyrophosphate is the condensing unit for carotene biosynthesis.

## INTRODUCTION

BY ANALOGY with terpene and steroid biosynthesis, Lynen and Henning[1] predicted the biosynthetic transformation of isopentenyl pyrophosphate ($C_5$) to phytoene ($C_{40}$) would proceed via geranyl pyrophosphate ($C_{10}$), farnesyl pyrophosphate ($C_{15}$) and geranylgeranyl pyrophosphate ($C_{20}$). Goodwin, Yokoyama *et al.*, Porter and Suzue have suggested, on the basis of experimental work, that a C-20 compound (geranylgeranyl pyrophosphate) formed by the coupling of farnesyl pyrophosphate to a C-5 unit is an obligatory intermediate in the reaction, prior to the formation of a C-40 chain (lycopene or phytoene).[2–5]

The enzymatic synthesis of geranylgeranyl pyrophosphate has been demonstrated by Grob[6] in yeast, Nandi and Porter[7] in carrot root and pig liver, and Kandutsch *et al.*[8] in *Micrococcus lysodeikticus*. There has, however, been no experimental proof that this compound is the condensing unit for carotenes.[2, 3]

A tomato enzyme system capable of synthesizing phytoene from isopentenyl pyrophosphate also synthesizes acid labile phosphorylated compounds. Upon gas chromatographic analyses of these compounds, Jungalwala and Porter[9] reported that an appreciable amount of radioactivity from labelled farnesyl pyrophosphate was found associated with geranyllinallol and geranylgeraniol. However, neither they nor workers in this laboratory were able to show that geranylgeranyl pyrophosphate was incorporated to any appreciable extent into

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[7] D. L. NANDI and J. W. PORTER, *Arch. Biochem. Biophys.* **105**, 7 (1964).
[8] A. A. KANDUTSCH, H. PAULUS, E. LEVIN and K. BLOCH, *J. Biol. Chem.* **239**, 2507 (1964).
[9] F. B. JUNGALWALA and J. W. PORTER, *Arch. Biochem. Biophys.* **119**, 209 (1967).

carotenes. There have been many experiments which show that the geranylgeranyl pyrophosphate is formed in systems capable of synthesizing carotenes and there is also some circumstantial evidence which indicates that a C-20 unit is the condensing unit, but no direct proof of the fact that geranylgeranyl pyrophosphate is the condensing unit has been forthcoming.

Recently, methods for an enzymatic synthesis of geranylgeranyl pyrophosphate by homogenates of endosperm tissue of immature *Echinocystis macrocarpa* seed was reported by Oster and West.[10] Using this system we were able to synthesize, separate and purify a relatively large amount of $^{14}$C-*trans*-geranylgeranyl pyrophosphate for use as a substrate for further studies on carotenoid biosynthesis.

In this paper we report using a partially purified cell-free enzyme system obtained from *Phycomyces blakesleeanus*[11] for the enzymatic conversion of $^{14}$C-geranylgeranyl pyrophosphate into phytoene, phytofluene, ζ-carotene, neurosporene, and lycopene and present evidence that geranylgeranyl pyrophosphate is the condensing unit for C-40 carotenoids.

## RESULTS AND DISCUSSION

### *Preparation of* $^{14}$C-trans-*Geranylgeranyl Pyrophosphate*

$^{14}$C-*trans*-geranylgeranyl pyrophosphate was enzymatically synthesized from 2-$^{14}$C-mevalonate using the *Echinocystis macrocarpa* system according to Oster and West.[10] Confirmatory assays were made in our laboratory with authentic samples kindly provided by Oster and West. The following assays showed the identity and purity of $^{14}$C-*trans*-geranylgeranyl pyrophosphate: (1) acid hydrolysis; (2) enzymatic hydrolysis; (3) thin-layer and paper chromatography; and (4) assay of pyrophosphate by the method of Rosenberg.[12]

### *Assay of Carotenes in* Phycomyces blakesleeanus $R_1$ *Mutant*

Carotenes from this mutant were isolated, purified and identified according to normal methods (see Experimental section). The carotene contents are shown in Table 1. In essence acyclic carotenes (lycopene, neurosporene, etc.) predominate, whereas cyclic carotenes (β-carotene, β-zeacarotene, etc.) are present only in trace amounts.

TABLE 1. CONCENTRATION AND ABSORPTION MAXIMA IN LIGHT PETROLEUM OF CAROTENOIDS FROM *Phycomyces blakesleeanus* $R_1$ MUTANT

| Carotenoid | $E^{1\%}_{1\,cm}$ | $\lambda_{max}$ (nm)† | Concentration μg/g dry weight |
|---|---|---|---|
| Phytoene | 1250 | 275, 285, 296 | 490 |
| Phytofluene | 1350 | 331, 348, 367 | 83 |
| ζ-Carotene | 2270 | 376, 399, 425 | 42 |
| Neurosporene | 2990 | 416, 440, 470 | 36 |
| Lycopene | 3450 | 446, 472, 505 | 975 |
| β-Zeacarotene* | 2570 | 406, 428, 454 | Trace |
| γ-Carotene | 3100 | 437, 462, 494 | Trace |
| β-Carotene | 2505 | 425, 451, 482 | Trace |

* In hexane.
† The center peak had the highest intensity in all cases.

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*Incorporation of* 2-$^{14}$*C Mevalonate and* $^{14}$*C*-trans-*Geranylgeranyl Pyrophosphate into Carotenes*

The total recovered carotenoids were counted in all cases. The counts for each compound therefore reflect the total incorporation less the percentage which was not recovered during crystallization or chromatography. These losses vary slightly between the individual carotenes, but generally do not exceed 20 per cent of the total counts.

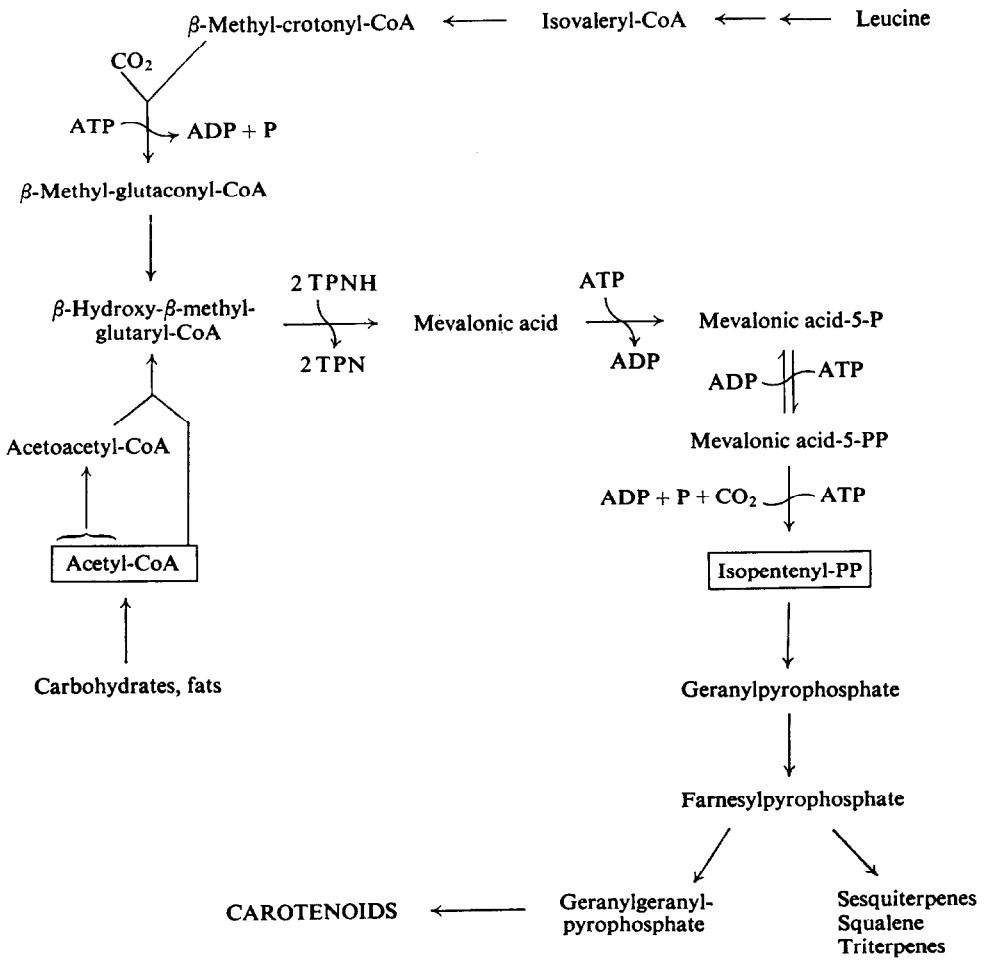


FIG. 1. POSTULATED INTERMEDIATES IN CAROTENOID BIOSYNTHESIS.

Experiments were performed as shown in Table 2. As seen from the results of experiments 2, 3 and 4 (Table 2), $^{14}$C-geranylgeranyl pyrophosphate was incorporated into phytoene, phytofluene, ζ-carotene, neurosporene and lycopene. The incorporation of radioactivity indicates that geranylgeranyl pyrophosphate can act as the condensing unit for carotene synthesis.

Comparing the results of experiment 1 with experiments 2 and 3 (Table 2), $^{14}$C-geranylgeranyl pyrophosphate was incorporated into carotenes much more efficiently than 2-$^{14}$C-mevalonate. When the relative utilization of mevalonate and geranylgeranyl pyrophosphate

were compared on the basis of total incorporation, geranylgeranyl pyrophosphate is eleven times as effective as mevalonate. This would confirm proposed pathways shown in Fig. 1, i.e. the steps of carotene biosynthesis, from mevalonate, proceed via isopentenyl pyrophosphate ($C_5$), geranyl pyrophosphate ($C_{10}$), farnesyl pyrophosphate ($C_{15}$), geranylgeranyl pyrophosphate ($C_{20}$) to phytoene ($C_{40}$).

TABLE 2. INCORPORATION OF 2-$^{14}$C-MEVALONATE AND $^{14}$C-*trans*-GERANYLGERANYL PYROPHOSPHATE INTO CAROTENES

| | | Experiment No.* | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| Radioactive substrate | | 2-$^{14}$C-MVA | $^{14}$C-GGPP | $^{14}$C-GGPP | $^{14}$C-GGPP | $^{14}$C-GGPP |
| Total activity added (d.p.m.)† | | $240\times10^3$ | $102\times10^3$ | $102\times10^3$ | $102\times10^3$ | $102\times10^3$ |
| Incubation period | | 24 hr | 24 hr | 25 hr | 10 hr | 24 hr |
| Total activity of non-saponifiable fraction (epiphase, carotene) (d.p.m.) | | 7,120 | 64,000 | 48,000 | 61,600 | 700 |
| % Incorporation yield into non-saponifiable fraction | | 2·9% (5·8%)‡ | 57% | 43% | 55% | — |
| Incorporation of radioactivity (d.p.m.) | Phytoene | 88 | 866 | 1,200 | 5,318 | — |
| | Phytofluene | 884 | 3,224 | 5,126 | 932 | — |
| | ζ-Carotene | 109 | 1,112 | 2,990 | 1,540 | — |
| | Neurosporene | 199 | 3,004 | 4,328 | 1,360 | — |
| | Lycopene | 846 | 9,082 | 5,424 | 3,688 | — |
| Incorporation into total carotenes (d.p.m.) | | 2,125 | 18,288 | 19,068 | 12,838 | 0 |
| % Incorporation into total carotene fractions | | 0·85% (1·70%)‡ | 17·9% | 18·7% | 12·6% | 0% |

* The incubation systems contained:

*Experiment* 1: The reaction flask contained 2000 μmoles of Tris-HCl buffer, pH 7·8; 100 μmoles of ATP; 15 μmoles of NADH; 15 μmoles of NADPH; 15 μmoles of NAD; 750 μmoles of $Mg^{2+}$; 500 μmoles of dithiothreitol; protein 680 mg; and 2-$^{14}$C-MVA ($240\times10^3$ d.p.m.), in a total volume of 40 ml. The incubation period was 24 hr at room temperature. At the end of the incubation period, nonradioactive carotenes were added before extraction of the pigments. *Experiment* 2: All contents are similar to Experiment 1 except the use of $^{14}$C-*trans*-geranylgeranyl-pyrophosphate (total activity $102\times10^3$ d.p.m.) instead of 2-$^{14}$C-mevalonate. *Experiment* 3: The reaction flask contained 2000 μmoles of Tris-HCl buffer, pH 7·8; 500 μmoles of dithiothreitol; 750 μmoles of $MgCl_2$; protein 680 mg; and $^{14}$C-geranylgeranyl-pyrophosphate (total activity $102\times10^3$ d.p.m.), in a total volume of 40 ml. The incubation period was 24 hr at room temperature. At the end of the incubation period, nonradioactive carotenes were added before extraction of the pigments. *Experiment* 4: All contents in the incubation flask are similar to Experiment 3. The incubation period was 10 hr. *Experiment* 5: All contents in the flask are similar to Experiment 3, with the exception that boiled (100°, 3 min) enzyme preparation was used.

† Disintegrations per min.

‡ One optical isomer utilized.

Comparing experiment 2 and experiment 3. It was shown that the cofactors required for the conversion of mevalonic acid to carotenes by the *P. blakesleeanus* enzyme system (ATP, NADH, NADP, NADPH are not essential for the conversion of geranylgeranyl pyrophosphate to carotenes. This is reasonable since these co-factors are needed in earlier condensing steps.

The maximal conversion was obtained at pH 7·8, Tris-HCl buffer at room temperature

for 24 hr, in the presence of $Mg^{2+}$ and a sulfhydryl reagent (dithiothreitol). As cited by Yokoyama *et el.*,[11] the stimulation by manganese in the same enzyme system for the conversion of mevalonate to $\beta$-carotene is of interest, since manganese accelerates both enzymatic and non-enzymatic decarboxylation;[13,14] stimulation by manganese of cholesterol synthesis from acetate and mevalonic acid has also been shown.[15] Magnesium could be substituted for manganese, but the activity of the system was decreased. This is in contrast with the case of acetate, which requires magnesium for the conversion of acetate to mevalonic acid.

Recently Jungalwala and Porter[9] reported that both $Mn^{2+}$ and $Mg^{2+}$ are required for the conversion of isopentenyl pyrophosphate to phytoene. However, only $Mg^{2+}$ is required when geranylgeranyl pyrophosphate is the substrate in tomato plastid enzyme systems.[16] Our result here is thus consistent with those demonstrated by Porter *et al.*

The presence of sulfhydryl stabilizing agent (GSH or dithiothreitol) presumably functions in maintaining sulfhydryl groups of the enzymes in the reduced state.

Experiments 3 and 4 (Table 2) show the distribution of radioactivity in carotenes. After 10 hr of incubation, phytoene contains the largest amount of radioactivity. At the end of 24 hr the radioactivity in phytoene decreases, whereas the radioactivity in phytofluene, $\zeta$-carotene, neurosporene, and lycopene all increases. It can thus be inferred that phytoene participates in the synthesis of the carotenes and quantitative analysis of the data suggests the postulation that phytoene is an intermediate in the synthesis of carotene is acceptable.

Porter and Lincoln[17] have suggested that lycopene formation in tomatoes proceeds through the stepwise dehydrogenation of phytoene, via phytofluene, $\zeta$-carotene and neurosporene to lycopene. This possibility has also been demonstrated in experiments here at this time by using $^{14}$C-geranylgeranyl pyrophosphate as a substrate for carotene formation in cell-free enzymes from *P. blakesleeanus* $R_1$ mutant.

Recent communication with Porter *et al.*[16] indicates they have also found a soluble tomato plastid enzyme system for the conversion of geranylgeranyl pyrophosphate into phytoene which was maximal at pH 7·6–7·8 with borate buffer at 25° for 4 hr. The conversion also depended upon the presence of $Mg^{2+}$ and a sulfhydryl reagent (dithiothreitol). The similarity between their system (enzyme from tomato plastid) and ours (enzyme from *P. blakesleeanus* $R_1$ mutant) thus indicates the ubiquity of this general conversion route occurring in different organisms.

## EXPERIMENTAL

### *Solvents*

Light petroleum (b.p. 30–50°), acetone, methanol, and diethyl ether, all analytical reagent grade, were redistilled and the light petroleum passed through silica gel (grade 12, Grace Division, Davidson Chemical Co., U.S.A.) prior to use.

### *Materials*

The *N,N'*-dibenzylethylenediammonium salt of 2-$^{14}$C-DL-mevalonic acid was obtained from New England Nuclear Corporation, Boston, Mass., U.S.A.

Authentic geranylgeranyl pyrophosphate and geranylgeraniol were gifts from Drs. M. O. Oster and C. A.

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[17] J. W. PORTER and R. E. LINCOLN, *Arch. Biochem. Biophys.* **27**, 390 (1950).

West, University of California, Los Angeles, U.S.A. Other chemicals, enzymes, and chromatographic adsorbents were obtained from commercial sources.

*Organisms*

*Phycomyces blakesleeanus* $R_1$ mutant was kindly provided by Dr. G. Meissner, California Institute of Technology, Pasadena, California, U.S.A. *Echinocystis macrocarpa* Greene seeds were collected during March from Carrol Canyon, Los Angeles, California, U.S.A.

*Preparation of $^{14}$C-Geranylgeranyl Pyrophosphate*

$^{14}$C-*trans*-geranylgeranyl pyrophosphate was synthesized enzymatically from 2-$^{14}$C-mevalonate by a soluble enzyme fraction of a homogenate of endosperm tissue of immature *E. macrocarpa* seed, according to Oster and West.[10] Purification was by (1) collidine (2-methyl-5-ethylpyridine) extraction of the incubation mixture; (2) transfer of the pyrophosphate ester to 0·01 M sodium bicarbonate solution from a collidine–ether mixture; and (3) chromatography of the bicarbonate extract on a DEAE-Sephadex A-25 column developed with an ammonium bicarbonate gradient. The identity of *trans*-geranylgeranyl pyrophosphate was shown by the following procedures:

*Acid Hydrolysis of Geranylgeranyl Pyrophosphate*

Aqueous samples were acidified with sufficient 6·0 M HCl to bring the final concentration of acid to 0·1 M. After heating (50–60°) ½ hr a 20 per cent excess of 10 M NaOH was added to neutralize the acid. The acid-released material was extracted with three 2-vol. portions of benzene–acetone (3:1).

*Enzymatic Hydrolysis*

Aqueous samples were adjusted to either 0·1 M or 0·5 M Tris-HCl, pH 8·3, with 1·0 M buffer and were treated with 0·1 mg of bacterial alkaline phosphatase per ml of sample. After 60 min of incubation at 30°, the liberated material was extracted from the mixture with benzene–acetone as described above.

*Thin-Layer and Paper Chromatography*

The products from incubation with geranylgeranyl pyrophosphate as the substrate were assayed by chromatography of the acetone or benzene–acetone extract or precoated thin-layer plates of silica gel F254 (Merck), or chromatographed on acid-washed paper. Solvents systems, developing procedure and detecting method were described by Oster and West.[10]

*Culture Condition*

The cultures of *P. blakesleeanus* $R_1$ mutant were grown aerobically on a medium of 2·5% D-glucose (w/v), 0·5% yeast autolysate (w/v), 6 mg thiamine-HCl/100 ml medium, $KH_2PO_4$ (0·15 g/100 ml medium), $MgSO_4$ (0·75 g/ 100 ml medium). Organisms inoculated to 250 ml medium in a 1000 ml Erlenmeyer flask were grown on shake cultures at 20°.

*Preparation of Cell-Free Enzyme Systems*

Detailed procedures were previously described.[11] 4–5-day-old mycelial mats of *P. blakesleeanus* were cooled to approximately 4°, cut into small pieces, and placed in a cold homogenizing medium containing 0·2 M Tris-HCl buffer, pH 8·0, 0·2 M nicotinamide, and 0·002 M glutathione. The mixture was ground for 30 sec in a very loosely fitting Potter–Elvehjem homogenizer containing 2 vol. of the suspending medium at approximately 500 rev/min. The unbroken cells and tissue debris were eliminated by centrifugation for 5–10 min at $4000 \times g$ at 4°. Dialysis (2 l. 0·002 M Tris changed twice for 10 hr at 4°), was used to remove the salts and low molecular weight substances. Since prolonged storage of the preparation at 4° was not feasible, it was used immediately after dialysis.

*Incubation of Homogenate*

The conditions and contents varied from experiment to experiment. The details are given in Table 2. The substrates and co-factors were added to incubation systems as concentrated stock solutions. Incubations were carried out at room temperature in air in a Laminar Flow Ultra-Clean workbench (Agnew-Higgins Inc., Garden Grove, Calif., U.S.A.) to prevent microbial contamination during long incubation periods. The lack of contamination was verified by microscopic examination before and after experiments. Incubations were stopped by the addition of 3 vol. of EtOH.

*Preparation of Carrier*

The quantity of carotene and related materials in the incubation mixtures was so small that often it became necessary to add inactive carrier to the mixture to prevent loss and to aid in the extraction of carotene and

related materials. 8–10-day-old mycelia of *P. blakesleeanus* $R_1$ mutant was disintegrated with glass beads in the Waring blender and centrifuged at about $15{,}000 \times g$ for 10 min. The supernatant was discarded and the pellets used as carriers.

*Isolation and Purification of Radioactive Carotenes and Related Polyenes*

After incubation and the addition of nonradioactive carrier mycelial pellets the reaction mixture was heated for 1 min at 100° and centrifuged for 5 min at $1000 \times g$. The supernatant fraction was discarded. Carotene and related polyenes were extracted from the pellet with MeOH and acetone (15:85 v/v). The hydrocarbons were transferred to light petroleum by the addition of water and saponified by the addition of equal volumes of 20% KOH–MeOH. After saponification, the epiphase was washed and dried ($Na_2SO_4$).

The pigment extract was chromatographed in a tightly packed $22 \times 200$ mm column of MgO-Hyflo Super-Cel (1:1, w/w). The column was developed with 0·5% acetone–99·5% light petroleum (P.E.) followed by 4 and 8% acetone. The column was allowed to run dry after pytoene and phytofluene had been eluted and the various other carotenoid bands were cut out and eluted with acetone and MeOH from the adsorbent. Phytoene and phytofluene were purified by rechromatographing on a 20 mm column (12 mm i.d.) of alumina. The column was developed according to the procedure described by Jungalwala and Porter.[18] Each compound was collected as a series of eluents of 5-ml fractions. The radioactivity of each fraction was determined by scintillation counting. Phytoene and phytofluene were therefore chromatographed twice, whereas the other carotenes were further purified by crystallization as below.

*Counting Technique*

In all experiments, the recrystallized samples of the carotenes (lycopene, neurosporere, and ζ-carotene) were added to a counting vial and dissolved in 3 ml of toluene, the samples previously having been crystallized from mixtures of MeOH and hexane. The mixture was bleached by using a 275 W General Electric Co. sunlamp for 1 hr beyond the complete disappearance of color. In counting purified colorless materials (phytoene, phytofluene) appropriate aliquots were added to vials, the solvent was evaporated, 3 ml of toluene were added, and the samples were bleached along with the colored carotenes. Double-strength scintillation solvent was added, and the resultant solution was counted in a liquid scintillation spectrophotometer (Packard Tricarb Model 314E, Packard Instrument Co., Inc., La Grange, Ill., U.S.A.). Solvent was prepared by dissolving 1 mg of 1,4-bis-2-(5-phenyloxazolyl-L-benzene) and 12 mg of 2,5-diphenyloxazole in 100 ml of toluene. Corrections for quenching were obtained through counts on internal standards. In order to allow extraneous counts to disappear, bleaching samples were generally held 24 hr. A blank solution of toluene was also irradiated with u.v., and the counting of the radioactive samples was not started until the extraneous counts in this sample had disappeared.

*Spectrophotometric Analysis*

Absorption spectra were made with a Cary 14 recording spectrophotometer (Cary Instruments, Monrovia, California, U.S.A.). Identification of the various carotenes was made on the basis of their position on the column and their absorption spectra.[19]

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