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SUBCELLULAR SITE OF CAROTENOID BIOSYNTHESIS IN *NEUROSPORA CRASSA*

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Key Word Index—*Neurospora crassa*; fungi; carotenoid biosynthesis; membranes; photoregulation; carotenogenic enzymes; cell-free system.

Abstract—A cell-free system prepared from *Neurospora crassa* mycelia was capable of incorporating radioactivity from [^{14}C]-mevalonic acid into phytoene and to a much lesser extent into more unsaturated carotenoids. Whereas carotenogenic activities were only minimal in extracts from dark-grown cultures, they were several-fold increased following a short *in vivo* illumination; this photo-induced increase was inhibited by cycloheximide. Subcellular localization of carotenogenic enzyme activities was investigated using incubations of particular isolated fractions, together with [^{14}C]-mevalonic acid and a geranylgeranyl-pyrophosphate-synthesizing system provided by the endosperm of maturing pumpkin seeds. Maximum carotenogenic activity (*ca* 80%) was localized in two membrane fractions previously shown to contain plasma membranes and, in particular, membranes of the endoplasmic reticulum. The lipid layer, containing the bulk of carotenoid pigments, possesses only trace amounts of enzyme activities.

INTRODUCTION

In a number of micro-organisms biosynthesis of carotenoids is photo-induced. From experiments using inhibitors, it has been concluded that in such organisms carotenogenic enzymes are most likely formed *de novo* as a consequence of illumination (for ref. and discussion, see [1, 2]). As a prerequisite for further elucidation of the photoregulatory mechanism, in particular for direct evidence of photo-induced *de novo* synthesis of the enzymes, the elaboration of a functional *in vitro* assay system is required for accurate estimation of enzyme activities. Carotenogenic enzyme activities, so far not calculable by simple enzyme tests, have been demonstrated in cell-free systems obtained from bacteria [3, 4], fungi [5, 6] and higher plants [7, 8]; the enzyme system derived from tomato fruit plastids is the only one that has been partially purified so far [9, 10]. However, these enzyme preparations are not suitable for investigations designated to study photoregulation because in the organisms where they originated carotenogenesis is not under strict photocontrol (except for the system from *Mycobacterium* sp. [11, 12]).

In higher plant tissues carotenogenic enzymes are assumed to be compartmented in the chloroplast [13, 14]. Studies on localization in fungi have been carried out only very recently. In a preceding paper [15] we presented data for carotenoid distribution in subcellular fractions of *Neurospora crassa*, a fungus which features photo-induced carotenogenesis [15–18]; carotenoids are mainly located in cell fractions enriched in membranes of the endoplasmic reticulum and in a supernatant lipid layer. Moreover, we reported in a preliminary form [19] on the elaboration of a crude cell-free system from mycelia of *N. crassa* capable of synthesizing phytoene and carotenoids from mevalonic acid, as well as on the localization of carotenogenic enzyme activity. Spurgeon *et al.* [20] also isolated a cell-free system from *N. crassa* which converted isopentenyl pyrophosphate into phytoene. Our present paper describes characteristics of the system, subcellular site of carotenogenesis and the photoregulation of enzyme activities.

RESULTS

Elaboration of cell-free system

Illuminated mycelia were homogenized and the resulting crude extract was incubated, after addition of cofactors, for varying lengths of time with ^{14}C-labelled mevalonic acid ([^{14}C]-MVA). For the preparation of the incubation mixture the cofactors described for other cell-free systems [5, 21] were added with only minor variations.

From the unsaponifiable lipid fraction, the ^{14}C-labelled substances were isolated and identified as described in the Experimental. Incorporation of radioactivity into the unsaponifiable lipid fraction was proportional to the amount of crude extract when up to 3 mg protein per ml incubation medium were added. Therefore, unless stated otherwise, the assay mixture contained *ca* 21 mg protein in a total volume of 7.5 ml.

The incorporation of radioactivity into phytoene and more unsaturated carotenoids was significantly affected by both the age of the mycelia and the time interval between illumination of the fungus and preparation of cell-free extracts. Maximum incorporation was obtained using homogenates prepared from mycelia 2 hr after start of the photoinduction, i.e. when carotenogenic enzymes were present in optimum quantities. This time also coincides with the period of highest rate of pigment accumulation *in vivo* [17, 22].

Incorporation of radioactivity from [^{14}C]-MVA into the unsaponifiable lipid fraction depends on the duration

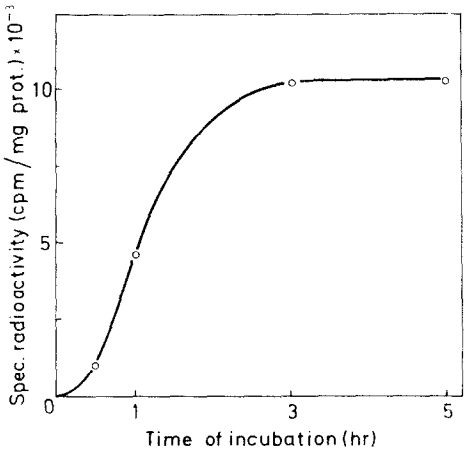

Fig. 1. Kinetics of incorporation of radioactivity from [2-^{14}C]-MVA into the unsaponifiable lipids using a crude extract prepared from illuminated mycelia.

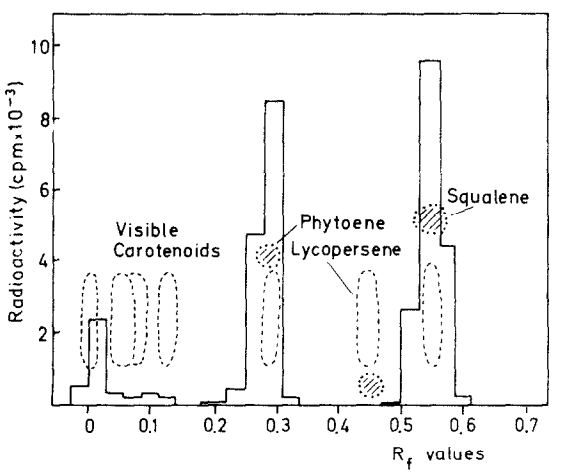

Fig. 2. Separation of a 2% Et_2O fraction (obtained by column chromatography) on Si gel plates using C_6H_6–petrol (1:14). Radioactivity was estimated by liquid scintillation spectrometry after scraping off the Si gel zones (continuous line). Visualization of substances by I_2 and UV staining is indicated by the dotted line; hatched spots show authentic standards.

of incubation as illustrated by Fig. 1. The shape of the curve indicates that, under the conditions used, the enzymes involved were active for 2–3 hr. Throughout the incubation period [^{14}C]-MVA was present in excess. The crude enzyme preparations incorporated up to 22% (*ca* 7 × 10^{-6} M) of the biologically active isomer of [^{14}C]-MVA into unsaponifiable lipids; as a control a boiled enzyme extract showed no incorporation.

The incorporation of radioactivity into different metabolites was determined after a 3 hr incubation and subsequent separation of the unsaponifiable lipids. The data compiled in Table 1 demonstrate that nearly 80% of the radioactivity was recovered from squalene and sterols and *ca* 12% from the phytoene fraction; only a minor amount of radioactivity was found in more unsaturated carotenoids. Moreover, after saponification, low radioactive label was found in prenols, e.g. farnesol (F) and geranylgeranyl (GG). Labelled phytoene and the other carotenoids (identified as described in the Experimental) could be unequivocally separated from contaminating compounds, such as squalene, by the use of TLC (Fig. 2).

Compared to *in vivo* studies, phytoene-synthesizing activity was found to be satisfactory, whereas biosynthesis of the more unsaturated carotenoids seemed to be poor in the crude enzyme system. Radioactivity recovered from sterols was found in several intermediates, while synthesis of ergosterol, the major sterol of *N. crassa*, was very low. No labelled prephytoene and lycopersene were detected, consistent with *in vivo* analysis.

Localization of carotenoid-synthesizing enzymes

In a preceding paper [15] we reported on the preparation of subcellular fractions by differential centrifugation of cell homogenates and on their enzymatic characterization and their carotenoid content. Several centrifugation steps yielded different pellet fractions (designated as P_x, x indicating $g \times 10^{-3}$), a final supernatant referred to as soluble fraction (S_{115}) and a lipid layer on top of it (LL). In order to investigate the subcellular localization of carotenogenic enzymes, the same fractions were prepared from mycelia 2 hr after start of illumination and incubated for 3 hr using the medium described for the crude extract. The incorporation of radioactivity into several metabolites was subsequently estimated (Table 2).

Similar activities as found in the crude extract were also exhibited in a system compiled of aliquots of all cell fractions isolated ('recombined system'); this indicates that preparation procedures did not cause severe damage to the enzymes. No radioactivity was incorporated when both recombined and single particulate cell fractions were incubated omitting the soluble fraction (S_{115}). Furthermore, in the soluble fraction and in the overlying lipid layer, labelling of carotenoids (including phytoene)

Table 1. Incorporation of [2-^{14}C]-MVA into different substances of the unsaponifiable lipid fraction mediated by a crude extract during incubation for 3 hr

	Carotenoids					
	Phytoene	Others	Geranylgeranyl	Farnesol	Squalene	Sterols
Total incorporation (cpm)	27 700	2060	11 000	4350	74 600	107 000
Specific incorporation (cpm/mg protein)	1260	90	500	200	3400	4860
% of total	12.1	0.9	4.8	1.9	32.6	46.6

Table 2. Incorporation of radioactivity from [2-^{14}C]-MVA into several metabolites by different cell fractions (3 hr incubation)

Fraction	Specific incorporation (cpm/mg protein)					
	Phytoene	Carotenoids	GG	Farnesol	Presqualene* Squalene	Sterols
Crude extract	1320	170	130	210	3150	5160
Recombined pellets	0	0	0	0	0	0
S_{115}	300	0	850	620	7540*	450
Lipid layer	250	0	310	450	8480*	370
Recombined system	2430	100	70	370	3740	3190

* Presqualene was identified in addition.

and sterols was strongly reduced, whereas incorporation of radioactivity into prenols and squalene was increased. Enzymes synthesizing presqualene and squalene seemed to be soluble. From these data we concluded that a GGPP-synthesizing system is present in the soluble cell fraction, while carotenogenic enzymes are located in the particulate fractions.

In order to test this assumption each of the different pellet fractions was incubated together with the 115000 g supernatant (S_{115}) and [^{14}C]-MVA for 3 hr. A high ratio of soluble to particulate bound protein (11:1) was chosen; additionally, an ATP-regenerating system (0.13 mM PEP; 100 μg pyrophosphate kinase) and potassium fluoride (13.3 mM) as an inhibitor of active phosphatases were added to the incubation medium. The results (Table 3) show that due to the supplementation with the soluble fraction, the different pellet fractions incorporated [^{14}C]-MVA into phytoene and, to a much lesser extent, also into more unsaturated carotenoids. Maximum synthesizing activity for carotenoids and also for sterols was present in the fractions P_{21} and P_{115}; these have been previously [15] characterized as microsomal-membrane fractions whereas P_{13} is enriched in mitochondria. However, the rates of incorporation of MVA into phytoene and other carotenoids appeared to be insufficient, possibly resulting from substrate (GGPP) deficiency.

In order to overcome this presumed deficiency by an increased supply of the system with [^{14}C]-GGPP (which is not commercially available), we prepared a supplementary GGPP-synthesizing *in vitro* system reported by Oster and West [23] as modified by Graebe [24]. This system consisted of the semi-liquid endosperm of maturing seeds of *Cucurbita pepo* (pumpkin) and cofactors as described in the Experimental. By adding the growth retardant AMO 1618 (1 μM; final concentration), an inhibitor also for cyclases [25], the conversion of MVA into kaurene was inhibited to some extent, thus presumably causing an increased accumulation of [^{14}C]-GGPP.

For investigation of carotenoid-synthesizing activity in the different particulate fractions of *N. crassa* under more optimal conditions, combined incubations were carried out in the following way: enzyme preparations from *Cucurbita pepo* endosperm (30 mg protein) were preincubated for 2.5 hr; the complete incubation medium included AMO 1618 and [^{14}C]-MVA. Subsequently, carotenoid synthesis was initiated by addition of the particular cell fractions from *N. crassa* (1 mg protein each), prepared from illuminated mycelia. A reaction time of 10 min proved to be optimal for both sufficient incorporation of radioactivity into carotenoids and prevention of substrate deficiency. As a control, an enzyme preparation from *Cucurbita pepo* endosperm was incubated without any *Neurospora* fraction. The results of these experiments (Table 4) show that the synthesizing capacity of enzymes from *Cucurbita pepo* endosperm was very low except for GG and kaurene, particularly when calculated on a 1 mg protein basis. Furthermore, comparison of the data obtained with the pure endosperm system on the one hand and the combined systems on the other hand demonstrates quite clearly that the incorporation capacity into kaurene, farnesol and squalene was exclusively due to the enzymes of the endosperm

Table 3. Synthesizing activities of different subcellular fractions prepared from illuminated mycelia using the soluble protein fraction as a precursor source (3 hr incubation)

Fraction	Specific incorporation (cpm/mg protein)*					
	Phytoene	Carotenoids	GG	Farnesol	Squalene	Sterols
$P_{2.3}$	940	20	190	550	6520	550
P_{13}	1460	20	250	470	5840	720
P_{21}	2460	80	100	280	2650	3130
P_{115}	1900	150	40	240	5010	2030

The protein concentrations used were: particulate bound protein (1.5 mg) and soluble protein from S_{115} (16.6 mg).

* Expressed cpm per mg total protein.

Table 4. Biosynthesis of several metabolites during 10 min incubation of different subcellular fractions (1 mg protein each) together with a GGPP-synthesizing system from *Cucurbita pepo* (30 mg protein). *N. crassa* fractions were prepared from illuminated mycelia

Fraction	Specific incorporation (cpm/mg protein)*						
	Phytoene	Carotenoids	GG	Kaurene	Farnesol	Squalene	Sterols
$P_{2,3}$	9730	240	9430	15100	530	1930	2990
P_{13}	67300	1300	11900	20100	560	2910	15400
P_{21}	226000	4650	13200	19100	460	3100	41500
P_{115}	205000	2760	11300	15900	420	2990	36600
S_{115} + lipid layer	1710	124	7430	18800	350	2370	1880
	Radioactivity (cpm)†						
Cucurbita endosperm	650	140	9260	28000	460	3030	2980

* Specific incorporation is given per mg *N. crassa* protein.
+ Incorporation of radioactivity in the control is shown in cpm.

whereas incorporation of radioactivity into phytoene, carotenoids and sterols was mediated by the subcellular fractions from *N. crassa*, predominantly by the fractions P_{21} and P_{115}. Consequently, synthesizing activity for all carotenoids and also for sterols can be unequivocally localized in the cell fractions P_{21} and P_{115}. Low carotenogenic enzyme activity in a mitochondrial cell fraction (P_{13}), might be due to a contamination with membranes [15]. Calculated on a percentage basis of total enzyme activity, about 80% of carotenogenic enzymes are located in the two membrane fractions P_{21} and P_{115}. Since the P_{115} fraction contains about 4 times more protein than the P_{21} fraction, the bulk of enzymes for biosynthesis of phytoene and other carotenoids seems to be localized in the P_{115} fraction.

Effect of in vivo *illumination on carotenoid-synthesizing activity in cell-free systems*

Subcellular fractions were prepared from both dark-grown and illuminated mycelia and incubated for 10 min in the combined system described above. Results illustrated in Fig. 3 show that in dark-grown cultures low levels of carotenogenic enzyme activities were present in the particular fractions indicating that minor amounts of enzymes are constitutive. Illumination of the mycelia resulted in a drastic increase of incorporation of radioactivity into phytoene (12-fold) and other carotenoids (8-fold), particularly in the subcellular fractions P_{13}, P_{21} and P_{115}. Labelling of FPP and GGPP does not seem to be under photoregulation; incorporation into sterols was found to be reduced after *in vivo* light treatment (data not shown).

Addition of cycloheximide (CHI) at a final concentration of 0.05 mM to mycelia prior to illumination completely inhibited photo-induced carotenoid accumulation [17]. As shown in Table 5 for crude extracts prepared from CHI-treated and untreated mycelia and incubated as described, CHI also completely inhibited the photo-induced increase of incorporation of radioactivity into phytoene and more unsaturated carotenoids. Direct inhibition of enzyme activities by CHI can be ruled out since MVA incorporation in crude extracts from CHI-treated mycelia is similar to that from mycelia which are not photo-induced.

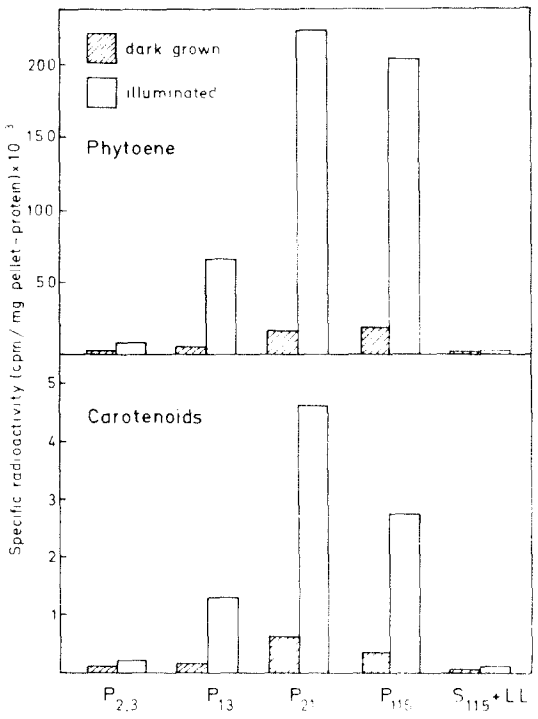

Fig. 3. Effect of an *in vivo* illumination (30 min) on the level of synthesizing activity for phytoene and more unsaturated carotenoids in different cell fractions of *N. crassa* (10 min enzyme reaction). Incubation conditions are given in the text.

DISCUSSION

A cell-free system was developed from mycelia of *Neurospora crassa* which is able to incorporate radioactivity from [^{14}C]-MVA into phytoene and to a much lesser extent into the more unsaturated carotenoids (Table 1) indicating the presence of carotenogenic enzyme activity. As might be expected, more label was recovered from squalene and sterols, because the enzymes of this biosynthetic pathway also use MVA as a precursor and the content of sterols is 20 times greater than that of

Table 5. Inhibition of photo-induced synthesizing activity for phytoene and carotenoids by addition of cycloheximide (CHI) in crude extracts from *N. crassa*

		Dark	Light	Light + CHI
Phytoene	Sp. inc.*	280	2620	230
	%†	2.7	22.7	2.2
Carotenoids	Sp. inc.*	0	219	0
	%†	0	1.9	0

The extracts were prepared from dark-grown (dark) or illuminated (light) mycelia. CHI was added 30 min prior to illumination to a final concentration of 0.05 mM.

* Specific incorporation (cpm/mg protein).

† Expressed as percentage of total [^{14}C].

carotenoids in mycelia of *N. crassa* [22, 26]. Furthermore, in homogenates of an albino mutant of this species, incorporation of radioactivity into sterol precursors using labelled MVA was substantial whereas that into phytoene was minimal [27]. Therefore, a relatively low labelling of tetraterpenes might be due to a limited availability of prenols, withdrawn by sterol biosynthesis.

As in several other micro-organisms (for ref. see [1, 2]) carotenoid accumulation in *N. crassa* is photo-induced. The results presented show that, in cell-free preparations from dark-grown mycelia, a low level of phytoene and also minor amounts of carotenoid-synthesizing activities are present; in agreement with the data of Spurgeon *et al.* [20] brief *in vivo* illumination resulted in 12- and 8-fold increases, respectively, of these activities.

From results of experiments using inhibitors of protein synthesis (e.g. cycloheximide), it has been concluded that photo-induced carotenoid accumulation is due to *de novo* synthesis of carotenogenic enzymes as a consequence of illumination. Most convincing evidence for such a conclusion has been obtained using cell-free extracts, so far only available from *Mycobacterium* sp. [11, 12]. Further support now comes from the results of Spurgeon *et al.* [20] and those presented in this paper (Table 5) showing that CHI applied to mycelia prior to illumination completely inhibited the increase of carotenogenic capacity tested in enzyme extract incubations.

For our investigations concerning subcellular localization of carotenogenic enzyme activities, the same fractions as reported for pigment localization [15] were used. The results presented clearly indicate that not all enzymes catalysing the formation of carotenoids from MVA are located in the same cell compartment. A GGPP-synthesizing system was present in the soluble cell fraction (Table 2); consequently, prenyltransferases from *N. crassa* apparently are also soluble as reported for bacteria and higher plants [12, 23, 28]. Enzyme activities for the biosynthesis of both phytoene and more unsaturated carotenoids could be located in particulate subcellular fractions. This was unequivocally demonstrated in experiments using incubations of *Neurospora* cell fractions together with endosperm of maturing pumpkin seeds which obviously provide sufficient amounts of GGPP (Table 4; Fig. 3). In the supernatant (S_{115}) as well as in the lipid layer, only trace amounts of carotenogenic activities were present; the bulk of carotenoid-synthesizing capacity was localized in two membrane fractions previously characterized as containing plasma membranes and, in particular, membranes of the endoplasmic reticulum (ER) [15]. Carotenogenic activity associated with a fraction enriched in mitochondria (P_{13}) might be due to contamination with membranes as indicated by the presence of marker-enzyme activity of ER in this fraction. In contrast to our results, Spurgeon *et al.* [20] reported that a 100000 *g* supernatant was most effective as an enzyme source in their experiments. However, there might be no disagreement since the authors pointed out that 'a turbid layer which sediments but does not pellet' is necessary for synthesis of phytoene, and therefore presumed a membrane bound enzyme.

There are some indications that localization of enzymes of the biosynthetic pathway of carotenoids might differ in bacteria, fungi and higher plants. For *Halobacterium* sp. it cannot be excluded that carotenogenic enzyme activities are present in the soluble cell fraction [3]; a partially purified enzyme system isolated from plastids of tomato fruits appeared to be a complex [9] but was shown to dissociate [10].

In several fungi the majority of carotenoids was recovered from the supernatant lipid layer [15, 29–31] which is essentially comprised of spherosomes [32]. Our data (Table 4) show that the lipid layer contained only trace amounts of carotenogenic activity which might be, moreover, due to contamination. Consequently, at least in *N. crassa*, compartments for biosynthesis and storage of carotenoids are different; spherosomes have only a storing function whereas enzymes for biosynthesis are localized in membranes presumably of the ER which, besides spherosomes contain substantial amounts of the pigments [15]. Whether localization of the carotenogenic enzymes in membranes may support the model of an 'enzyme aggregate', as postulated from genetical and physiological results on carotenoid biosynthesis in *Phycomyces* (for ref. see [33]) needs further clarification.

EXPERIMENTAL

Standard compounds. The chemicals used were of the highest grade of purity available. Ergosterol, geraniol, farnesol, lanosterol and squalene were obtained commercially. DL-[2-^{14}C]-Mevalonic acid lactone was from Amersham–Buchler Corp. Geranylgeranyl and lycopersene were generous gifts from Hoffmann–La Roche u.Co., Ltd. Basel, Switzerland.

Biological material, growth conditions and photo-induction of carotenogenic enzymes. N. crassa (wild type strain, ATCC 10816) was grown as liquid cultures for 22 hr in the dark, harvested, resuspended in a buffer–glucose soln and illuminated (with white light) under experimental conditions employed in earlier

studies [15]. Time of illumination was 30 min with an incident fluence rate of 60 W/m^2 which is sufficient for the first satn level of the photoreaction [34] thus inducing satisfactory amounts of active enzymes during the subsequent incubation in the dark at 22° for 2 hr on a rotary shaker.

Preparation of cell-free extracts. Two hr after photo-induction mycelia were harvested as described previously [15], mycelial pads were resuspended (0.5 g wet wt/ml) in ice-cold homogenization buffer containing 0.2 M Tris·HCl, (pH 8), 0.2 M nicotinamide and 2 mM GSH [5], and ground with sand in a mortar for 8 min. The homogenate was filtered through 2 layers of gauze and then centrifuged at *ca* 700 *g* for 20 min, thus removing cell debris and spores. The resulting supernatant was used as crude enzyme extract in subsequent expts. All procedures were carried out at 0–4°. Influence of light was minimized by working under dim light conditions. For fractionation of the crude extract, centrifugation steps used were similar to those described previously [15]: 2300 *g*, 10 min; 13000 *g*, 12 min; 21000 *g*, 30 min; 115000 *g*, 2.5 hr. The resulting enzyme prepns were incubated as soon as possible, but all at the same time. Protein concn was determined by the method of ref. [35], with BSA as standard.

Incubation system. DL-[2-^{14}C]-MVA lactone (sp. act. 10.9 mCi/mmol) was converted prior to incubation to its K salt with aq. KOH according to ref. [36]. In the incubation mixture (final vol. 7.5 ml) concns of components were: DTT, 12.5 mM; ATP, 5 mM; $MgCl_2$, 18.8 mM; $MnCl_2$, 1.3 mM; NAD(P) and NAD(P)H, 0.373 mM each; FAD, 0.2 mM; [2-^{14}C]-MVA, 0.015 or 0.03 mM (1.2 or 2.5 μCi); finally 21 mg protein were added. Incubations were carried out in 18 ml gas wash flasks at 25° in a H_2O bath shaker under weak aeration and in dim day light. Reactions were stopped by addition of EtOH and Me_2CO, 5 ml each. Any variations in incubation conditions are given in the Results. Samples were either immediately saponified or stored at −20° in a N_2 atmosphere.

Extraction of unsaponifiable lipids. KOH soln (in MeOH, 60% w/v) was added to the samples (0.1 ml KOH/ml) and the mixture saponified overnight at room temp. After addition of unlabelled carotenoids as carriers (*ca* 5 μg per sample), the unsaponifiable lipids were extracted according to ref. [24] with slight modification: Protein was ppted with Me_2CO and centrifuged for 5 min at 500 *g*; from the ppt. lipids were extracted (×4) with Me_2CO–MeOH (3:1). This led to a residue ppt. free from radioactivity. From the combined aq. Me_2CO supernatants, lipids were extracted with petrol (×5). The petrol extract was washed, dried (Na_2SO_4), evapd to dryness and redissolved in petrol (20 ml); radioactivity was determined in an aliquot (0.5 ml).

Column chromatography of radioactive terpenols. Preliminary separation of the unsaponifiable lipids was carried out on 14 × 0.5 cm columns of Al_2O_3 (Woelm neutral, Brockmann activity grade III) using increasing concns (v/v) of Et_2O in petrol (40–60°) slightly modified according to ref. [37]. The substances eluted by each fraction are given in parentheses: 50 ml 0% (squalene, phytoene and more saturated carotenoids), 50 ml 2% (more unsaturated carotenoids), 80 ml 12% (prenols, sterols), 50 ml 100% Et_2O–petrol (ergosterol). The different lipids were identified by their chromatographic behaviour compared with authentic compounds and by their absorption spectra. The identity of phytoene was confirmed, in addition, by chromatography on a column of Al_2O_3 (Brockmann activity grade I–II) modified according to ref. [38]. Phytoene, present as the 15-*cis* isomer, has been found on the same column, but with Brockmann activity grade I according to ref. [39]. Sterols were identified, in addition, by the Liebermann–Burchard test according to ref. [38].

TLC of terpenols. The lipid fractions eluted by column chromatography were further separated using various TLC systems. Squalene, lycopersene and phytoene were usually separated from more unsaturated carotenoids on activated Si gel 60, F 254 (Merck) with C_6H_6–petrol (1:24) as developing solvent; the method was slightly varied according to ref. [24]. The R_fs were increased (R_f phytoene 0.28; lycopersene 0.45; squalene 0.5–0.6) using unsaturated chambers, thus yielding better separation of substances. Marker spots of authentic samples were applied on chromatograms for comparison. Substances were located under both day and UV light, and by exposure to I_2 vapor. Prenols were separated from sterols using paraffin-impregnated Si gel layers (obtained by dipping in a 4% v/v soln of liquid paraffin in petrol (40–60°) with Me_2CO–H_2O (13:7) as solvent system, slightly modified according to ref. [40]. Sterols remaining at the plate origin were eluted and re-chromatographed on activated Si gel 60, F254 with EtOAc–C_6H_6 (1:5) according to ref. [37]. Authentic standards were co-chromatographed.

Radioactivity determinations. Radioactivity on TLC plates was detected by scanning. Radioactive positions were determined by scraping the Si gel layer off in sections and counted in a liquid scintillation spectrometer using toluene–butyl-PBD scintillation mixture; counting efficiency was 96%.

Calculation of data. Data in figures and tables represent mean values of 2–3 expts, samples run in duplicate.

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