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A Carotenogenic Enzyme Aggregate in *Phycomyces:* Evidence from Quantitive Complementation

(heterokaryons/mutants/lycopene/phytoene)

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ABSTRACT **Wild-type *Phycomyces blakesleeanus* accumulates β-carotene, while the mutant strain C2 is unable to synthesize carotenoids, and the mutant strain C9 accumulates lycopene. Heterokaryons containing a proportion, p, of C2 nuclei and $(1 - p)$ of C9 nuclei accumulate lycopene, γ-carotene, and β-carotene in the relative amounts $(1 - p)$, $p(1 - p)$, and p^2, respectively, for different values of p.**

It is shown that these results are expected from the operation of a carotenogenic enzyme aggregate that works as an assembly line and contains two copies of a cyclase, which is defective in strain C9, as well as other enzymes.

The biosynthesis of carotenoids is thought to proceed according to the scheme depicted in Fig. 1. From the first 40-carbon compound, phytoene, a series of four dehydrogenations leads to the production of lycopene, two further cyclizations convert it to β-carotene. β-Zeacarotene substitutes for lycopene as an intermediate in an alternative proposal, which changes the order of the last dehydrogenation and the first cyclization (1, 2).

The discovery of multienzyme systems in the synthesis of fatty acids and other compounds (3–5) has led to speculations (6) that carotenoid synthesis might be organized in an analogous way, but no evidence has yet been found for such a multienzyme aggregate for carotenoids.

The wild types of the fungus *Phycomyces blakesleeanus* accumulate large amounts of β-carotene, resulting in bright yellow mycelia, sporangiophores, and young sporangia. Red mutants (accumulating lycopene) and white mutants (accumulating phytoene or unable to synthesize carotenoids altogether) have been isolated (7, 8).

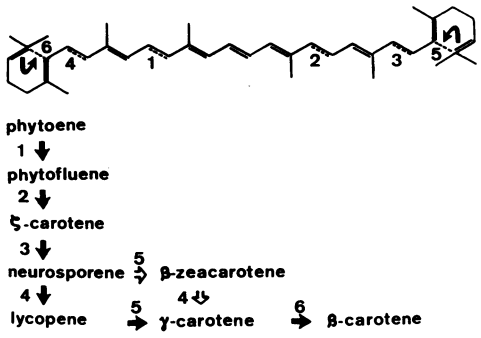

FIG. 1. Biosynthesis of β-carotene from phytoene. *Vertical arrows* indicate dehydrogenations, with formation of a double bond; *horizontal arrows* indicate cyclizations. The *hollow arrows* represent an alternative proposal.

Phycomyces heterokaryons containing two types of nuclei, one type from a strain failing to produce carotenoids and the other from a strain accumulating lycopene, are of many different shades of color, from whitish to orange, according to the proportion of the two types of nuclei (9). This observation led us to think that perhaps a mixture of carotenoids was resulting from the operation of an enzyme complex.

We report now on the results of quantitative analyses of the carotenoids (lycopene, γ-carotene, and β-carotene) produced by such heterokaryons. They coincide with the expected result if the synthesis is organized by enzyme complexes operating as assembly lines, and permit the description of certain features of such complexes.

MATERIALS AND METHODS

P. blakesleeanus, strain C2, carrying the mutation *car-5* (previously designated Alb. 5) produces a white mycelium and white fruiting bodies and contains only about 1% of the usual concentration of β-carotene and no other carotenoids. Strain C9, carrying mutation *carR21* (previously R1) is bright red, because of lycopene accumulation (8). Both were isolated from wild type strain NRRL1555, sexual type (−), after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (7).

A heterokaryon C2 * C9, containing nuclei of both strains, was constructed artificially. The nuclei of the heterokaryon are randomly distributed into multinucleate spores, which can give rise to either type of homokaryon or to heterokaryons of different nuclear proportions. A heterokaryon may be propagated with a constant nuclear proportion by plating a small piece of young mycelium onto a new medium (7).

Mycelia are grown on glucose–asparagine–yeast solid medium (9) at room temperature (18–23°C) under normal daylight conditions.

For chemical analysis, the mycelium grown 4 days in a Petri dish is scraped off with a spatula, carefully cleaned with tweezers of bits of agar and young sporangiophores, and stored in the dark at −20°C if the analysis is not done immediately. The mycelium is thawed at room temperature, dried with filter paper, cut to small pieces, and divided into two weighed portions, one for determination of dry weight by exposure to 105°C for 1 hr and the other for carotenoid extraction. The latter portion (usually 0.2 g dry weight) is blended in a Sorvall Omni-Mixer with 20 ml of methanol and

20 ml of petroleum ether (boiling point 50–70°C) for 3 min; the operation is repeated twice after changing the petroleum ether layer, and the three resulting petroleum ether fractions are mixed and centrifuged at 2000 rpm to remove any gross particles. The supernatant is concentrated to about 5 ml under a stream of nitrogen, and is chromatographed on a MgO–diatomaceous earth column (8). The spectra, taken with a Beckman DK-2A recording spectrophotometer, and the chromatographic properties of lycopene, γ-carotene, and β-carotene coincide with published data (10, 11). The concentrations of these pigments are calculated from their absorptivities (12); their sum is taken as total carotenoid content since no other carotenoids were found in appreciable amounts.

The nuclear proportions in a heterokaryotic mycelium are deduced from the proportions of white (C2), red (C9), and yellow (heterokaryotic) mycelia arising from the spores of such a heterokaryon by a procedure described previously (7, 9). The tubes are scored as containing yellow (alone or mixed), red, white, red and white, or no mycelium. The average concentration of C2 and C9 homokaryotic spores is determined from their Poisson distributions in tubes not containing yellow mycelia. The value of the proportion of C2 nuclei in the heterokaryon is then obtained from the theoretical curve reproduced in Fig. 2.

RESULTS

The results of genetic analyses of nuclear proportions are given in Fig. 2. The average of several independent determinations is taken as the proportion of C2 nuclei in the heterokaryon. Table 1 gives quantitative analyses of carotenoids in heterokaryotic mycelia with different nuclear proportions; each analysis refers to material from a different Petri dish.

INTERPRETATION

The results fit the expectations from the hypothesis that carotenogenesis is performed by a linearly-organized enzyme aggregate working under the following rules:

Each enzyme receives its substrate from the previous one and passes its product over to the next enzyme. Intermediate substrates cannot be transferred from one aggregate to another, nor taken up from the surrounding cytoplasm.

The mutant genes in strains C2 and C9 produce defective enzyme molecules that substitute for the normal ones in the aggregate. If one of the enzyme molecules is defective, the corresponding intermediate substrate is released from the aggregate.

The gene products of the many nuclei present in a mycelium combine randomly to form the aggregates.

Each aggregate contains two copies of a cyclase, performing the cyclization steps. This enzyme is defective in strain C9. The aggregate may also contain one copy of the enzyme that is defective in strain C2 and is involved in an unknown step before the formation of phytoene.

Fig. 3 diagrams the expected activity of the carotenogenic aggregates in homokaryons C2 and C9 and heterokaryons C2 * C9. Aggregates blocked before the formation of phytoene, because they received the faulty enzyme from C2, are unable to synthesize carotenoids. The other aggregates synthesize lycopene if the first cyclase is copied from C9 (irrespective of whether the second is copied from C2 or C9), γ-carotene if the first is copied from C2 and the second from C9, and β-carotene if both are copied from C2. The expected

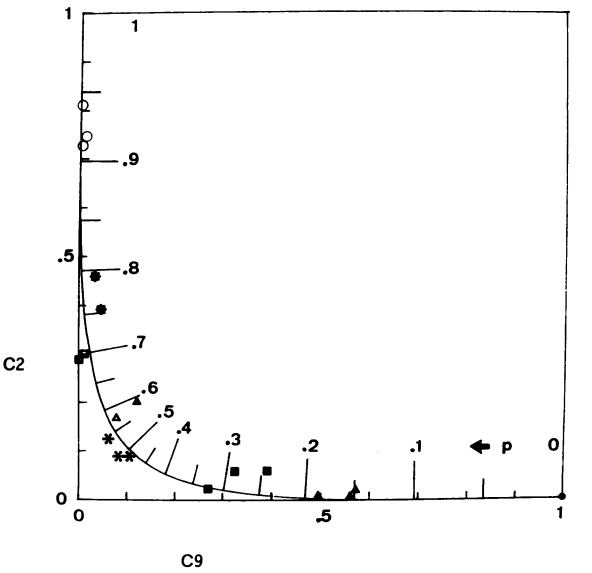

FIG. 2. Proportions of C2 and C9 homokaryotic spores produced by C2 * C9 heterokaryons. Identical symbols refer to genetic analyses performed on different samples of the same heterokaryon. The continuous curve represents theoretical expectations and its sidelines point to corresponding values of the proportion of C2 nuclei in the heterokaryon, p.

proportions of these pigments in the total carotenoid content as a function of the proportion of C2 nuclei, p, are $(1-p)$ for lycopene, $p(1-p)$ for γ-carotene, and p^2 for β-carotene. The predicted and the experimental values are shown in Fig. 4. The concordance is excellent and constitutes strong sup-

TABLE 1. *Quantitative analyses of carotenoids in heterokaryotic C2 * C9 mycelia with different proportions of C2 nuclei, p (averaged from Fig. 2)*

p	Total carotenoid content (μg/g dry weight)	Relative proportions		
		Lycopene	γ-Carotene	β-Carotene
0.000	415	1.0	0.0	0.0
	300	1.0	0.0	0.0
	560	1.0	0.0	0.0
	500	1.0	0.0	0.0
0.167	311	0.82	0.11	0.06
	337	0.78	0.15	0.07
	337	0.81	0.12	0.07
	491	0.86	0.10	0.04
0.290	204	0.70	0.16	0.14
	422	0.72	0.17	0.11
	221	0.68	0.18	0.14
0.513	54	0.48	0.27	0.25
	77	0.45	0.26	0.30
0.580	201	0.37	0.26	0.36
0.695	73	0.31	0.22	0.48
	62	0.32	0.22	0.47
	63	0.36	0.26	0.38
0.775	171	0.26	0.18	0.56
0.923	43	0.20	0.19	0.61

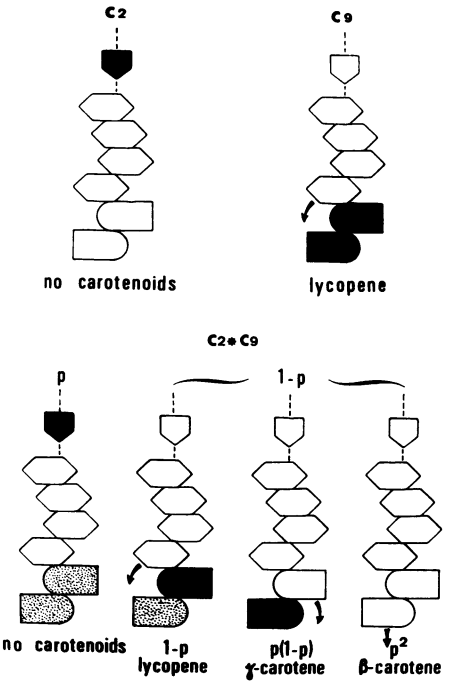

FIG. 3. Operations performed by the carotenogenic aggregates of homokaryons C2 and C9 and heterokaryons C2 * C9, indicating the expected proportion of each product according to the rules specified in the text. *White* symbols represent active enzymes, *black* symbols, defective enzymes, *dotted* symbols, either active or defective.

port for the proposed model of cyclization. The total carotenoid content should be proportional to $(1-p)$, in the absence of unknown regulations or uncontrolled environmental influences. The experimental results (Fig. 5) suggest the integration in the aggregate of the enzyme defective in C2.

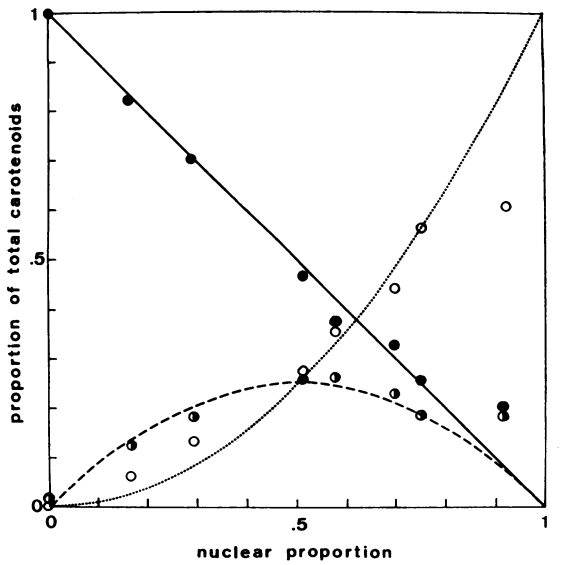

FIG. 4. Average proportions of lycopene, γ-carotene, and β-carotene in the total carotenoid content of heterokaryotic mycelia as a function of the proportion of C2 nuclei in the heterokaryon. *Circles* indicate experimental results; *continuous lines*, the theoretical expectations from Fig. 3. Lycopene, ●—; γ-carotene, ◑– – –, and β-carotene, ○· · · · ·

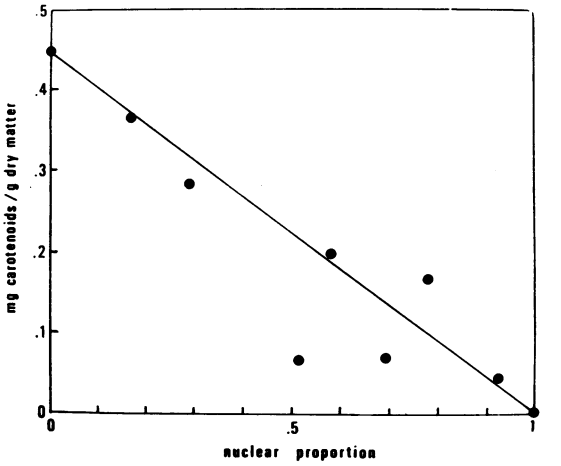

FIG. 5. Total carotenoid content in the heterokaryotic mycelia as a function of the proportion of C2 nuclei in the heterokaryon.

DISCUSSION

The results definitively eliminate many simple models of carotenogenesis, particularly the random action of free enzymes. They establish the sequential action of two copies of a cyclase, which are part of a multienzyme aggregate. Each cyclase cannot be a multimer with identical subunits coming from different nuclei. Various steric designs are compatible with the data, for example, branching aggregates with several two-cyclase teams connected to a single chain of early enzymes.

Further support for the hypothesis comes from our observation that 2-(4-chlorophenylthio)triethylamine hydrochloride (13) and 2-(*p*-diethylaminoethoxibenzal)*p*-methylacetophenone (received from Dr. H. Yokoyama) provoke in the wild type the accumulation of lycopene, γ-carotene, and β-carotene. The action of both compounds may be interpreted as cyclase inhibition.

The dehydrogenations may be explored by the analysis of the carotenoids in heterokaryons where one component is blocked in a dehydrogenation. Preliminary results with heterokaryons of C5 (which accumulates phytoene) and C6 (which produces no carotenoids) indicate that phytoene, phyto-

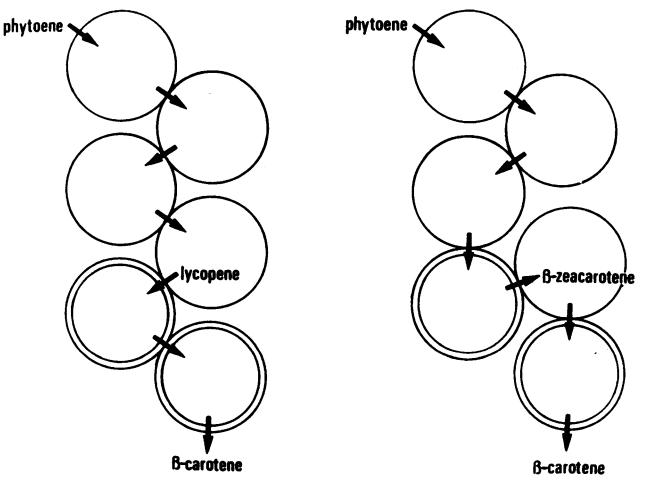

FIG. 6. Hypothetical representation of the carotenogenic aggregate (*left*) indicating that a slight structural modification (*right*) might alter the sequence of the reactions and have β-zeacarotene as an intermediate instead of lycopene.

fluene, ζ-carotene, neurosporene, and β-carotene are present in the proportions expected if the aggregate contains four copies of a dehydrogenase that is deficient in C5.

It has long been debated whether the β-carotene pathway passes through lycopene or β-zeacarotene. Our results are easier to accommodate with the first suggestion and explain some observations supposed to favor the second. In *Rhizophlyctis rosea*, an aquatic fungus, the lycopene made in the earlier part of its life has been shown not to be a precursor for the later synthesis of γ-carotene (1). We would predict that the earlier carotenogenic aggregates have no cyclase and produce lycopene; later a single copy of cyclase is added to each aggregate, and the new aggregates make γ-carotene. However, they cannot use the previously made lycopene as a substrate, just as lycopene made in cyclase-deficient aggregates in *Phycomyces* is not transferred to other aggregates. If the spatial structure of Phycomyces aggregates resembles the one depicted in Fig. 6, *left*, a slight structural change may lead to the structure represented in Fig. 6, *right*, which will produce β-zeacarotene as an intermediate. Perhaps the dehydrogenase inhibitor, diphenylamine, induces such a change, resulting in the observed accumulation of β-zeacarotene and other intermediates (1).

As suggested above for *Rhizophlyctis*, the structure of the aggregates may depend on the species and even on the developmental stage. Quantitative complementation studies should be feasible in *Neurospora* and other fungi, and the central point, $p = 0.5$, should be readily observed in heterozygous diploids.

The results reported here depend on the random assortment of gene products to form the aggregate and the lack of transfer of intermediate substrates. These features may not be universal, and they seem not to apply to the *arom* aggregates in *Neurospora* (14). In our case, it is likely that water-soluble compounds, like the cyclase inhibitors, have access to the aggregates from the cytoplasm, while the water-insoluble carotenoids do not. It is then difficult to predict the usefulness of quantitative intergenic complementation in studying the organization of other cell functions. *Phycomyces* is particularly well suited for such studies because of the stability of the heterokaryons and the easy determination of nuclear proportions.

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