GENETIC AND NON-GENETIC FACTORS IN PIGMENTATION OF NEUROSPORA CRASSA

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THE biochemical genetics of carotenoid pigments has been studied in the tomato (LEROSEN *et al.* 1941; MACKINNEY and JENKINS 1949; PORTER and LINCOLN 1950), corn (MANGELSDORF and FRAPS 1931) and red yeast (BONNER *et al.* 1946). HAXO (1949) worked out the carotenoid components of the wild type Neurospora. The present paper deals with genetic and biochemical studies of a modifier system for pigmentation in Neurospora and the effect of light on this system.

MATERIALS AND METHODS

In the genetic analysis, spores were isolated on complete medium including nutritionally normal strains, since unmistakable judgment of all the color types can be made only on complete medium. For biochemical studies of pigments four nutritionally wild-type color types of the same mating type *a* from a cross of a valine-isoleucineless mutant 16117*A* to albino 15300*a* were used. The medium for mass culture was HAXO's enriched medium (HAXO 1949). 3L-Fernbach flasks containing 300 ml of agar medium were used for mass culture. Mold from 5 to 15 such flasks was used for each experiment depending upon the pigment content of the strain. The cultures were incubated for four days at 25°C in a dark room, followed by ten days at room temperature (25 ± 2°C) illuminated with a 14 watt daylight fluorescent lamp at a distance of 50–60 cm.

The procedure of extraction and chromatography adopted by HAXO (1949) was followed except for minor modifications. Extraction was done with the aid of a Waring blender. The residue was saved for dry weight determination. The same amount of acetone was used in the extraction for all the color types irrespective of their color intensity taking the most intensely colored type as a standard.

Columns of size 35 × 250 mm and 20 × 200 mm were used for chromatography. Hyflo super cel instead of celite was used as the filter aid. Identification and quantitative estimation of the carotenoids were made exclusively with a Beckman spectrophotometer.

GENETICS

When spores from crosses of the valine-isoleucineless 16117*A* or the wild-type *crassa* 1*A* to the albino strain 15300*a* were isolated on complete medium,

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asci of two and four color types could be distinguished in the fully grown cultures. In the latter the color types were salmon (the wild-type color), pink, peach and white (the albino color). In the former they may be either salmon and white or pink and peach. A two factor hypothesis was proposed. Accordingly the genotype of the parents may be represented as *C* (or $+^{al_2}$, the major color gene) *I* (the intensifier) and *c* (or al_2) *i*. The four color type ascus therefore would be the tetratype and the two-color type ascus, the parental and non-parental ditypes (PERKINS 1949).

This hypothesis was tested by making further crosses. Thus, crosses of *cI* × *CI*, *ci* × *Ci* and *ci* × *cI* gave exclusively ditype in eleven asci, while the cross of *Ci* × *cI* gave one tetratype in two asci.

An analysis of 51 asci, 6 from a cross of *crassa* 1*A*, 35 from valine-isoleucineless 16117*A* to the albino strain 15300*a*, and 10 from Chilton *a* to 15300*A*, gave the centromere distance for the *C* gene as 25 map units, as compared with the published value 24.6 (HOULAHAN *et al.* 1949), and that of the *I* gene 20 map units.

Among 41 asci the numbers of tetratypes, parental and non-parental ditypes were 23, 11 and 7, respectively. This indicates that *C* and *I* are not linked. If two genes are linked then the number of asci of parental ditype should be more than that of non-parental ditype, because both noncrossover and two strand doubles would give rise to the parental ditype while only four strand doubles would give rise to the non-parental ditype. Conversely, if two genes are not linked then the parental and non-parental ditypes should always be equal, and the number depends upon the centromere distance (PERKINS 1949 table 3).

ANALYSIS OF PIGMENTS

A preliminary experiment was carried out with the wild type *crassa* 1*A*. The preliminary chromatogram and the chromatogram of the major sections were in essential agreement with the results of HAXO (1949). One extra component was identified tentatively by its absorption maxima and minima (max. 377, 397, 422, 452; min. 385, 410, 445) as pigment B described in the red yeast (BONNER *et al.* 1946, max. 377, 399, 424; min. 386, 411). On the preliminary chromatogram it was barely visible on the column lying between δ-carotene (sec. D, table 1) and β-carotene (sec. F). No quantitative estimation of the pigments in *crassa* 1*A* was attempted.

The colors of the extracts of the four types, *CI*, *Ci*, *cI* and *ci*, were golden yellow, reddish orange, red, and light yellow. The results of the chromatograms of the epiphase of the four types before developing further with 3 percent acetone in petroleum ether are shown in table 1, the *CI* type being identical with that of *crassa* 1*A*.

The major sections of the *CI* type after rechromatography again gave chromatograms essentially identical with those of *crassa* 1*A*. The quantity of pigments eluted from the chromatograms of all the color types is shown in table 2. The quantities of isomers which could be the results of isomerization in the

TABLE 1

Major sections of chromatograms of the epiphase of the four color types. Figures in parentheses represent the breadth of the section, in mn.

Major section (major pigment)	CI	Ci	cI	ci
A (spirilloxanthin)	rose red (3)	same (3)	faint red (3)	faintly colored streak[1]
B (lycopene)	reddish orange (8)	same (3)		
C (neurosporene)	lemon yellow (9)	same (6)	faint yellow (5)	....
D (γ-carotene)	orange (5)	same (4)	same (1)	....
(δ-carotene)	faint red (14)	....	....	....
	colorless (15)	same (20)	same (20)	....
E (pigment B)	very faint yellow (7)	same (7)	....	....
	colorless (20)	same (35)		
F (β-carotene)	pale orange (15)	same (10)	very faint (10)	....
G (phytofluene)	fluorescent (20)	same (15)	....	....

[1]No movement of the streak occurred on prolonged development with 3 percent acetone in petroleum ether.

TABLE 2

Carotenoid content of four color types of N. crassa grown in the light. Figures represent mg carotenoid per 100 gm dry wt. of mold with the exception of δ-carotene, pigment B and the acidic hypophasic pigment, which are in relative concentrations. Figures in parentheses represent dry wt. of mold used in each experiment.

Carotenoid	CI (22.5 gm)	Ci (21 gm)	cI (42 gm)	ci (45 gm)[4]
Spirilloxanthin	2.45	0.19	....	....
(Neo-A)	(0.25)	(0.08)		
Lycopene	3.72	0.14	....	....
(Neo-A)	(0.42)	(0.05)		
Neurosporene	5.0	0.23	....	....
γ-Carotene	8.8	0.50	0.024	....
β-Carotene	0.99	0.04	0.011	....
Sum	20.96	1.10	0.035	....
Phytofluene	2.5	0.41	....	....
	2.6[2]	0.27[3]		
δ-Carotene[1]	1.43	0.15	....	....
Pigment B[1]	12.2	1.26	....	....
	17.6[2]	0.55[3]		
Hypophase[1]	10.91	5.66	0.84	....
Relative amt. of epiphasic pigments	1	1/10–1/28	1/90–1/600	....

[1]Relative concentration expressed in volume of the pigment × dilution × E, at the main peak/dry wt. of the mold.
[2]Dry wt. of the mold 9.2 gm in duplicate experiment.
[3]Dry wt. of the mold 7 gm in the duplicate experiment.
[4]Dry wt. of the mold 38 gm in the duplicate experiment.

experimental processes have been added to the main pigments and are indicated in parentheses. Only the main component of the hypophase is included in the table. The blanks indicate the absence of pigment. It is evident from the data presented in table 2 and our knowledge about the position of genetic blocks, which will be discussed later, that both the main color gene and the modifier have similar effects, though different in magnitude, and that their joint effect is non-additive.

EFFECT OF LIGHT ON THE CAROTENOID CONTENT

The wide occurrence of the colorless carotenoid phytofluene in plants is of interest because of its possible role as an intermediate in the biosynthesis of carotenoid pigments (ZECHMEISTER and SANDOVAL 1945; BONNER *et al.* 1946). HAXO (1949) reported a two-fold decrease of total carotenoid pigments and a slight increase in phytofluene when the cultures were kept in darkness. It was observed that cultures of the *CI* type wrapped in tin foil appeared almost colorless. Experiments were performed, therefore, to test the effect of complete darkness on the carotenoid content of the *CI* strain. The culture flasks were painted with aluminum paint, wrapped in black cloth and kept in a closed cabinet. The results are shown in table 3.

TABLE 3

Carotenoid content of cultures grown in light and in darkness. Figures represent mg carotenoid per 100 gm dry weight of mold. Figures in parentheses represent dry weight of the mold used in each experiment.

Carotenoid	*crassa* 1A (Haxo 1949) (Average of two lots)			CI (single lots except those marked with superscript one.)		
	In light	In dark	Ratio	In light (22.5 gm)	In dark (39.5 gm)	Ratio
Spirilloxanthin	6.60	1.95		2.45	0.13	
Lycopene	9.40	4.35		3.72	0.06	
Neurosporene	5.55	5.95		5.00	0.47 0.30[2]	
γ-Carotene	7.60	4.30		8.80	0.15	
β-Carotene	0.33	0.15		0.99	0.02	
Sum of pigments	29.48	16.70	0.57	20.96	0.83	0.04
Phytofluene	2.95	3.70	1.25	2.50 2.60[1]	1.98 1.30[2]	0.79
Sum of total carotenoids	32.43	20.40		23.46	2.81	
Ratio pigments / phytofluene	10.0	4.5		8.4	0.4	

[1]Dry weight of the mold 9.2 gm in duplicate experiment.
[2]Dry weight of the mold 8.5 gm in the duplicate experiment.

DISCUSSION

It has been reported that the biosynthesis of phytofluene runs parallel with the gradual formation of carotenoid pigments during the ripening of Pyracantha berries (ZECHMEISTER and SANDOVAL 1945). Attempts to demonstrate the accumulation of phytofluene in slightly colored or colorless mutants of red yeast met with failure (BONNER *et al.* 1946). This may be due to early genetic blocks before the formation of phytofluene, as suggested, and seems to be true also for the present case.

An analysis of the data listed in table 3 strongly supports the notion that the phytofluene could be the precursor of the carotenoid pigments. The agreement between our values and those of BONNER *et al.* on cultures grown in the light is reasonably close. The low values from our dark experiments could be due to the complete absence of light in our experiments. There is an increase of 25 percent in the amount of phytofluene in dark over that in light when the interference in the pigment formation is moderate, as in HAXO's experiments. In our experiments the accumulation of phytofluene is not apparent; in fact, there is a 22 percent decrease in absolute amount. However, when we take into consideration the tremendous decrease in the total amount of carotenoids and the change in the ratio of pigments to phytofluene from 8.4 to 0.4, the relative amount of phytofluene increases by a factor of 21. In short, light affects not only the conversion of phytofluene to carotenoid pigments but also, to a certain extent, the formation of phytofluene or its precursor just as the genes C and I do. A single experiment also was carried out on the non-allelic albino strain al_1 (4637A). Neither pigment nor phytofluene could be found in the extract of 8 gm dry weight of the mold. The following scheme of the biosynthesis of carotenoid pigments involving the genetic and non-genetic factors is therefore proposed.

$$\xrightarrow[\uparrow\ I]{} \xrightarrow[\uparrow\ +^{al_1}]{} \xrightarrow[\uparrow\ C(\text{or } +^{al_2})]{} \xrightarrow{\downarrow\ \text{light}} \longrightarrow \text{phytofluene} \xrightarrow{\downarrow\ \text{light}} \text{carotenoid pigments}$$

The sequence of the genes $+^{al_1}$, C $(+^{al_2})$ and I and the effect of light before the formation of phytofluene is purely arbitrary and a branching scheme is not excluded. The location of genetic blocks of gene c and i before phytofluene, but not after, is further supported by the fact that the decrease of amount of different pigments is of the same order (table 2). The present study supports the scheme of biosynthetic pathways of carotenoids proposed by PORTER and LINCOLN (1950). The different magnitude of effect on the hypophase is not explained.

SUMMARY

The genetic data show that the four-color asci found in certain crosses can be explained by a C (major color gene)–I (intensifier) system. Experimental

data show that the more interference with the formation of carotenoid pigments, the greater the amount of phytofluene formed, relative to the amount of pigment when the cultures were grown in darkness. Phytofluene, therefore, is very likely a precursor of carotenoid pigments. Because of the fact that in the four-color types phytofluene runs parallel with pigment content, the genetic block associated with the genes *c* (al_2), *i* and al_1 must occur before the formation of phytofluene.

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