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Studies in Carotenogenesis

12. THE EFFECT OF TEMPERATURE AND THIAMINE CONCENTRATION ON CAROTENOGENESIS BY *PHYCOMYCES BLAKESLEEANUS*

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In *Rhodotorula glutinis*, variations in the temperature of cultivation have a profound qualitative effect on carotenogenesis (Skoda, 1951; Nakayama quoted by Mackinney, 1952). Low-temperature cultivation (5°) results in the production of yellow cultures containing predominantly α - and β -carotene, whilst at higher temperature (25°) the cultures become red owing to the production of the more unsaturated xanthophylls, torulin and torularhodin. In *R. sarniei*, however, temperature changes between 14 and 28° have no qualitative effect on pigmentation (Fromageot & Tschang, 1938).

Turian (1953) has recently also observed a temperature effect on carotenogenesis by *Mycobacterium phlei*. At 30° leprotene, γ -carotene, a 'rhodopin-like' carotene, a hydroxycarotene and traces of phytofluene are produced together with very small amounts of the acidic chrysophlein. At 37° only small amounts of these neutral compounds were formed, but the synthesis of chrysophlein was considerably increased.

Starr & Saperstein (1953) observed that *Corynebacterium poinsettiae* cultured in a medium low in thiamine (0.1 μ g./100 ml.) is pink and contains mostly spirilloxanthin and lycioxanthin. On a high-thiamine medium (100 μ g./100 ml.) orange colonies are formed which contain much less spirilloxanthin, the same amount of lycioxanthin and some cryptoxanthin, which was not present in the organisms grown in the low-thiamine medium.

The present paper describes an investigation undertaken to find out the effects of variation in both the temperature of cultivation and in the thiamine content of the medium on carotenogenesis in *Phycomyces blakesleeanus*.

EXPERIMENTAL

Cultures. *Phycomyces blakesleeanus* (—strain) was grown in 8 oz. medicine bottles in 15 ml. of our standard medium. This medium is that described by Schopfer & Jung (1935), but contains 2.5% instead of 10% (w/v) glucose; this concentration was found to give maximal β -carotene production (Garton, Goodwin & Lijinsky, 1951). In the experiments on temperature variation, two glass incubators (Garton *et al.* 1951) were placed close together in the laboratory, one being kept at 25° and the other at either 20 or 30°. Thus both the control (25°) and the experimental runs were always carried out under comparable conditions of illumination. The experiments at 3–5° were carried out in the dark in a 'warm' corner of a large refrigerator. It was not possible to illuminate the cultures in these experiments.

In the experiments on thiamine-deficient media, inoculation was not carried out by the usual means of a spore suspension made by shaking up a stock agar culture of *Phycomyces* with sterile water (Garton *et al.* 1951), but by a very small direct transfer with a needle from an agar slope, care being taken not to transfer any agar. This precaution was necessary to eliminate the possibility of carrying over significant amounts of thiamine. That this was achieved, was shown by the fact that no growth (or occasionally very little) occurred on media containing no thiamine.

On repeating the experiments on thiamine-low media some time after the first series was carried out, it was found that no growth could be obtained. This failure was eventually traced to the age of the parent culture. Inoculations from cultures up to 6 months old will grow quite well on the standard medium, but in order to get growth in thiamine-low media, young parent cultures (less than 4 weeks old) must be used.

Determinations of dry weight and carotene. Dry weight and carotene determinations were carried out according to the methods described by Garton *et al.* (1951). The chromatographic separation of the carotenoids was carried out according to the method of Goodwin (1952).

In the experiments on the low-thiamine media the dry-weight production per medicine bottle was often very small (approx. 5 mg.). In order to reduce the error in the dry-weight and β -carotene determinations at this low level, mycelia from five to six bottles were bulked for each determination.

RESULTS

Effect of temperature on growth, lipogenesis and carotenogenesis. Two experiments were carried out at 20, 25 and 30° and the results of one are given in Table 1. Each figure is the result of two determinations on the combined mycelia of two bottles. It will be seen that the temperature of cultivation has no obvious effect on production measured as dry weight, that lipogenesis decreases somewhat with increasing temperature and that β -carotene synthesis was greatest at 25°, being less at both 20 and 30°. The possibility existed that the 30° cultures had passed their maximal dry weight production before 4 days. This was considered unlikely, the value for 4 days (101 mg.) at 30° being probably rather exceptional. To check this, however, two

further experiments were carried out at 30° to determine whether maximal dry-weight production occurred before 4 days. The results were very similar in both experiments, and the mean values are recorded in Table 2. It will be seen that in this experiment the dry-weight production was rather less than in the experiments recorded in Table 1. Maximal dry-weight production occurred 6 days after inoculation, growth was by no means complete on the fourth day and insufficient mycelium was produced on the third day to warrant examination. It will be seen that compared with results in Table 1, lipid and carotene synthesis is still somewhat lower at 30° than at 25°. A direct comparison could not, however, be made because no controls at 25° were run, as the main interest was in the time at which maximal dry-weight production occurred. At 3–5° growth was very slow and sufficient amounts of mycelia for examination were produced only 20 days after inoculation, even then growth was not complete (see Table 3). It did however appear to be complete after 26 days. It will be seen that very

Table 1. *Growth, carotene and lipid production by Phycomyces cultured at different temperatures*

Standard medium: all quantities expressed as amounts produced on 15 ml. medium in 8 oz. medicine bottles.

Age of culture (days)	20°				25°				30°			
	Total dry wt. (mg.)	Lipid (mg.)	Lipid concn. (%)	Carotene concn. (mg./100 g. dry wt.)	Total dry wt. (mg.)	Lipid (mg.)	Lipid concn. (%)	Carotene concn. (mg./100 g. dry wt.)	Total dry wt. (mg.)	Lipid (mg.)	Lipid concn. (%)	Carotene concn. (mg./100 g. dry wt.)
4	111	34.7	31.3	13.2	109	35.0	32.4	37.3	101	24.7	24.5	60.3
6	119	35.0	29.4	39.2	114	29.0	25.4	59.8	88	20.9	23.8	55.4
8	114	34.1	29.9	66.0	106	25.1	23.7	113.1	91	18.2	20.0	87.8
10	101	26.7	26.4	82.3	103	21.8	21.2	129.0	96	17.1	17.8	79.9

Table 2. *Growth, β -carotene and lipid production by Phycomyces blakesleeanae cultured at 30°*

Standard medium: all quantities expressed as amounts produced in 15 ml. medium in 8 oz. medicine bottles.

Age of culture (days)	Dry wt. mycelium (mg.)	Lipid (mg.)	Lipid concn. (%)	β -Carotene (μ g.)	β -Carotene concn. (mg./100 g. dry wt.)
3	Insufficient growth for analysis				
4	38.8	10.4	26.8	15	38.7
5	60.6	15.6	25.8	35	57.8
6	76.8	14.9	19.4	54	70.3
7	78.3	14.7	18.8	56	71.5

Table 3. *Dry weight, lipid and β -carotene synthesis in Phycomyces blakesleeanae grown at 3–5° in the dark*

15 ml. of standard medium in 8 oz. medicine bottles, each figure represents the mean of 4 determinations.

Age of culture (days)	Dry wt. mycelium (mg.)	Lipid (mg.)	Lipid concn. (%)	β -Carotene (μ g.)	β -Carotene concn. (mg./100 g. dry wt.)
20	61.4	14.2	23.2	14	22.8
26	91.0	21.8	24.0	13	14.3
Control* (mean 8–13 days in dark at 25°)	88.0	15.8	18.0	76	86.4

* From Garton *et al.* (1951).

Table 4. *Growth and carotene synthesis by Phycomyces blakesleeanus on media low in thiamine*

Standard medium containing varying amounts of thiamine: 6-day cultures; temp. 25°. All quantities expressed as amounts produced by 15 ml. medium in 8 oz. medicine bottles; five to six cultures bulked and analysed at each concentration of thiamine.

Thiamine concn. (mg./100 ml.)	Dry wt. mycelium (mg.)		β -Carotene (μ g.)		β -Carotene concn. (mg./100 g. dry wt.)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0.02	5.2	2.1	1.7	0.5	32.3	24.6
0.04	4.7	—	1.3	1.9	27.5	—
0.08	9.4	6.9	6.4	4.1	67.5	60.4
0.10	8.2	7.8	4.8	4.9	58.0	62.7
0.12	11.7	9.7	8.9	8.9	78.5	91.6
0.16	13.1	11.6	10.4	12.1	79.0	104.9
0.20	12.9	10.3	11.1	9.6	86.0	92.6
Control (25)	—	91.7	—	85.0	—	92.6

little β -carotene is produced at this low temperature, although the dry weight of the mycelium eventually reaches a normal value. Lipid synthesis is a little higher than observed earlier in the dark at 25° by Garton *et al.* (1951).

Chromatography of the total carotene fractions produced at different temperatures showed that all were quite normal in pigment distribution and that even at low temperatures, when β -carotene synthesis was considerably reduced, there was no corresponding increase in the synthesis of unsaturated polyenes such as phytoene and phytofluene.

Effect of thiamine on carotenogenesis. It is well known that, at concentrations of thiamine less than 2.0 μ g./100 ml. of medium, the growth of *Phycomyces* is proportional to the amount of thiamine (see Schopfer, 1942). Preliminary experiments indicated that the concentration of carotene in the mycelium was reduced when *Phycomyces* was cultured on media containing less than 0.6 μ g. thiamine/100 ml.

The effect of these low levels of thiamine on the production of carotene was investigated in greater detail. The usual 6-day cultures were used in these experiments because Schopfer (1942) has shown that, although the final growth is reduced on low-thiamine media, the rate of growth is little affected. In order to minimize errors, the 6-day cultures from five to six bottles were bulked for the dry-weight determination and the β -carotene analysis. This meant that the total dry weight measured was at least 10 mg. and the β -carotene 5–10 μ g.

The results of two experiments are given in Table 4. It will be seen that in confirmation of the preliminary experiments at very low concentrations of thiamine, there does appear to be an inhibition of the synthesis of β -carotene, although the carotene production appears to reach normal levels at slightly lower thiamine concentrations (0.12–0.16 μ g./100 ml.) than was first indicated (0.6 μ g./100 ml.).

Chromatography of these extracts did not reveal any differences from normal extracts; in particular there did not appear to be any increase in the production of unsaturated polyenes.

It is also obvious from Table 4 that, on thiamine-low media, the carotene concentration is normal long before growth reaches its maximal value. As stated previously, this was found to occur at a thiamine level of 2.0 μ g./100 ml.

DISCUSSION

The effect of temperature variations on carotenogenesis by *P. blakesleeanus* is quantitative only, and no differences in the types of polyenes synthesized were noted over the range 5–30°. There is, however, an optimum temperature for carotene synthesis at about 25°. The qualitative observations are similar to those of Fromageot & Tschang (1938) who found no changes in the pigments produced by *Rhodotorula sarnnei* over the temperature range 14–28°. In the closely related *R. glutinis*, however, the pigments produced at low temperatures (5°) were different from those produced at normal temperatures (Skoda, 1951; Mackinney, 1952). There are no reports of quantitative experiments on *Rhodotorula* spp.

P. blakesleeanus in its reaction to variations of temperature, also differs from *Myco. phlei* in which a lowering of the incubation temperature from 37 to 30° considerably affects the relative amounts of the various carotenoids produced (Turian, 1953).

As in the case of the experiments in which the cultivation temperature was altered, it was found that quantitative effects on carotenogenesis could be obtained by lowering the thiamine levels of the medium. This occurred only at the lowest levels examined, 0.02–0.16 μ g. thiamine/100 ml. There was no indication, however, of any qualitative changes similar to these observed in *C. poinsettiae* cultured on media containing different levels of thiamine (Starr & Saperstein, 1953).

It is interesting that in the low-thiamine media pyruvate accumulates in considerable amounts (Griffiths, 1953). Whether this means that pyruvate is an intermediate in the formation of β -carotene is not known, but when tested under other conditions pyruvate did not stimulate pigment synthesis (Goodwin, Lijinsky & Willmer, 1953).

SUMMARY

1. β -Carotene synthesis by *Phycomyces blakesleeanus* was greater at 25° than at 3–5, 20 or 30°. At all temperatures, β -carotene was the major pigment produced. There was no indication of the synthesis of enhanced amounts of more saturated polyenes at temperatures where β -carotene synthesis was reduced.

2. β -Carotene synthesis is inhibited in *Phycomyces blakesleeanus* grown on media containing less than 0.2 μ g. thiamine/100 ml., whilst growth inhibition begins below a much higher level (2.0 μ g./100 ml.). No qualitative changes in polyene synthesis were associated with this inhibition of carotenogenesis.

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Chemical Analysis of Human Foetal Skull Bones

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Pressures exerted on the foetal head during labour cause it to alter its shape, and this ability of the head to 'mould' in response to external pressure becomes less marked as the foetus ages. The present work was an attempt to correlate changes in chemical composition of the bones with the increasing 'hardness' of the maturing foetal head.

There have been several investigations into the chemical composition of foetal bones, but the only reports referring to the cranial bones are those of Yoshida (1930) and Toverud & Toverud (1933). Yoshida analysed the parietal bone for calcium and phosphorus, and related his findings to the length of gestation. These showed that whereas the absolute amounts of phosphorus and calcium in this bone increased as pregnancy advanced, the percentage of phosphorus decreased while that of calcium increased. This finding was surprising as was the fact that at a foetal age of 10 months the Ca/P ratio of the parietal bones was over four.

Toverud & Toverud (1933) analysed a small portion (2 × 1.5 cm.) of the parietal bone for the percentage calcium per dry weight of the sample, and the percentage calcium and phosphorus of ash.

The findings were related to the length of gestation of the foetus, and no obvious relationship between percentage of calcium and foetal age was noted. The authors, however, found a sudden increase in percentage of calcium in the section of parietal bone of full-term babies as compared with the values found for the premature infants up to and including 38 weeks.

METHODS

Material

The bones for this study were obtained from still-births or from infants who had died very soon after birth. The age of the foetus was estimated from the menstrual history of the mother, only singletons were used and any foetus which showed obvious signs of possible skeletal deformity was discarded.

The bones were removed *post mortem* and kept in 0.9% (w/v) NaCl in a refrigerator until they were prepared for examination, which was always within 5 days of death. The parietal and frontal bones only were used. The medial border of each parietal bone was not included in the specimen, nor was the orbital part of the frontal bones included. Before the bones were examined the soft tissues on each surface of the bone were removed by stripping.