SUMMARY

1. The amount of pseudo-cholinesterase in the livers of adult male rats decreased from 96 to 70 units/100 g. initial body weight during a 6-day fast, but the activity per g. liver changed very little, and the activity of the serum enzyme remained constant at 7·1 units/ml. serum.

2. The total pseudo-cholinesterase in the livers of female rats amounted to 330 units/100 g. initial body weight. Two days' starvation reduced this figure to 110, and 6 days' starvation to 96. There was little change per g. liver after the second day. The activity in the serum fell from 41 to 23 units/ml. in the 6 days.

The authors wish to thank Prof. R. A. McCance for his valuable advice and criticism during this investigation.

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Studies in Carotenogenesis

1. GENERAL CONDITIONS GOVERNING β-CAROTENE SYNTHESIS BY THE FUNGUS *PHYCOMYCES BLAKESLEEANUS* BURGEFF

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Information concerning carotenogenesis in both Phanerogams and Cryptogams is limited, and that which does exist is often far from unequivocal (see Goodwin, 1951, for a full discussion). In order to simplify the experimental approach as much as possible by eliminating photosynthesis, it was decided to investigate carotenogenesis in a fungus; *Phycomyces blakesleeanus* was an obvious choice, for it is easily cultured on simple aqueous media, requiring only aneurin as a growth factor (Schopfer, 1934), and produces considerable amounts of a single carotenoid, β-carotene (Schopfer & Jung, 1935; Karrer & Krause-Voith, 1947; Albrecht, 1948; Bernhard, 1948) together with traces of α-carotene Karrer & Krause-Voith, 1947; Albrecht, 1948; Bernhard, 1948) and, possibly, lycopene (Albrecht, 1948; Bernhard, 1948); no xanthophylls have ever been detected. *Phycomyces blakesleeanus* is a heterothallic fungus existing in two well defined strains, (+) and (−) (Blakeslee, 1904). According to Schopfer (1935), cultures of the (+) strain are generally yellower than those of the (−) strain, an observation which suggests that the (+) strain produces the greater amount of carotene.

The only quantitative information concerning carotenoid formation in *Phycomyces* is that obtained by Schopfer (1935); he records the carotene concentration as being about 0·09% on dry weight.

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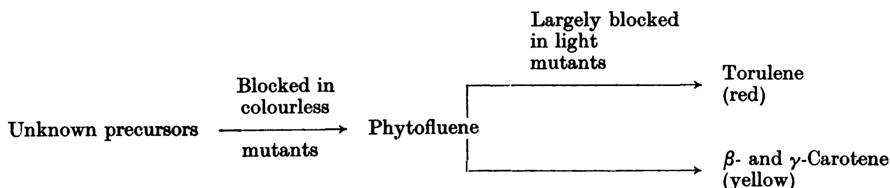
Further information is restricted to short statements by Schopfer (1934) in a paper mainly concerned with other aspects of the biochemistry of *Phycomyces*. He noted that the C:N ratio of the medium was an important factor, and that glycine and asparagine were about equally effective in promoting carotenogenesis, but ammonium nitrate was better than either; further, he claimed that, when cultured in the dark or in red light, the fungus produces no β-carotene whilst in daylight or violet light it does.

Equally meagre is the information available concerning carotenoid synthesis by other fungi. Glycerol is the most effective single source of carbon for carotenogenesis by *Rhodotorula sanniei*; a mixture of lactic acid and glucose is equally effective, but glucose alone does not support pigment formation (Fromageot & Tchang, 1938). Light is apparently necessary for the biosynthesis of carotenoids by *Neurospora sitophila* (unpublished work of Deventer, quoted by Zechmeister, 1944), but is not necessary in the case of *N. crassa*, although it does stimulate production (Haxo, 1949). Bonner, Sandoval, Tang & Zechmeister (1946), in a study of carotenoid synthesis by seven ultraviolet mutants of *Rhodotorula rubra*, found that in light-coloured mutants synthesis of torulene (Lederer, 1933) was considerably restricted whilst that of the other carotenoids, including the colourless polyene phytofluene (a possible precursor of carotenoids), was unchanged; in the colourless mutants all carotenoids including phytofluene disappear. On the basis of these observations Bonner *et al.* (1946) put forward the following tentative metabolic scheme:



In order to have a firm foundation on which to base future work, a full investigation has been carried out on the general factors concerned with carotenogenesis by *Phycomyces*. A short account of part of this work has already been reported (Garton, Goodwin & Lijinsky, 1950).

EXPERIMENTAL

Cultural methods. Phycomyces blakesleeanus Burgeff, (+) and (−) strains, were obtained on malt agar slopes from the Centraalbureau voor Schimmelcultures, Baarn. They were maintained on malt agar, subcultured every 2 months and stored in the dark at room temperature. In the first part of the investigation both (+) and (−) strains were used, but later only the (−) strain, which produces more carotene, was used. Except in experiments involving medium changing, the cultures were grown in 8 oz. medicine bottles laid flat and containing 15 ml. of the appropriate liquid medium to which 3 ml. of a spore suspension had been added from an automatic pipette. The bottles were plugged with non-absorbent cotton wool and the plugs covered with cellophan caps. In medium-changing experiments 250 ml. Erlenmeyer flasks were used instead of medicine bottles.

Spore suspensions were made by taking a mature medicine-bottle agar slope (1 month old; 15 ml. of agar) adding 150 ml. of distilled water, shaking vigorously and decanting through a plug of glass wool into the reservoir of the automatic pipette. All operations were carried out under sterile conditions. The cultures were incubated in glass cabinets maintained at 25°, the temperature of optimum growth of *Phycomyces*. The cabinets received illumination during the normal hours of daylight. The glass cabinets were constructed by removing the sides from obsolete incubators and replacing them by plate glass. In order to maintain an even temperature throughout the cabinet, it was found necessary to put a small electric fan into the base of the cabinet; the electrical circuit was so arranged that when the heaters were on the fan revolved quickly and when they were off it revolved slowly. This arrangement was quite effective and even when the cabinets were full (100–120 bottles) a temperature difference greater than 2° between the top and bottom of the cabinets was rarely encountered. In one cabinet the heaters were 60 W. lamps encased in metal containers and in the other General Electric Company 60 W. enclosed heater units.

Sterilization of apparatus was carried out at 15–20 lb. pressure for 15 min. Media were sterilized either in the same way or by filtering through a Pyrex SF. 3 A. porosity 5/3 filter.

The standard culture medium used was that recommended by Schopfer (1934), containing glucose (10%), L-asparagine (0·2%), $MgSO_4.7H_2O$ (0·05%), KH_2PO_4 (0·15%) and aneurin (0·025 mg. %), (all w/v), made up in glass-distilled water. Appropriate experimental media were made up as required by varying the glucose concentration, altering the main C source, or altering the N source. The amino-acids and glucose (A.R.) used were obtained from British Drug Houses Ltd. and the other sugars from T. Kerfoot and Sons; the inorganic salts were of A.R. grade.

Chemical determinations. The fungus was harvested by shaking a culture bottle until the mycelium had formed a compact mass; this was then drawn out of the bottle with a small spatula and transferred to a small Hirsch funnel attached to a water pump. The medium was then filtered through the same funnel to collect any small fragments of mycelium which might have become detached from the main mycelial mat. Total dry weight, ether-extractable lipids and β-carotene were determined on each sample in the following manner. The mycelial mat, dried as much as possible by suction, was transferred to a mortar and the lipids and β-carotene extracted by grinding under successive portions of ether freshly redistilled over reduced iron. The combined

Table 1. *The production of dry weight, lipids and β-carotene by the (−) strain of* Phycomyces blakesleeanus

(Cultured on Schopfer's medium (10% (w/v) glucose) in normal daylight at 25°. The amounts recorded are those produced in one 8 oz. medicine bottle lying horizontally and containing 15 ml. of medium.)

Time after inoculation (days)	Total dry wt. (mg.)	Lipids		β-Carotene		
		Amount (mg.)	Percentage of dry wt.	Amount (μg.)	Percentage of total dry wt.	Percentage lipids
1						
2	11·2	Trace	—	5	0·045	—
3	48	8	16·7	17	0·035	0·212
4	68	14	20·1	40	0·059	0·286
5	67	16	23·9	92	0·137	0·575
6	89	21	23·7	93	0·105	0·443
7	79	20	25·3	125	0·158	0·625
8	89	18	20·3	141	0·158	0·783
9	88	16	18·2	127	0·144	0·794
10	76	13	17·1	103	0·136	0·794
11	88	14	15·9	116	0·132	0·828
12	100	18	18·0	100	0·100	0·556
13	91	16	17·6	122	0·134	0·763
14	94	19	20·2	102	0·109	0·537
15	101	15	15·0	103	0·102	0·688
16	82	16	19·5	87	0·106	0·543
17	95	15	15·8	97	0·102	0·646
18	99	17	17·1	96	0·097	0·564
19	108	20	18·5	96	0·089	0·480
21	106	22	20·8	64	0·060	0·291
22	91	13	14·3	79	0·087	0·608
23	96	20	20·8	62	0·065	0·310
24	105	17	16·2	57	0·054	0·335
26	99	16	16·2	45	0·046	0·281
27	96	19	19·8	30	0·031	0·158
28	90	13	14·4	34	0·038	0·261
30	126	22	17·5	43	0·034	0·195
35	103	17	16·5	30	0·029	0·176
Means (6–35 days)	**95**	**18**	**19**			

Table 2. *The production of dry weight, lipids and β-carotene by the (+) strain of* Phycomyces blakesleeanus

(Grown under the same conditions as those outlined in Table 1.)

Time after inoculation (days)	Total dry wt. (mg.)	Lipids		β-Carotene		
		Amount (mg.)	Percentage of dry wt.	Amount (μg.)	Percentage of total dry wt.	Percentage of lipids
2	19	—	—	4	0·021	—
3	55	18	33	11	0·020	0·061
4	78	18	23	42	0·054	0·233
5	86	22	26	76	0·088	0·346
6	82	20	24	58	0·071	0·290
7	80	22	27	83	0·104	0·377
8	84	13	15	60	0·071	0·461
9	77	13	17	58	0·075	0·447
10	72	20	28	79	0·110	0·395
13	85	18	21	60	0·071	0·333
15	93	14	15	31	0·033	0·222
16	81	15	19	45	0·056	0·300
17	97	19	20	56	0·058	0·295
19	93	22	24	85	0·091	0·386
21	88	17	19	26	0·030	0·153
26	114	17	15	33	0·029	0·194
27	110	22	20	58	0·053	0·264
35	96	16	17	31	0·032	0·194
Means (5–35 days)	**90**	**18**	**20**			

extracts were filtered through a tared G4 sintered-glass crucible and the ether-insoluble fraction then transferred quantitatively to the crucible with the aid of a further small volume of ether. The extract containing lipids and β-carotene was reduced in volume by distillation at normal pressure in dim light on a gently boiling water bath; the concentrated extract (about 10–20 ml.) was transferred quantitatively to a tared 25 ml. Erlenmeyer flask, the remaining solvent removed in a stream of N_2, and the weight of the lipid residue determined with as little delay as possible in order to eliminate the possibility of the destruction of β-carotene. The lipid residue was then dissolved in an appropriate volume of light petroleum (A.R., b.p. 40–60°), a few drops of absolute ethanol added, and the β-carotene determined by measuring E 450 mμ. in a Beckman photoelectric spectrophotometer. No chromatographic removal of the small amounts of α-carotene always present (not more than 5 %) was carried out for routine analysis. The dry weight of the ether-extracted thallus was obtained by drying it in the sintered crucible at 80° for 2 hr.

Chromatographic separation of the carotenoids in Phycomyces. The lipids were extracted as already described, saponified and the unsaponifiable matter obtained using the procedure general in this laboratory (Goodwin & Morton, 1946). The residue was dissolved in light petroleum (b.p. 40–60°) and chromatographed on alumina (Spence, Grade O), slightly deactivated with methanol (Goodwin & Taha, 1950). On developing the chromatogram with light petroleum (40–60°) containing 1 % (v/v) acetone, two coloured and one colourless zones were obtained, the latter being detected by its greenish-blue fluorescence in ultraviolet light.

The most weakly adsorbed zone was yellow and always constituted about 5 % of the total carotenoids present. Its chromatographic behaviour suggested that it was α-carotene and this was confirmed by measuring its absorption spectrum in a number of solvents; the spectra in all cases were in accord with those reported for authentic α-carotene. The main yellow-orange zone was identified as β-carotene by its absorption spectrum and by a mixed chromatogram with an authentic specimen of crystalline β-carotene. The colourless fluorescent zone has not yet been investigated in detail, but by rechromatographing on $CaCO_3$ it could be resolved into a number of bands. The possible importance of these fluorescent substances as precursors of carotenoids must not be overlooked. It should be noted that it was not possible to detect lycopene in our extracts, although Albrecht (1948) and Bernhard (1948) considered that it probably occurred in their extracts.

RESULTS

The carotenoids in Phycomyces. It has been confirmed that β-carotene is the predominating pigment, although α-carotene also occurs in small amounts; no xanthophylls or lycopene were detected.

Production of dry weight, lipids and β-carotene under standard conditions (Schopfer's medium). A large number of experiments have been carried out to determine the dry weight, lipid and β-carotene production by the (−) strain of *Phycomyces* as a function of time and the mean values obtained are recorded in Table 1. It will be seen that maximum growth and lipid production occur within 5–6 days of inoculation, but that maximum carotene production does not occur until a few days later (7–9 days). During the ageing of the culture no change in dry weight or fat occurs, though about 14–16 days after inoculation β-carotene begins gradually to disappear from the mycelium.

Similar experiments using the (+) strain of the fungus showed exactly the same pattern, with the exception that the β-carotene level only reached about one-half that of the (−) strain (Table 2).

Table 3. *The production of dry weight, lipids, and β-carotene by the* (+) *and* (−) *strains of* Phycomyces blakesleeanus *grown in complete darkness*

(Conditions, otherwise standard, outlined in Table 1.)

Time after inoculation (days)	Total dry wt. (mg.)	Lipids: Amount (mg.)	Lipids: Percentage of dry wt.	β-Carotene: Amount (μg.)	β-Carotene: Percentage of total dry wt.	β-Carotene: Percentage of lipids
(−) Strain						
4	65	13	18·5	14	0·022	0·117
6	80	13	16·3	31	0·039	0·238
7	127	29	22·8	74	0·058	0·255
8	86	14	16·3	87	0·104	0·622
9	87	14	16·1	76	0·087	0·543
13	93	19	20·4	64	0·069	0·337
16	112	23	20·5	41	0·037	0·178
19	106	15	14·1	55	0·052	0·367
Mean (6–19 days)	**99**	**18·1**	**18·3**			
(+) Strain						
7	92	10	10·9	16	0·017	0·0160
9	93	16	17·2	23	0·025	0·0144
13	101	22	21·8	25	0·025	0·0114
16	122	24	19·7	26	0·021	0·0108
Mean (7–16 days)	**102**	**18**	**17·4**			

Table 4. *The effect of varying light conditions on the growth, lipid production, and β-carotene production by* Phycomyces blakesleeanus

Conditions	Time after inoculation (days)	Total dry wt. (mg.)	Lipid (mg.)	β-Carotene (μg.)
Culture bottles wrapped in red cellophan (Fig. 1)	10	108	19	120
Culture bottles wrapped in green cellophan (Fig. 1)	10	106	16	126
Culture bottles wrapped in colourless cellophan	10	104	21	136
Cultured for 4 days in dark and then transferred to light	7	105	24	110
Cultured for 4 days in light and then transferred to dark	7	89	19	126

Role of light in β-carotene production. When grown in the dark the dry weight and fat production by both strains of *Phycomyces* were indistinguishable from those obtained with cultures kept in the light. In both cases, however, the β-carotene production was only one-half of that produced in cultures grown in the light (Table 3).

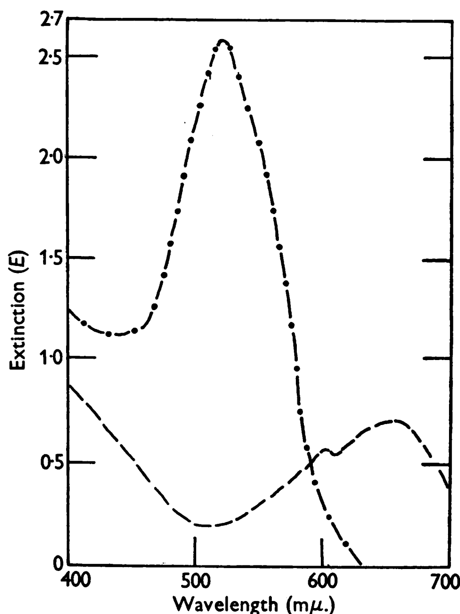


Fig. 1. Absorption spectra of the cellophans used in covering the culture bottles. The *E* values are those obtained using a single thickness of cellophan. – – –, green cellophan; —·—·—·, red cellophan.

In order further to investigate the action of light, cultures were grown in bottles wrapped in either red, green or colourless cellophan. The coloured cellophans exhibited absorption spectra with maxima at 520 mμ. (red) and 605 and 660 mμ. (green), respectively (Fig. 1); The colourless cellophan reduced the intensity of light equally over the range 400–700 mμ. to the extent of 8–10 %. Table 4 indicates that as long as the fungus is exposed to light, it produces normal amounts of β-carotene irrespective of the wavelength of the illumination. This is confirmed by the observation that no changes in β-carotene synthesis were noted in cultures grown in normal daylight in June and December.

A further experiment, also recorded in Table 4, indicated the stimulatory action of light. Cultures grown in the dark for 3 days, and thus containing very little β-carotene, when subsequently placed in the light for 7 days produced an amount of β-carotene very similar to that produced during the same period by a culture grown in light all the time. Conversely, when cultures kept in daylight for 3 days are placed in the dark they continue to produce β-carotene at the 'daylight' rate.

Variation in N *source in the culture medium.* The results obtained with *Phycomyces* cultured on media containing nitrogenous compounds other than L-asparagine (Table 5) show that of the amino-acids investigated glycine, L-valine and L-leucine produced normal amounts of dry weight and lipids. Dry-weight production was normal with L-alanine, but there was a slightly lowered fat production. Glycine, however, produced more β-carotene than the other amino-acids, which produced the same amounts as did L-asparagine. Growth on L-isoleucine was poor, all three constituents being produced in amounts about one-eighth of that in control cultures. When the organism was cultured on inorganic N sources, production of dry weight and β-carotene followed a normal pattern, but fat synthesis was reduced on ammonium acetate, whilst on ammonium nitrate growth ceased when all three constituents had reached about one-quarter or one-fifth the normal values.

Some experiments were carried out using well-established mycelia, which were transferred to a medium containing no source of nitrogen, but which contained all the other constituents of the control medium. 3-Day old mycelial mats were produced

Table 5. *Dry weight, lipid and β-carotene production by* Phycomyces blakesleeanus *grown on different nitrogen sources, but under otherwise identical conditions*

(See Table 1 for growth on asparagine.)

Time after inoculation (days)	Total dry wt. (mg.)	Lipids (mg.)	β-Carotene (μg.)	Total dry wt. (mg.)	Lipids (mg.)	β-Carotene (μg.)	Total dry wt. (mg.)	Lipids (mg.)	β-Carotene (μg.)	Total dry wt. (mg.)	Lipids (mg.)	β-Carotene (μg.)
		Glycine			Alanine			Valine			Leucine	
3	32	5	9	52	6	13	28	9	24	22	3	8
4	48	10	38	70	14	42	—	—	—	—	—	—
5	49	11	42	—	—	—	—	—	—	—	—	—
6	92	22	122	80	13	86	79	18	96	28	4	24
8	65	11	149	89	13	112	—	—	—	—	—	—
10	95	17	194	96	12	90	—	—	—	—	—	—
11	97	19	168	93	10	101	—	—	—	—	—	—
12	115	22	201	—	—	—	95	24	80	84	23	111
13	93	19	145	97	12	84	—	—	—	—	—	—
18	110	28	79	101	14	73	100	19	90	96	26	98
22–24	102	17	81	93	11	65	94	9	88	74	17	76
Mean (8–24 days)	**97**	**20**		**91**	**13**		**92**	**17·5**		**85**	**22**	
		Isoleucine			NH_4NO_3			Ammonium acetate				
3	28	4	3	19	5	18	—	—	—			
4	—	—	—	19	4	19	72	10	19			
5	—	—	—	22	5	35	69	10	26			
6	18	5	9	23	5	37	80	14	59			
8	—	—	—	21	3	34	82	10	59			
10	—	—	—	22	3	25	—	—	—			
11	—	—	—	22	3	28	91	16	104			
12	11	3	16	19	2	25	—	—	—			
13	—	—	—	23	5	17	90	9	89			
18	19	7	15	27	4	25	90	10	82			
22–24	22	10	19	22	4	9	—	—	—			
Mean (8–24 days)	**16**	**7**		**22**	**3·5**		**88**	**10**				

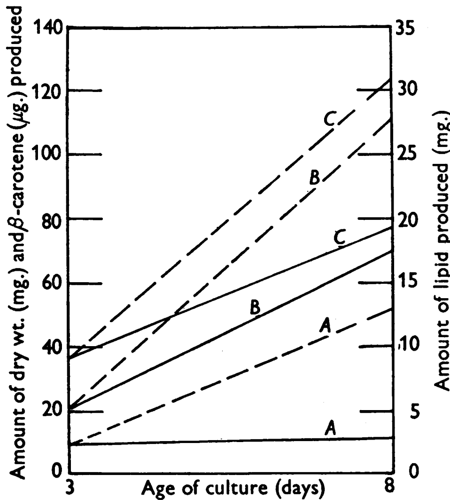


Fig. 2. Dry weight, lipid, and β-carotene production by *Phycomyces* in the presence and absence of nitrogen. – – –, on N-containing medium; ——, on N-free medium. *A*, β-carotene; *B*, lipid; *C*, dry weight.

in the normal way and then the medium was removed, the mat carefully washed with sterile distilled water and the N-free medium added: control experiments were carried out in which the normal medium was removed and replaced by a fresh sample of the same medium. The results are recorded graphically in Fig. 2. It will be seen that mycelia which are not growing, but are only dissimilating glucose, increase in dry weight and fat content, but do not produce any carotene. Whether this type of experiment is applicable to substrates other than glucose is under investigation.

Table 6. *β-Carotene production by* Phycomyces *grown on different carbohydrate sources* (3%), *but under otherwise identical conditions*

(The dry weight and lipid production are not recorded because in each case they are indistinguishable from those obtained using glucose.)

Time after inoculation (days)	β-Carotene produced (μg.) Maltose	Fructose	Xylose	Glucose
4	6	13·5	4	40
6	42	27	26	93
10	111	62	49	103
13	122	57	69	96
18	96	61	74	77
23	69	45	64	62

Variation in carbon source. The ability of various carbon compounds to replace glucose was investigated. Lactose and glycerol did not support growth, whilst mycelia grown on maltose, fructose and xylose produced dry weights and lipid contents indistinguishable from the values obtained with glucose. The production of β-carotene was, however, quite definitely reduced using fructose or xylose, but was normal with maltose.

Variation in the C:N *ratio of the medium.* Two experiments were carried out to observe the effect of changing the C:N ratio of the medium on the growth, lipid and β-carotene production of *Phycomyces*. (The C value used is the total carbon in the medium, i.e. that from glucose and amino-acid.) In the first experiment the N concentration was kept constant (0·2 % L-asparagine) and the glucose concentration varied and in the second the glucose was kept constant (2·5 % glucose) whilst the N concentration was altered; the mycelia were harvested and examined at intervals from 4 to 25 days after inoculation. The effect of altering the C:N ratio can most clearly be demonstrated diagrammatically; in Figs. 3 and 4 mean values for the dry weight, lipids and β-carotene for 6–20-day-old mycelia are plotted against the C:N ratio of the medium. It will be seen that in the first experiment (N constant) (Fig. 3) dry weight increases directly with increasing C:N ratio until a value is obtained which cannot be improved by increasing the glucose concentration of the medium. Lipid production increases rather slowly at first with increasing C:N ratio and then accelerates until a maximum value is obtained at a ratio of 25:1. β-Carotene increases only very slowly until a ratio of 15:1 is reached; between 15:1 and 25:1 there is a remarkable increase (about 400 %) in production. When the glucose content is constant and the N concentration so altered to obtain the same C:N ratios as those used in the previous experiment, the results obtained were markedly different (Fig. 4). Apart from the culture containing 0·1 % of L-asparagine,

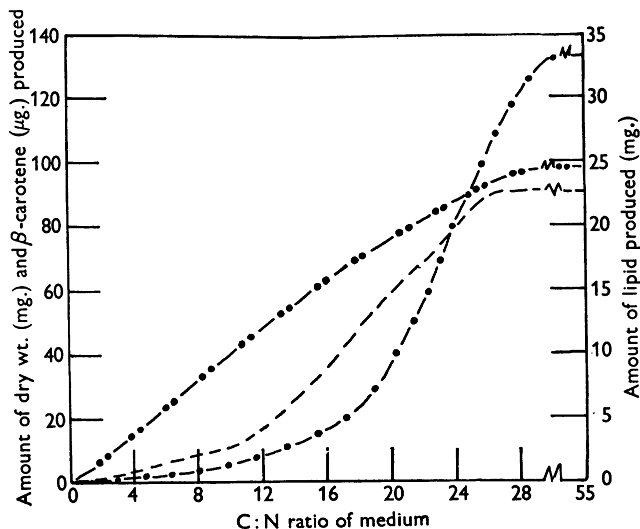


Fig. 3.

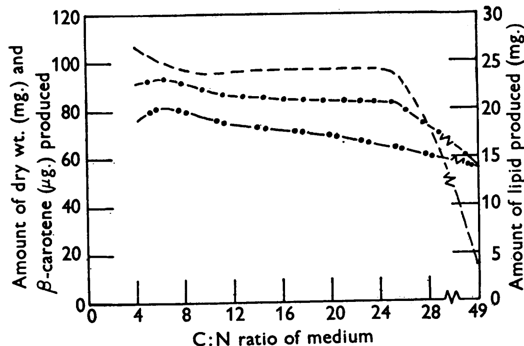


Fig. 4.

Fig. 3. The variation in the production of dry weight, lipids, and β-carotene by *Phycomyces* cultured on media containing 0·2 % (w/v) of L-asparagine and varying amounts of glucose. —·—·—, β-carotene; – – –, lipid; —··—··—, dry weight.

Fig. 4. The variation in the production of dry weight, lipids, and β-carotene by *Phycomyces* cultured on media containing 2·5 % (w/v) of glucose with varying amounts of L-asparagine. – – –, β-carotene; —··—··— lipid; —·—·—, dry weight.

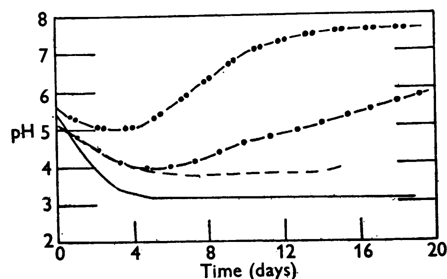


Fig. 5. The pH-time curve for different media on which *Phycomyces* was growing. – – –, Schopfer's medium (high C:N ratio); —·—·—, Schopfer's medium, but concentrations of glucose and L-asparagine altered to give medium C:N ratio; —··—··—, as above, but with low C:N ratio; ——, Schopfer's medium with L-asparagine replaced by NH_4NO_3.

the dry-weight, lipid and β-carotene production was essentially the same for all C:N ratios. The differences between these two experiments have important theoretical implications and are discussed later.

Variations in pH *of culture media.* During growth of the mycelium the pH of the medium undergoes changes and these differ according to the type of medium in use. Changes in pH observed with the media employed in this investigation are recorded in Fig. 5. For brevity, if the changes observed with a certain medium were indistinguishable from those of the control medium, they are not recorded. It will be seen that, in most cases, the pH drops from its initial value of about 5·0 to about 3·7. In other cases, following a preliminary drop, a rise is observed in the pH which at times can reach a value of 8·0: only cultures utilizing ammonium nitrate as N source ever drop below pH 3·5.

DISCUSSION

The results reported in this paper show that, on the medium recommended by Schopfer (1934), β-carotene formation is much slower in the early stages of growth than is the production of lipids and dry weight, but after lipid and dry-weight values have reached their maxima, β-carotene production continues for some days, reaching a maximum concentration of about 0·14 and 0·7 % on dry weight and lipids, respectively (cf. Schopfer's value of 0·09 % on dry weight). In ageing cultures, the pigment gradually disappears, although the fat and dry weight remain constant. The reason for this disappearance of carotene is unknown, but the possibility of the presence of an active lipoxidase should not be overlooked.

With the various nitrogen sources examined, carotene concentration, based on either dry weight or lipid, was the same in all cases except with glycine; this amino-acid specifically stimulated carotenogenesis, producing a mycelium containing about 0·2 % of dry weight (or 1·0 % of lipid) of β-carotene. The full explanation of this must await further work, but the carbon residue remaining after the deamination of glycine must be an extremely active intermediate; further, it must be a compound which can be produced from glucose only relatively slowly; unless this were so the overwhelming preponderance of glucose in the medium (10 % compared with 0·2 %) would preclude this effect. A possible metabolic scheme can be represented thus:

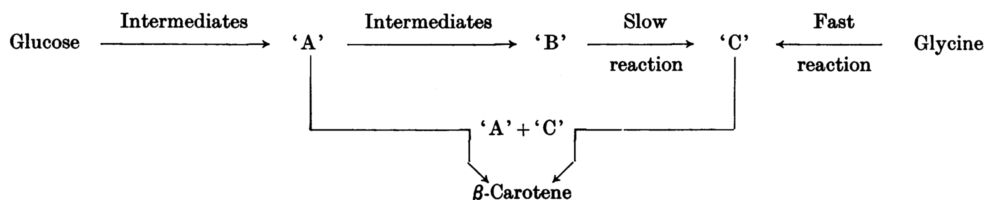
The fact that glycerol is the most effective carbon source for carotenoid production in *Rhodotorula* must be taken at the moment to indicate different anabolic requirements of the fungi rather than two separate routes for carotenoid synthesis. Failure of *Phycomyces* to produce β-carotene on glycerol is primarily a failure of the fungus to grow on this carbon source.

It is significant that L-valine, which could theoretically yield β-methylcrotonic acid (a suggested intermediate), does not stimulate carotenogenesis under the present conditions. This does not rule out β-methylcrotonic acid as an intermediate, but does indicate that if it is an intermediate it can be easily formed from glucose.

It is interesting to note a differential β-carotene production in the (+) and (−) strains, the (−) strain producing, on the average, almost twice as much as the (+) strain. This observation does not agree with that of Schopfer (1935), who found the (+) strain to contain more carotene, an observation in agreement with an earlier report on carotenoids in the (+) and (−) strains of *Mucor hiemalis* (Chodat & Schopfer, 1927); whether this differential distribution has any reproductive significance remains to be seen.

Our results concerning the action of light on carotene production are also at variance with those of Schopfer (1934, 1943). He reported no pigment formation in the dark or in red light; in the present investigation experiments in which all possible precautions were taken to exclude light completely, it was found that 'dark' cultures always produced carotene to about one-half the extent of that produced by 'light' cultures, and that maximum production was achieved as soon as visible light of any wavelength was allowed to fall on the cultures. The stimulation of synthesis appears to be permanent, for 'light' cultures transferred to the dark continue to produce β-carotene at the usual rate. These observations agree with those of Haxo (1949) who found that light was not essential for carotenoid production in *Neurospora crassa*, but that it had a stimulatory effect. The observation that only half the normal amount of β-carotene is produced in the dark strongly suggests that there may be two synthetic routes, one of which is photochemical.

In order to differentiate between carotenoid production during normal growth from production during dissimilation of a carbon substrate, the effect of maintaining young mycelial mats (3 days old) on



nitrogen-free media was observed. The results (Fig. 2) indicate that with glucose as sole source of carbon, no β-carotene is formed although fat and dry weight are produced: apparently the fungus must metabolize an exogenous nitrogen source before β-carotene synthesis from glucose can take place.

It has been stated without any convincing experimental proof that the C:N ratio of the medium is the governing factor in carotenogenesis in *Phycomyces* (Schopfer, 1943) and in algae (Chodat, 1938). The present results (Figs. 3 and 4) show that under certain conditions the C:N ratio can assume a spurious significance. As long as there is sufficient nitrogen present to allow maximal growth, the C:N ratio is not important; what is important is the amount of assimilable carbon available, i.e. under most conditions, the concentration of glucose in the medium. This is well illustrated in Fig. 4; the amount of glucose present is always 2·5 % (an amount just sufficient to produce maximal β-carotene production when the asparagine concentration is 0·2 %), but reducing the C:N ratio by increasing the nitrogen concentration has no significant effect on carotene synthesis, or for that matter, on lipid or dry-weight production. From the pH changes in the medium it appears that the excess amino-acid is deaminated and the ammonia excreted into the medium. In this case the C:N ratio of the medium has no meaning, because it is quite different from the 'physiological' C:N ratio which can be considered as being the ratio of the amounts of carbon and nitrogen incorporated into the mycelium. Similar pH changes occur when the C:N ratio is low and the nitrogen is kept constant (Fig. 5); again excess nitrogen is discarded, but insufficient carbon is present to stimulate accumulation of carotenoid. An important point emerges from Fig. 4, namely that the values for dry weight and β-carotene obtained at a very high C:N ratio (33:1) are all low. In this case the concentration of L-asparagine was insufficient to produce maximal growth (under our conditions a fully developed mycelium contains about 4·7 mg. of nitrogen; 15 ml. of 0·2 % L-asparagine provides slightly more than necessary (5·6 mg.), but 0·1 % L-asparagine is inadequate). All possible growth with the amount of nitrogen available was achieved, and, as glucose was in excess, the maximal concentration of β-carotene (as opposed to amount) was achieved.

It is thus clear that the idea that the C:N ratio of a medium is the most important factor in carotene production, is of no fundamental significance in the case of *Phycomyces*, although the 'physiological' C:N ratio may be important. Provided sufficient nitrogen is present for the production of maximal growth, β-carotene synthesis only occurs to any great extent when the glucose concentration is above a certain level.

Dry-weight and lipid production. Although the main purpose of this investigation was a study of carotenogenesis, sufficient information has been obtained on the production of dry weight and lipids to make it possible to compare our results with other published data; only relative values can be compared, for varying cultural conditions may considerably affect absolute production. Bernhard & Albrecht (1948) have previously reported on the fat content of *Phycomyces* and their values, which range from 17·9 to 21·5 %, are comparable with those reported here.

Concerning the effect of varying the nitrogen source on the dry-weight production, it was found that ammonium nitrate and isoleucine promoted only about 20 % of the growth obtained with either L-asparagine, glycine, L-valine, L-leucine or ammonium acetate, all of which had similar growth-promoting abilities. Albrecht (1948) found L-alanine and ammonium acetate to be 82 and 61 %, respectively, as effective as L-asparagine; the difference between his figure and ours for ammonium acetate is outside expected variations; at the moment only one explanation can be put forward to account for this. Albrecht harvested his cultures after 5 or 10 days; our experience indicates that growth on ammonium acetate is slower than on asparagine, although it eventually reaches the same level; thus Albrecht's ammonium acetate cultures may not have been fully grown. A further interesting result of Albrecht's work is that growth on DL-leucine is only 26 % of that on L-asparagine. It might be considered that the difference between our values using L-leucine and those using DL-leucine occurred because of the better utilization of the L-isomer; however, this explanation appears unlikely for, according to Albrecht, DL-asparagine is 88 % effective. Turning to Schopfer's (1934) results, we agree with him in observing that L-asparagine and glycine are equally effective, whilst ammonium nitrate is much inferior. Leonian & Lilly (1940) also found L-asparagine and glycine equally effective. The poor growth-promoting activity of ammonium nitrate is probably due to pH changes; for the first 2–4 days after inoculation, growth is normal, but thereafter it ceases; this coincides with a drop in pH below the usual value of 3·7 to 2·9.

Dry-weight production obtained using various carbon sources differs considerably in this investigation from that recorded by Schopfer (1934). The relative growths obtained by Schopfer on glucose, xylose, maltose, fructose, glycerol and lactose were 100:92:147:226:62:4. Our results show that the first four carbon sources were equally effective, and it is difficult to explain why Schopfer obtained such a stimulation of growth on maltose and fructose. We agree with Schopfer in finding lactose a very poor medium, but again differ from him with regard to glycerol, for we could obtain no mycelial growth when this was the sole carbon source.

SUMMARY

1. It has been confirmed that the major carotenoid of *Phycomyces blakesleeanus* Burgeff is β-carotene; small amounts of α-carotene are also present, but no trace of lycopene or xanthophylls was evident.

2. The dry-weight, lipid (ether-soluble) and β-carotene production by the (+) and (−) strains of *Phycomyces* has been examined under a variety of conditions.

3. On Schopfer's (1934) medium, maximal production of dry weight and lipid occurred after 5–6 days and of β-carotene after 5–9 days. β-Carotene, but not dry weight or lipid, decreased in ageing cultures. The (−) strain produces twice as much β-carotene as does the (+) strain, although lipid and dry-weight production is the same in both.

4. Light has no effect on lipid and dry-weight production; but β-carotene production is twice as great in cultures exposed to light as in those kept in the dark. When cultures exposed to light are placed in the dark they continue to produce β-carotene at the 'light' rate.

5. Substituting either glycine, L-alanine, L-leucine, or ammonium nitrate for L-asparagine has no effect on dry weight, and little on lipid production; glycine, however, specifically stimulated β-carotene production. Growth is poor on L-isoleucine and ammonium nitrate. In the absence of nitrogen from the medium no β-carotene is formed, although glucose is being metabolized.

6. When the nitrogen source is kept constant (0·2 % L-asparagine), variation in the glucose concentration of the medium affects β-carotene synthesis to a much greater extent than it affects dry-weight and lipid production, but little variations are noted when nitrogen is varied and glucose kept constant (2·5 %). The C:N ratio appears to be less important in controlling β-carotene production than was previously thought.

7. Substituting either xylose, fructose or maltose for glucose had no effect on lipid or dry-weight production; β-carotene production was normal with maltose, but reduced with xylose or fructose. Neither lactose nor glycerol supports mycelial growth.

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