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Studies in Carotenogenesis

2. CAROTENE PRODUCTION BY *PHYCOMYCES BLAKESLEEANUS*: THE EFFECT OF DIFFERENT AMINO-ACIDS WHEN USED IN MEDIA CONTAINING LOW CONCENTRATIONS OF GLUCOSE

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In a previous investigation (Garton, Goodwin & Lijinsky, 1951) it was shown that, using a medium containing 3 % (w/v) glucose, carotene synthesis in *Phycomyces blakesleeanus* was independent of the amino-acid used as the nitrogen source with the exception that glycine enhanced carotene production. Amongst the amino-acids which had no effect was L-valine, from which a hypothetical five-carbon building unit such as β-methylcrotonaldehyde (Karrer, Helfenstein & Widmer, 1928) could conceivably arise and promote carotene synthesis. The only other naturally occurring amino-acid containing a terminal dimethyl grouping is L-leucine. Such a grouping would appear to be essential in an active repeating unit acting as a carotene precursor, but in media containing 3 % (w/v) glucose L-leucine was as ineffective as L-valine.

It was possible, in media containing high concentrations of glucose, for stimulation of carotene synthesis by L-leucine or L-valine to be masked; it was therefore decided to investigate the action of these amino-acids in media containing sufficient glucose to produce a reasonable mycelial growth, but insufficient to produce more than small amounts of carotene. The observations of Garton *et al.* (1951) indicated that the most useful glucose concentration would be 1 % (w/v); this produces about 75 % of the maximal mycelial growth, whilst the carotene production is less than 10 % of the maximal.

EXPERIMENTAL

The general cultural and analytical procedures employed were described in detail in the first paper of this series (Garton *et al.* 1951). The various media were made by altering the glucose concentration of the standard medium, generally to 1 % (w/v), and varying the N source according to requirements; the salt and aneurin concentrations were unaltered. All experiments were carried out under natural illumination.

Replacement cultures. Well-developed mycelia which could be easily handled and transferred to fresh media were obtained by culturing the fungus on filter paper (Whatman no. 50) in Petri dishes (11 cm. diameter). Small glass beads (2–3 mm. diameter) were added in sufficient numbers to cover the bottom of the dishes and 45 ml. of the medium added to each dish; this is just sufficient to cover the beads. The filter papers were then placed in position on the beads and the dishes sterilized. Inoculation was carried out by pipetting the spore suspension on to the filter paper. When the mycelia were at the appropriate stage of growth they were transferred to a new medium in another Petri dish by lifting out the filter paper and adhering mycelium, washing it with sterile water in an inoculating cabinet and placing it carefully on the new medium. Glass beads are not essential in the replacement media, but they are necessary in the first cultures in order to obtain reproducible mycelial growths. Glass beads are much better for supporting the filter paper than sand, which had previously been suggested for this purpose by Raaf (1941). Small amounts of sand inevitably get into the mycelia and, as they are impossible to remove completely, dry weight determinations cannot be carried out. After growth is completed, the filter paper can be removed by peeling it away from the fungal mat, which can then be examined in the usual way.

RESULTS

In the first experiment three media were used; all contained 1 % (w/v) of glucose, whilst the N sources were either L-leucine, L-asparagine or L-valine (*c*, 0·35, 0·2 and 0·676 % (all w/v) respectively). It

was intended that the nitrogen content of the media should remain constant at 0·0374 % (w/v); this was possible with the first two amino-acids, but the growth rate on the equivalent amount of L-valine was found to be so small that the nitrogen concentration had to be doubled in order to get a reasonable growth rate. The results obtained on these media (Table 1) show that the growth rate is very much less on L-leucine and L-valine than on L-asparagine, 10–14 days being required to reach maximal mycelial dry weight compared with 4 days. The final growth is also somewhat reduced. Carotene synthesis, on the other hand, is considerably increased both relatively (from 0·03 to 0·07–0·09 % dry weight) and absolutely (from 18 to 32–41 μg.); in young cultures lipid production is more rapid than general mycelial growth, but the final amount produced is not significantly different from that formed on L-asparagine.

Table 1. *Dry weight, lipid and carotene production by* Phycomyces *on media containing* 1 % (*w/v*) *glucose and either* 0·2 % (*w/v*) (= 0·0374 % N) L-*asparagine*, 0·35 % (*w/v*) (= 0·0374 % N) L-*leucine, and* 0·624 % (*w/v*) (= 0·0748 % N) L-*valine*

(The amounts are those produced in a standard culture flask (8 oz. medicine bottle containing 15 ml. of medium).)

Period of growth (days)	Amino-acid in medium: L-Asparagine Dry wt. (mg.)	L-Asparagine Lipid (mg.)	L-Asparagine Carotene (μg.)	L-Valine Dry wt. (mg.)	L-Valine Lipid (mg.)	L-Valine Carotene (μg.)	L-Leucine Dry wt. (mg.)	L-Leucine Lipid (mg.)	L-Leucine Carotene (μg.)
4	66	15·8	14	—	—	—	21	7·9	5
5	65	15·9	11	—	—	—	23	7·3	7
7	60	10·0	18	16	5·3	6	36	12·5	19
10	52	5·4	12	37	11·5	23	46	14·2	41
14	46	4·1	10	50	9·0	32	46	13·8	34

It was then decided to find out (*a*) if L-leucine and L-valine stimulated carotenogenesis when the fungus was growing at its normal rate, and (*b*) if glycine, which stimulates synthesis in glucose-rich media, is also effective in media low in glucose, and (*c*) if mixtures of glycine and either L-leucine or L-valine were more effective than the corresponding mixtures with L-asparagine. Media containing the following amino-acid constituents were used: (*a*) L-asparagine, (*b*) glycine, (*c*) L-asparagine + glycine, (*d*) L-leucine + L-asparagine, (*e*) L-leucine + glycine, (*f*) L-valine + L-asparagine and (*g*) L-valine + glycine. All the media contained 0·0374 % (w/v) of nitrogen; in those containing two amino-acids one-half of the nitrogen was provided by each component. As changes in the carbon content of the medium in the concentration range used in this experiment can affect the amount of carotene synthesized (Garton *et al.* 1951), the concentration of carbon in all these media was kept at 0·464 % (w/v); this was achieved by adding the amino-acids at levels equivalent to a nitrogen concentration of 0·0374 % (w/v) and then adding sufficient glucose to provide a final carbon concentration of 0·464 % (w/v).

The results of a typical series of experiments with these media are recorded in Table 2. Dry weight and lipid production are approximately the same in all media, whilst more carotene is produced on media containing L-leucine or L-valine than on any of the others; under these conditions glycine has no stimulatory action on carotene production either in the presence or absence of L-leucine or L-valine. When the concentrations of carotene are considered, the stimulation by L-leucine and L-valine is very obvious (Fig. 1), the values in the presence of these amino-acids being two to four times greater than those obtained on the other media. It will also be noted that the effect persists throughout the growth period examined (4–14 days) and that L-leucine is considerably more effective than L-valine.

The stimulation recorded in the experiments just described appears to be the maximal effect obtainable with L-leucine and L-valine, because it was found that doubling the concentrations of these amino-acids had no further effect on carotene production.

In order to find out if the effect of L-leucine and L-valine was observed in cultures which were growing only very slightly, as well as in actively growing mycelia, a series of experiments was carried out using replacement cultures. The fungus was cultured on the standard medium (2·5 % (w/v) glucose and 0·2 % (w/v) L-asparagine) on filter paper supported by glass beads, in Petri dishes; when the mycelia were 4–5 days old, i.e. when almost fully grown but containing very little carotene, they were transferred to fresh media. In the first series of transference experiments the media used contained 1 % (w/v) glucose and 0·2 % (w/v) of either L-valine, L-leucine or L-asparagine. The mean base-line levels of dry weight, lipid and carotene were obtained by analysing a number of 4- to 5-day-old mats inoculated the same time as, and cultured alongside, the mats which were transferred. The results are re-

Table 2. *Dry weight, lipid and carotene production by* Phycomyces *on media containing glucose and amino-acids*

(The concentrations of the amino-acids were so arranged that the N content of all the media was 0·0374% (w/v) and in those media containing two amino-acids each provided one-half of the N. The total C content of all the media was kept constant by adding sufficient glucose to provide a final C content of 0·464% (w/v). The amounts are those produced in a standard culture flask (8 oz. medicine bottle containing 15 ml. of medium).)

Period of growth (days)	Amino-acid in medium																					
	L-Asparagine			Glycine			L-Asparagine + glycine			L-Leucine + L-asparagine			L-Leucine + glycine			L-Valine + L-asparagine			L-Valine + glycine		
	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)
4	68	19	9	43	6	12	67	10	11	58	11	24	42	8	16	50	12	13	30	5	10
6	54	10	9	60	10	6	43	12	8	53	7	23	56	10	24	48	9	16	53	12	13
8	54	9	10	52	9	9	54	9	7	47	5	21	51	7	24	43	7	17	44	8	14
10–11	51	6	6	46	6	8	43	4	8	44	3	21	49	5	22	41	5	15	43	7	13
13–14	48	5	7	41	2	5	46	4	6	44	3	14	43	6	13	37	3	10	37	4	10

corded in Table 3 and show that whilst the total dry weight of the transferred cultures has increased 3 days after transference on all the media it was greatest on the medium containing L-asparagine; the carotene production, however, was very much greater on the media containing the other amino-acids, whilst the amount of lipid produced had not appreciably altered in any culture. The dry weight

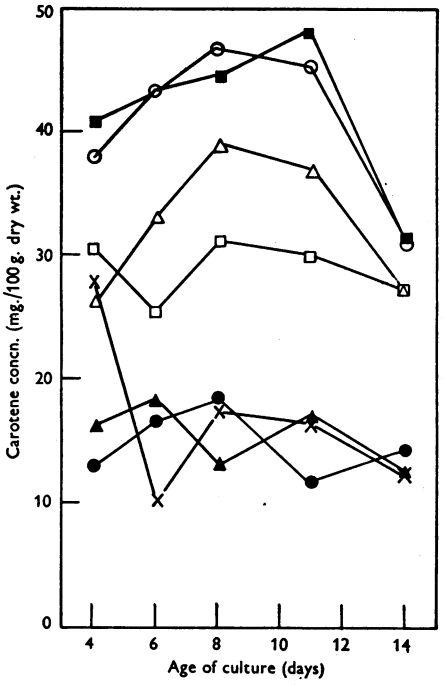


Fig. 1. The concentration of carotene produced by *Phycomyces* at various stages of growth when grown on media containing 0·0374% (w/v) of N and 0·464% (w/v) of C. The N applied as different amino-acids either singly or as binary mixtures, the C (other than that in the amino-acids) as glucose. ●—●, L-Asparagine; ×—×, glycine; ▲—▲, L-asparagine + glycine; ■—■, L-leucine + L-asparagine; ○—○, L-leucine + glycine; △—△, L-valine + L-asparagine; □—□, L-valine + glycine.

had dropped somewhat on all the media 5-6 days after transference, mainly owing to the disappearance of lipid from the mycelia; the carotene content had also dropped a little in the media containing L-asparagine and L-valine, but continued to increase on the L-leucine medium. The very great stimulatory effect of L-leucine and L-valine in these conditions can be appreciated by reference to Fig. 2, in which the concentrations of carotene in the three mycelia are compared. This figure also emphasizes again the superiority of L-leucine over L-valine in this respect.

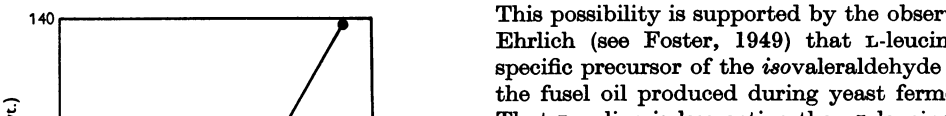
In the final experiment, well formed mycelial mats were transferred to media containing no glucose but

Table 3. *Dry weight, carotene and lipid production by well developed mycelia*

(Mycelia, 4 days old; grown on a medium containing 2·5% (w/v) of glucose, transferred to media containing 1% (w/v) of glucose and 0·2% (w/v) of either L-valine, L-leucine or L-asparagine. The amounts are those produced in an 11 cm. Petri dish containing 45 ml. of medium.)

	Amino-acid in medium								
	L-Asparagine			L-Valine			L-Leucine		
Time after transference (days)	Total dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Total dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Total dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)
0	273	77	56	273	77	56	273	77	56
3	401	76	163	374	97	273	350	81	364
5	370	53	135	347	70	229	319	48	441

only the amino-acids L-asparagine, L-leucine, L-valine or glycine as the sole nitrogen and carbon sources. Table 4 shows that under these conditions, the media containing 0·2% L-leucine or L-valine synthesized a little carotene whilst those containing L-asparagine or glycine synthesized none. Synthesis took place to a slight but definite extent on all the media when the amino-acid concentration was raised to 1% (w/v); no differences between the amino-acids could, however, be observed. The increases noted are very small compared with those obtained when small amounts of glucose are present (Table 3). In all cases the dry weight of the mycelia dropped after transference mainly owing to the rapid disappearance of the lipid fractions.



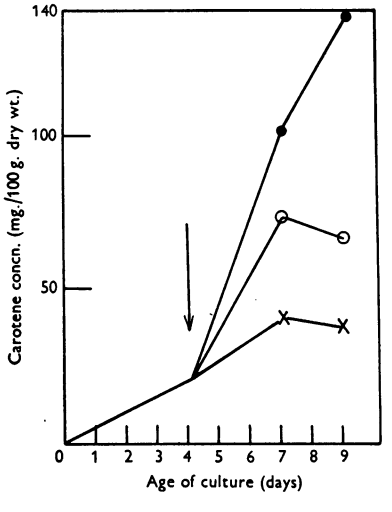
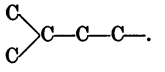


Fig. 2. The concentration of carotene produced by fully grown mycelial mats of *Phycomyces* transferred to media containing 1% (w/v) glucose and either 0·2% (w/v) L-valine, L-leucine or L-asparagine. The arrow indicates the day the mycelia were transferred. ●—●, L-Leucine; ×—×, L-asparagine; ○—○, L-valine.

DISCUSSION

The demonstration that L-valine can, in the absence of excess glucose, stimulate carotenogenesis in *Phycomyces* strongly suggests that the repeating unit in the synthesis contains the C_5 skeleton:



L-Leucine is even more active than L-valine and may furnish the C_5 residue by losing its carboxyl carbon; in this way a decarboxylation following oxidative deamination would yield *iso*valeraldehyde, which could function as the repeating unit, thus:

This possibility is supported by the observation of Ehrlich (see Foster, 1949) that L-leucine is the specific precursor of the *iso*valeraldehyde found in the fusel oil produced during yeast fermentation. That L-valine is less active than L-leucine may be because *iso*valeraldehyde is more easily formed from L-leucine than from L-valine. Whether this compound is, in fact, the active repeating unit and, if so, whether it is dehydrogenated before or after condensation is under investigation. It is of considerable importance to settle this because Bonner & Arreguin (1949), studying the biogenesis of rubber, an analogous problem, concluded that the unsaturated compound β-methylcrotonic acid was the active repeating unit; whilst implicit in the ideas of Porter & Lincoln (1950) on carotenoid synthesis in tomatoes is the assumption that the repeating unit is a saturated compound. They suggest that carotenoids are synthesized in tomatoes by the stepwise removal of four hydrogen atoms from a saturated C_{40} precursor.

As *Phycomyces* grows at almost the same rate on a medium containing NH_4^+ as the sole nitrogen source as it does on a medium containing L-asparagine or glycine, it must be concluded that the very

Table 4. *Dry weight, carotene and lipid production by well developed mycelia*

(Mycelial mats (4–5 days old) of *Phycomyces* transferred to media containing no glucose but one of the four amino-acids, L-leucine, L-valine, L-asparagine, and glycine, at a concentration of 0·2 or 1·0 %, w/v.)

Time after transference (days)	Amino-acids in media																	
	At 0·2 % (w/v) level								At 1 % level									
	L-Valine			L-Leucine			L-Asparagine			Glycine			L-Valine			L-Leucine			L-Asparagine		
	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)
0	206	48	46	206	48	46	206	48	46	206	48	46	273	77	56	273	77	56	273	77	56
3	187	33	56	199	24	53	191	29	39	177	28	33	244	42	76	241	43	93	245	51	87
6	170	18	53	133	11	44	149	8	44	154	16	45	231	36	88	220	28	86	221	32	72

slow growth on L-leucine or L-valine is due to the slow rate of deamination of these amino-acids; for on them growth does eventually reach a level almost equal to that on other amino-acids (Garton *et al.* 1951). The rate of glucose catabolism by *Phycomyces* developing on a medium containing only L-leucine or L-valine as the nitrogen source probably provides a sufficient concentration of active intermediates to fix, as cellular material, all the NH_3 arising from the slow deamination of the amino-acids. The carbon residues from these amino-acids (probably α-keto-*iso*valeric and -*iso*caproic acids) would thus be utilized for cellular growth only to a minor extent and be mostly available for, *inter alia*, carotene synthesis. In the case of rapidly deaminated amino-acids (e.g. L-asparagine) the carbon residue would much more likely be mainly utilized for general growth purposes. In the case of media containing a mixture of L-leucine or L-valine and L-asparagine or glycine, growth is normal because of the presence of the latter two amino-acids, but carotene synthesis is above normal because of the presence of L-leucine and L-valine.

It is important to realize that if L-leucine and L-valine had supported a normal growth rate of *Phycomyces*, the fact that they can provide an active intermediate for carotene synthesis might have gone unsuspected, for a rapid provision of ammonia would have probably resulted in the utilization of most of the keto acids for synthesis of cellular material. When well formed mycelia are transferred to fresh media containing 1 % (w/v) of glucose and 0·2 % (w/v) of an amino-acid as the nitrogen source, much less growth occurs as measured by nitrogen assimilation (Goodwin & Willmer, 1951) than in newly germinated mycelia. Under these conditions if a deaminated amino-acid were a specific intermediate in carotene synthesis some increase in pigment production should be obtained whatever the rate of deamination of that amino-acid. The observation that L-leucine and L-valine are still active in replacement media whilst the other amino-acids remain inactive, emphasizes the specificity of these two amino-acids in stimulating carotenogenesis; if L-asparagine or glycine had any specific stimulatory effect one would have expected it to show up in replacement cultures. These replacement experiments also show that carotenogenesis can be separated completely from lipogenesis, for the lipid content of the transferred mycelia always decreased although carotene production was proceeding rapidly.

The disappearance of the stimulatory action of L-leucine and L-valine when the culture media contain high concentrations of glucose (Garton *et al.* 1951) indicates that the specific unit provided by these amino-acids is readily formed during the dissimilation of the excess glucose.

The failure of L-leucine and L-valine to form appreciable amounts of carotene in replacement cultures in the absence of glucose is taken to indicate that energy is required for one or both of two reactions: (*a*) to transfer the amino-acids into the fungal cells, as is the case with certain bacteria (Gale, 1951); (*b*) to effect the condensation or the dehydrogenation, or both, of the repeating units; glucose dissimilation appears to be necessary in order to provide this energy.

The role of glycine in the biogenesis of carotenoids remains obscure. It is the only amino-acid which stimulates synthesis in the presence of high concentrations of glucose (Garton *et al.* 1951), but no such effect is noted in the presence of only 1 % (w/v) of glucose.

Lipid production. Although not primarily concerned with lipogenesis this investigation has brought out two important points which must be briefly considered. In the first place *Phycomyces* cultured on media containing only 1 % (w/v) of glucose contain, as soon as they are fully grown, a normal concentration of lipid (20–30 g./100 g. dry weight), but this is quickly lost as the mycelia age; on the higher glucose concentrations the disappearance of the lipid is much slower (Garton *et al.* 1951). The utilization of the mycelial lipid also occurs when well formed (4-day-old) mats are transferred to media containing only 1 % (w/v) of glucose. Secondly, when the culture medium contains 1 % (w/v) of glucose and either L-leucine or L-valine as the sole nitrogen source, the total mycelial growth rate is much more reduced than is the rate of lipid synthesis, so that in young cultures the concentration of lipid is much greater than normal (about twice). This rate is relatively not as great as the rate on media supporting normal rate of total growth; further, the amount of lipid synthesized on L-leucine and L-valine is never more than in other media, so that when maximal growth is attained the lipid concentration is not significantly greater than normal. This further demonstrates that the stimulatory effect of L-leucine and L-valine is confined to carotene; they stimulate the relative rate of lipogenesis but have no effect on the amount synthesized.

SUMMARY

1. The effect on the carotene production by *Phycomyces blakesleeanus* of using various amino-acids as the nitrogen source in media containing 1 % (w/v) glucose has been studied.

2. L-Valine and L-leucine whether used alone or in conjunction with glycine or L-asparagine stimulate carotenogenesis, in some cases as much as four-fold. Glycine and L-asparagine either alone or together have no such effect.

3. Transference of well formed mycelial mats to media containing 1 % (w/v) glucose and various amino-acid combinations also results in considerably increased carotene production only on those media containing either L-leucine or L-valine.

4. The relative rate of lipogenesis is increased in young mycelia growing on media containing 1 % (w/v) glucose and either L-leucine or L-valine. The total amount of lipid synthesized is, however, not different from that produced on media containing other amino-acids.

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