ponents of lower molecular weight estimated by quantitative paper-partition chromatography.

3. The significance of these findings is discussed in relation to the structures proposed by Dedonder (1951*a*) and Bacon & Edelman (1951) for the tuber carbohydrates and a suggestion is made as to the nature of their metabolic interconversion.

We are very grateful to Dr R. Dedonder for sending us a copy of his Thesis on 'Les glucides du topinambour', which contains much experimental detail not yet published.

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Studies in Carotenogenesis

4. NITROGEN METABOLISM AND CAROTENE SYNTHESIS IN *PHYCOMYCES BLAKESLEEANUS*

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Preliminary experiments on the relationship between nitrogen metabolism and carotenogenesis in *Phycomyces blakesleeanus* (Garton, Goodwin & Lijinsky, 1951) suggested that carotene was not synthesized when well formed mycelial mats were transferred to a medium containing glucose but no assimilable nitrogen. The present paper records a full investigation into this aspect of the problem of carotene formation in *Phycomyces*.

Part of this work has already been briefly described (Goodwin, Lijinsky & Willmer, 1951).

EXPERIMENTAL

The general cultural conditions, the technique of mycelial transfer, and the analytical methods for dry weight, lipid, and carotene determinations have previously been described in detail (Garton *et al.* 1951; Goodwin & Lijinsky, 1951). Nitrogen determinations were carried out using the micro-Kjeldhal method of Markham (1942).

RESULTS

Carotene production and nitrogen assimilation in growing cultures

Mycelia of *Phycomyces* cultured on media containing 0·2% (w/v) of L-asparagine and either 2·5, 1·5 or 1·0% (w/v) of glucose (unless otherwise stated the salt and aneurin concentrations are those used by Garton *et al.* 1951) were harvested at various times after inoculation and analysed for dry weight, lipid, carotene and nitrogen. The results obtained using the medium containing 2·5% glucose are recorded in Fig. 1. From this figure it will be seen

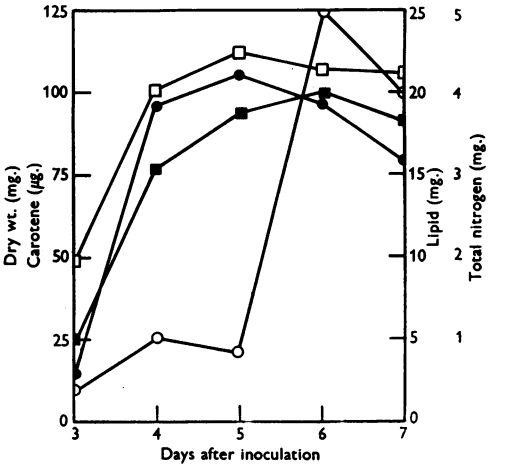

Fig. 1. The dry weight, lipid, carotene and nitrogen content of developing *Phycomyces* cultured in an 8 oz. medicine bottle on 15 ml. of a standard medium (Garton *et al.* 1951) containing 2·5% (w/v) glucose and 0·2% (w/v) asparagine. □—□, dry weight; ○—○, carotene; ■—■, lipid; ●—●, nitrogen.

that, assessed by cessation of either dry weight production or nitrogen assimilation, growth must be complete before carotene synthesis proceeds at all

rapidly. Growth is complete within 4 or 5 days, but at this time carotene formation is only about 16% of the final amount, which is rapidly synthesized within the next 2–3 days. Very similar results, which are not recorded here, were obtained using the medium containing 1·5% (w/v) of glucose, except that the amount of carotene synthesized was not as great. As would be expected from the previous results of Garton *et al.* (1951), after growth had ceased no further carotene was produced on the medium containing 1% of glucose owing to the absence of dissimilable glucose.

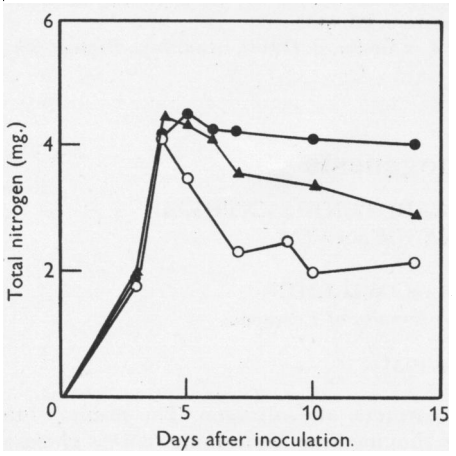

Fig. 2. The nitrogen content of developing *Phycomyces* cultured on media containing 0·2% (w/v) of L-asparagine but varying amounts of glucose. ○—○, 1% glucose; ▲—▲, 1·25% glucose; ●—●, 2% glucose.

Table 1. *Total nitrogen concentration in mycelia of* Phycomyces *grown on media containing varying amounts of glucose*

Age of culture (days)	Nitrogen concentration (%) in medium containing 1% (w/v) glucose	1·5% (w/v) glucose	2·5% (w/v) glucose
3	6·8	8·7	7·9
4	12·3	6·1	5·4
5	5·17	4·3	4·5
6	5·8	5·3	4·3
7	4·3	4·8	4·6
10	4·9	5·0	3·7
14	5·2	4·8	4·0

The results obtained for the nitrogen uptake on the different media are worthy of separate consideration. Fig. 2 shows that the maximal nitrogen content occurs 3–4 days after inoculation, and is the same irrespective of the glucose concentration of the medium. In the case of mycelia grown on media containing excess glucose (2·5%, w/v) most of this nitrogen is retained in the mycelia as they age. If, however, the media contain insufficient glucose it is clear from Fig. 2 that, as the mycelia mature, a considerable amount of the nitrogen is lost to the medium. Because dry weight production is directly proportional to, whilst the nitrogen uptake is independent of, the glucose concentration of the medium, young mycelia growing on a glucose-poor medium have a very high nitrogen concentration. This can be seen from Table 1 which also demonstrates that owing to the different rates at which nitrogen is lost from the mycelia, its concentration in all mycelia tends to be the same after about 7 days' growth. In older cultures, the nitrogen concentration of the mycelia grown on the glucose-rich medium again tends to be lower; this is due to the maintenance of the mycelial weight in old cultures when sufficient glucose is present. On the glucose-poor media old cultures tend to decrease in dry weight.

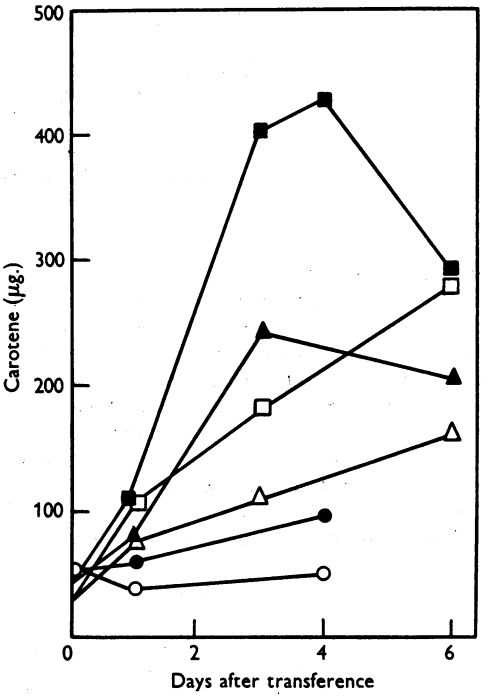

Fig. 3. The carotene content of mycelia after transference, when 4 days old, to hard media containing 2·5% glucose and either: ■—■, 0·2% (w/v) L-asparagine (unbuffered medium); ▲—▲, no nitrogen (unbuffered medium); ●—●, 0·2% (w/v) L-asparagine (buffered medium, pH 7); ○—○, no nitrogen (buffered medium, pH 7); □—□, 0·2% (w/v) L-asparagine (medium with high salt concn. see Table 2); △—△, no nitrogen (medium with high salt concn.).

Carotene synthesis in the presence and absence of assimilable nitrogen

The results just described suggest that the failure of Garton *et al.* (1951) to observe carotene synthesis by well developed mycelial mats dissimilating

glucose in the absence of nitrogen was due to a factor other than the absence of nitrogen from the medium. The only cultural difference in the transference experiments of Garton *et al.* (1951) compared with the other experiments described by them was that the formed mycelia were transferred to the standard medium buffered at pH 7·0; all other media were unbuffered. A series of experiments was thus undertaken to test whether the buffering of the medium inhibited carotenogenesis under conditions known to be normally favourable to synthesis. As the buffering of the medium necessitated a considerable increase in salt concentration of the medium, the effect of increasing this, irrespective of buffering power, was also investigated. The concentrations of the salts used to prepare the various media are recorded in Table 2 and the experimental results in Fig. 3 and Table 3. Fig. 3 shows that carotene synthesis on an unbuffered medium with a normal salt concentration is considerable, whether the medium contains nitrogen or not. On buffered media, on the other hand, no carotene is synthesized on the nitrogen-free media (there is actually a slight loss of carotene during the first day after transference), whilst only a very small amount is synthesized when nitrogen is present. On media with only slight buffering capacity but with twice the salt concentration of the buffered media, i.e. ten times the normal salt concentration, carotenogenesis still takes place, but to a somewhat lesser degree compared with the standard medium. Table 3 shows that dry weight production is not appreciably affected by the ionic condition of the medium; lipogenesis, on the other hand, is stimu-

Table 2. *Salt concentration in media used to investigate the effect of buffering and salt concentration on carotene synthesis*

	Concentration of salt % (w/v)				
Constituent	Standard medium	Medium buffered to pH 7·0	Unbuffered medium with salt concn. equal to twice that of buffered media	Medium buffered to pH 6·0	Medium buffered to pH 5·2
$MgSO_4.7H_2O$	0·05	0·05	0·5	0·05	0·05
KH_2PO_4	0·15	0·363	1·5	0·817	0·885
Na_2HPO_4	0·00	0·568	0·00	0·094	0·0237

Table 3. *Amounts of dry weight, lipid and carotene produced by well formed (4-day-old) mats of* Phycomyces *after transference to buffered and unbuffered media containing 3·0 % (w/v) of glucose and either no nitrogen or 0·2 % (w/v) of* L*-asparagine as the sole nitrogen source*

(The salt concentrations of the various media are given in Table 2. The mats were grown on filter paper in Petri dishes (7·5 cm. diameter) containing 45 ml. of medium and transferred to similar dishes containing the same amount of medium (see Goodwin & Lijinsky, 1951).)

Time after transference (days)	With nitrogen				Without nitrogen			
	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (p.p.m.)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (p.p.m.)
				Standard medium				
1	383	79	20·6	210	113	68	60·2	500
3	387	74	19·1	1060	103	35	34	2070
5	294	45	15·3	1070	84	21	25	2410
				Medium buffered at pH 7·0				
1	142	42	29·6	—	34	18	52·9	0
2	331	110	33·2	45	101	41	40·6	0
3	356	122	34·2	73	72	39	54·1	0
4	341	125	36·6	111	—	—	—	0
5	382	98	34·6	74	71	32	45·1	0
				Medium with high salt concentration (unbuffered)				
1	237	70	29·5	250	(−17)	(−2)	—	—
3	317	87	27·5	420	—	15	25·4	1110
5	369	112	30·4	630	80	80	37·5	1380

Table 4. *Dry weight and lipid production of* Phycomyces *cultured on the standard medium (containing 3 % (w/v) glucose) buffered at various* pH *values*

Age of culture (days)	Medium buffered at pH 7·0			Medium buffered at pH 6·0			Medium buffered at pH 5·2			Unbuffered medium		
	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)
4	57·5	17·4	30·1	48·5	—	25·6	59·1	17·0	28·8	63·5	17·5	27·5
7	103·0	34·7	33·7	109·2	36·4	33·3	98·3	28·5	28·9	83·7	14·3	17·1
11	104·8	33·6	32·0	104·9	26·3	25·0	97·7	21·7	22·1	69·7	10·7	15·3
17	93·4	14·1	15·0	91·8	16·8	18·3	96·5	18·2	19·0	—	—	—

lated on media containing a high salt concentration probably irrespective of pH (see later). Lipogenesis was also stimulated in the absence of nitrogen; up to 50 % of the dry weight formed by a well developed mat, after transference to a medium not containing nitrogen and buffered at pH 7·0, is lipid.

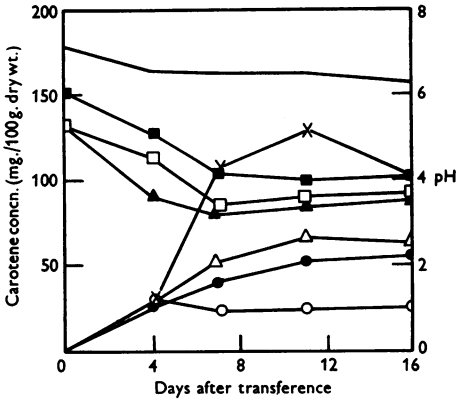

Fig. 4. The concentration of carotene in mycelia grown on standard medium buffered at various pH values; ○—○, medium buffered at pH 7·0; ●—●, medium buffered at pH 6·0; △—△, medium buffered at pH 5·2; ×—×, unbuffered medium.

Also the corresponding changes in the pH of the media during growth; ——, medium initially buffered at 7·0; ■—■, medium initially buffered at 6·0; □—□, medium initially buffered at 5·2; ▲—▲, unbuffered medium.

On unbuffered media the synthesis of carotene by developed mats is relatively greater in the absence of nitrogen (Table 3), for the concentration of carotene in the dry matter produced by a mat dissimilating glucose in the absence of nitrogen is about twice that observed when nitrogen is present in the medium.

The results of a series of experiments carried out to see if the effect of buffering the medium, just described for transferred cultures, is observed in ordinary cultures are recorded in Table 4 and Fig. 4. Three buffered media were used at pH 7·0, 6·0 and 5·2 (see Table 2 for the salt concentration). It will be seen from Fig. 4 that the pH of the medium buffered at 7·0 remained almost constant throughout the experiment at about 6·5 (the pH of the medium after inoculation with spore suspension), whilst those of the other buffered media fell to 4·1 and 3·8 respectively; the final pH of the control (unbuffered) medium was 3·2. Carotene synthesis varied inversely with the pH of the medium. The total dry weight production was not affected by the pH of the medium, whilst, as in the case of transferred cultures, lipogenesis was affected in exactly the opposite way to carotenogenesis, being considerably higher in media of high pH.

DISCUSSION

The results recorded in Fig. 1 show that, in the presence of excess glucose and a source of readily assimilable nitrogen, the major portion of carotene synthesis of *Phycomyces* occurs after growth (dry weight production and/or nitrogen assimilation) is complete. It appears, then, that most of the carotene is synthesized from the products of the dissimilation of glucose, quite probably under anaerobic conditions, for carotene accumulates in the surface of the mycelium in contact with the medium and, as the cultures are static, conditions in this region must be almost completely anaerobic. Carotenogenesis in *Phycomyces* could thus be an example of fermentative assimilation, a type of synthesis known to occur in yeasts (Clifton, 1946).

The question naturally arises as to how the observation of Goodwin & Lijinsky (1951), that, in the presence of suboptimal amounts of glucose, L-valine and L-leucine stimulate carotenogenesis, fits in with these just reported. It will be seen from Fig. 1 that a small amount of carotene is always synthesized as the mycelium is growing, thus indicating that the enzyme systems necessary for this synthesis are present in growing mycelia. If the amino-acid being used as the nitrogen source yields on deamination a carbon residue readily incorporated into the carotene molecule, then obviously the amount of carotene synthesized as the fungus is growing will be increased; this is what occurs with L-valine and L-leucine.

There is now no doubt that the inability of Garton *et al.* (1951) to observe carotenogenesis in *Phycomyces* mats dissimilating glucose in the absence of nitrogen

was due to their media being buffered at about pH 7. The present results show that in any medium in which the pH is not allowed to fall to 3·2, carotene synthesis is considerably inhibited. A probable explanation of this is that, assuming that the pigments are produced by fermentative assimilation, the changes in pH alter the pathway of glucose dissimilation, and the production of the building units required for carotene synthesis is reduced. The classic example of this is the very sharp optimal pH for the production of citric acid by *Aspergillus niger*, and the increased production of acids other than citric at other pH values (Bernhauer, 1926). The reduced synthesis by mats transferred to unbuffered media with a high salt concentration (Fig. 3) is probably due to the slight buffering power of this medium rather than to any osmotic effects, for it was noted that during the experiment the pH of these media never fell below 3·8. The pH of normal media falls to 3·2 (Fig. 4).

The present work has demonstrated that lipid production in *Phycomyces* depends also on the pH of the medium, the higher the pH the greater the lipogenesis. Again, this can be explained on the assumption of variation in the products of glucose dissimilation according to the pH of the medium. Similar variations in lipid production have been observed in *A. niger* (Pontillon, 1930) and *A. fisheri* (Prill, Wenck & Peterson, 1935).

Nitrogen metabolism

The observations reported here on the assimilation of nitrogen by *Phycomyces* fall into line with those by Steinberg (1939) on *A. niger*, i.e. the amount of nitrogen removed from the medium is very much the same regardless of the glucose concentration of the medium. The present work further shows that on media containing only a low concentration of glucose a considerable part of the nitrogen assimilated during the early stages of growth is rapidly lost as the mycelia age, probably owing to the utilization of nitrogen-containing material for the production of energy. On media containing adequate amounts of glucose little or no excretion takes place.

SUMMARY

1. The major portion of the carotene synthesized by *Phycomyces blakesleeanus*, using a readily assimilable source of nitrogen which does not also provide a specific carotene precursor, is produced only after the mycelial mat is fully grown, as measured by dry weight production and/or nitrogen assimilation.

2. Media buffered at high pH values (5·2–7·0) support normal growth of *Phycomyces*, although carotenogenesis is almost completely inhibited. Lipogenesis, on the other hand, is stimulated under these conditions.

3. Well formed mats of *Phycomyces* dissimilating glucose can synthesize relatively more carotene in the absence of assimilable nitrogen than in its presence. Under these conditions lipid synthesis is also relatively greater on the nitrogen-free media.

4. Nitrogen uptake by *Phycomyces* is independent of the glucose concentration of the medium. When the glucose concentration of the medium is low a considerable amount of the assimilated nitrogen is lost as the mycelia age.

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