Studies in Carotenogenesis

7. FURTHER OBSERVATIONS CONCERNING THE ACTION OF DIPHENYLAMINE IN INHIBITING THE SYNTHESIS OF β-CAROTENE IN *PHYCOMYCES BLAKESLEEANUS*

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Kharasch, Conway & Bloom (1936) observed that diphenylamine inhibited chromogenesis in a number of bacteria and fungi. Some of the organisms so affected are known to contain carotenoids, e.g. *Rhodospirillum rubrum* (van Niel & Smith, 1935), *Micrococcus pyogenes* var. *aureus* (Chargaff & Lederer, 1935), *Rhodotorula rubra* (Lederer, 1938), and Turian (1950) showed conclusively that diphenylamine inhibited carotenogenesis in *Mycobacterium phlei*. He suggested that it functioned by inhibiting the dehydrogenation of more saturated polyenes, such as phytofluene, which were assumed to be precursors of the carotenoids, although such compounds had not at that time been demonstrated in *Mycobact. phlei*. Recently, however, traces of phytofluene have been found in some strains of this organism (Goodwin & Jamikorn, 1952*a*).

Goodwin (1952) found that diphenylamine also inhibited the synthesis of the most unsaturated polyenes (α-, β- and γ-carotenes and lycopene) in the fungus *Phycomyces blakesleeanus*; simultaneously there was an increased synthesis of the more saturated polyenes, ζ-carotene, neurosporene, phytofluene and (probably) phytoene. Although these observations appeared at first sight to confirm Turian's suggestion, it was pointed out that an equally plausible explanation was that the inhibitor caused an accumulation of some unknown precursor common to both β-carotene and the more saturated polyenes. As the route to β-carotene was blocked this precursor was diverted to the synthesis of phytofluene, etc. On this argument these polyenes need not be intermediates in the synthesis of β-carotene.

Recently, Turian (1951) has stated that phenol has the same action as diphenylamine on carotenogenesis in *Mycobact. phlei*, especially when glucose is the carbon source, that resorcinol is 2–3 times less effective than phenol, that quinol and pyrocatechol are too easily oxidizable to be effective and that 1-naphthylamine, thiourea, potassium cyanide and salicylaldoxime are almost inactive.

The present paper records further work designed to show how diphenylamine inhibits the synthesis of β-carotene in *Phycomyces*. Some of these results were briefly reported to the 2nd International Congress of Biochemistry (Goodwin, Griffiths, Jamikorn & Willmer, 1952).

EXPERIMENTAL

The cultural methods used were those described by Garton, Goodwin & Lijinsky (1951) and the transfer technique that of Goodwin & Lijinsky (1952). The standard medium used throughout was that of Garton *et al.* (1951) with the exception that the glucose concentration was reduced from 10% (w/v) to 2·5% (w/v). This was because Garton *et al.* (1951) found that carotene synthesis was maximal at a concentration of 2·5% (w/v). Dry weight, lipid and total carotene determinations were carried out according to Garton *et al.* (1951) and the chromatographic separation of the constituent polyenes and the determination of their relative abundance according to the method of Goodwin (1952).

RESULTS

The rate of production of the various polyenes on diphenylamine-containing media

Phycomyces cultured on the standard medium (Garton *et al.* 1951) containing diphenylamine were analysed at varying times after inoculation. The results of a typical experiment are given in Fig. 1 and Table 1. It will be seen from Fig. 1 that the pattern of synthesis of the more saturated polyenes is quite different from that of β-carotene. Maximal synthesis of phytofluene occurs in very young cultures, i.e. within 3–4 days of inoculation when growth is only about 30–50% complete. Synthesis of ζ-carotene and neurosporene follow a somewhat similar pattern, although their maximal production is a little later (4–5 days). β-Carotene, on the other hand, is synthesized to a very limited extent in growing cultures and reaches its maximum only after growth has been completed (7 days). This difference in rate of synthesis is reflected in the concentration of the polyenes in mycelia of different ages (Table 1); very high concentrations of the more saturated components are obtained in young cultures and these diminish markedly with age. The concentration of β-carotene, on the other hand, gradually increases with age. The amounts of the

more saturated polyenes in cultures not containing diphenylamine are quite small, but the general pattern of synthesis is the same as in the presence of diphenylamine. This is illustrated in Figs. 4 and 5, which give the results of an experiment designed for another purpose (see later).

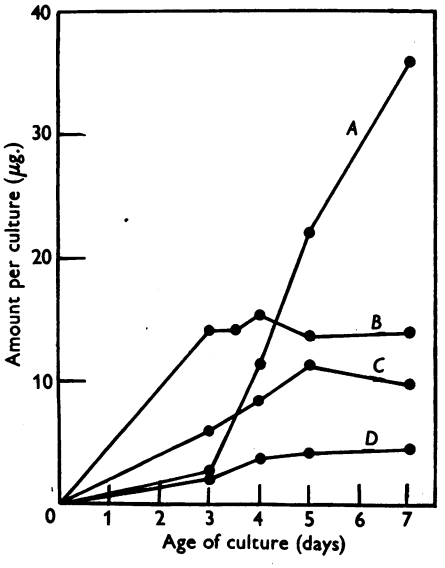

Fig. 1. The rate of synthesis of the various polyenes by *Phycomyces* when cultured on the standard medium (Garton *et al.* 1951) containing 1/70 000 (w/v) of diphenylamine. *A*, β-carotene; *B*, phytofluene; *C*, ζ-carotene; *D*, neurosporene.

Effect of varying amounts of diphenylamine. Cultures were set up containing amounts of diphenylamine varying from 1/35000 to 1/560000 (w/v). Concentrations higher than 1/35000 could not be used owing to the insolubility of diphenylamine. The mycelia were harvested 7 days after inoculation and examined quantitatively for polyenes. The results are recorded in Fig. 2. This clearly shows that the effect of diphenylamine on the inhibition of β-carotene and the stimulation of the other polyenes is directly proportional to its concentration.

Attempts to find inhibitors with an action similar to diphenylamine

A number of compounds of similar structure to diphenylamine together with phenol (reported by Turian (1950) to have the same effect as diphenylamine in *Mycobact. phlei*) were examined. It will be seen from the results given in Table 2 that, with one exception, there was no effect on carotene synthesis, or on the synthesis of the more saturated polyenes. Many of these results were obtained by Dr W. Lijinsky, whom we thank for allowing us to quote them here.

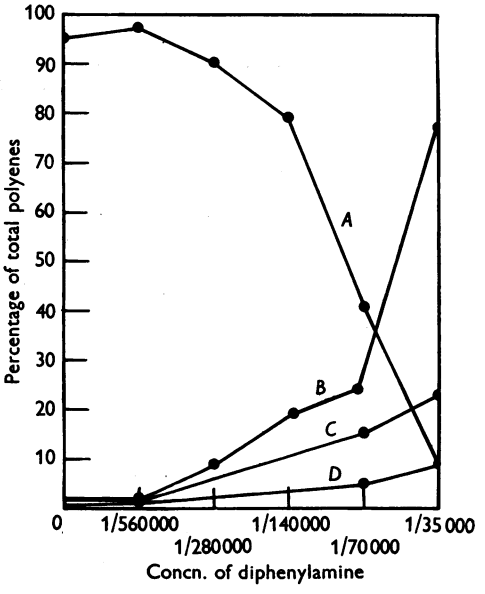

Fig. 2. The variation in polyene production by *Phycomyces* cultured for 7 days on the standard medium (Garton *et al.* 1951) containing varying amounts of diphenylamine. *A*, β-carotene; *B*, phytofluene; *C*, ζ-carotene; *D*, neurosporene.

The exception mentioned above is 2-naphthylamine which produces cultures with very high concentrations of β-carotene. This appears to be due to the fact that 2-naphthylamine inhibits growth but not the synthesis of carotene: 1-naphthylamine also

Table 1. *The dry weight, lipid and carotene production by* Phycomyces *cultured on standard medium (Garton* et al. 1951*) containing diphenylamine* (1/70000 *w/v*)

(The amounts are those produced in an 8 oz. medicine bottle containing 15 ml. of medium.)

Days after inoculation	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Phytofluene (p.p.m.)	β-Carotene (p.p.m.)	ζ-Carotene (p.p.m.)	Neurosporene (p.p.m.)
3	27	2·9	10·8	519	85	222	78
4	60	15·6	26·0	257	192	143	63
5	87	19·1	22·0	155	244	136	47
7	99	19·1	19·3	143	364	97	48

Table 2. *The effect of various compounds related to diphenylamine on the synthesis of β-carotene by* Phycomyces

(The fungus was cultured on the standard medium (Garton *et al.* 1951) containing 2·5% (w/v) glucose to which the test compounds were added as 1/40000 (w/v). Age of culture 10 days. The amounts produced are per 15 ml. medium.)

Substance tested	Dry wt. (mg.)	Lipid (mg.)	Lipid (concn. %)	Carotene (μg.)	Carotene (p.p.m.)
Phenol	91·5	19·2	21·0	105	1150
Indole	101·4	19·5	19·2	98	970
1-Naphthylamine	77	22·2	28·8	89	1150
2-Naphthylamine	51	12·7	24·8	103	2020
Methylaniline	91	26·1	28·7	86	950
Diethylamine	100·7	19·6	19·5	106	1050
Dimethylaniline	96·0	19·3	20·1	98	1020
Aniline	99	14·2	14·3	86	870
Control	104·9	23·1	22·0	106	1010

inhibits growth but carotene synthesis is inhibited equally. It is interesting that the following substances at about M/4000 completely inhibited sporulation of *Phycomyces*: hydroxylamine, chloral hydrate, Nembutal (pentobarbitone), safranine, neutral red, methylene blue, tannic acid and fumaric acid. (Further investigations on the effect of well known metabolic inhibitors on carotenogenesis are at present in progress.)

It was thought that compounds with oxidation-reduction potentials similar to that of diphenylamine might cause a similar inhibition of carotenogenesis. Setopaline and diphenylaminesulphonic acid were tested as well as diphenylbenzidine (reported to be the functional form of diphenylamine in oxidation-reduction reactions). None of these compounds had any effect on carotenogenesis. The results of these experiments are, therefore, not recorded here.

Counteracting the effect of diphenylamine

Turian (1950) reported that the addition of ferrous sulphate even at 0·0001% completely counteracted the effect of diphenylamine in inhibiting carotenogenesis in *Mycobact. phlei*. It was found impossible to repeat Turian's observations using *Phycomyces*, even when ferrous sulphate was added at 100 times the concentration recommended by Turian (1951).

It was thought that the tendency to increase the production of more saturated polyenes and to decrease that of β-carotene might be due to an inhibition of a dehydrogenase system by diphenylamine. It was therefore decided to see if addition of the coenzyme components of a known dehydrogenase system, e.g. triphosphopyridine nucleotide (TPN) [as a mixture of diphosphopyridine nucleotide (DPN) and adenosinetriphosphate (ATP)] would have any effect on the diphenylamine inhibition.

It was found that the presence of a mixture of DPN and ATP (in twice the molar concentration of diphenylamine) returned the synthesis of β-carotene and the rate of growth of the fungus to normal, but did not reduce the synthesis of the more saturated polyenes to normal. Further experiments showed that all the activity in counteracting diphenylamine could be ascribed to adenylic acid (AMP, adenosine-5-phosphate, muscle adenylic acid). The results of a series of experiments with this substance are given in Figs. 3–5. It will be seen from Fig. 3 that diphenylamine reduces the growth rate of the fungus but not the final amount of mycelium produced; this confirms our earlier report (Goodwin, 1952). The addition of AMP completely overcomes this inhibition of growth rate, whilst by itself it does not increase growth rate above normal. Fig. 4 shows the production of the more saturated polyenes on media containing AMP with and without the addition of diphenylamine. Not included in Fig. 4 are the values for the content of carotenoids in normal media; these are not detectably different from those obtained in the presence of AMP. It will be seen that AMP has no effect either on the normal levels or on the raised levels obtained in the presence of diphenylamine. Furthermore, no inhibition of phytofluene synthesis occurs in young cultures grown in the presence of diphenylamine and AMP: there is, however, some tendency for phytofluene to disappear more quickly from older cultures in the presence of AMP. Even so, the phytofluene levels are never reduced to anywhere approaching their normal values even in older cultures. The situation with regard to β-carotene is entirely different (Fig. 5). The presence of AMP restores the synthesis to normal, although it should be observed that by itself AMP has no stimulatory effect on carotenogenesis.

AMP and diphenylamine either together or singly have no effect on lipogenesis in *Phycomyces*; this can be seen from the data recorded in Table 3.

As shown in Table 4, adenine has no action in counteracting diphenylamine, whilst adenosine shows a very slight action. This is probably due to a limited synthesis of AMP from adenosine. Furthermore, in the absence of diphenylamine it was found that the addition of either adenine or adenosine to the medium had no effect on any aspect of the growth of the fungus.

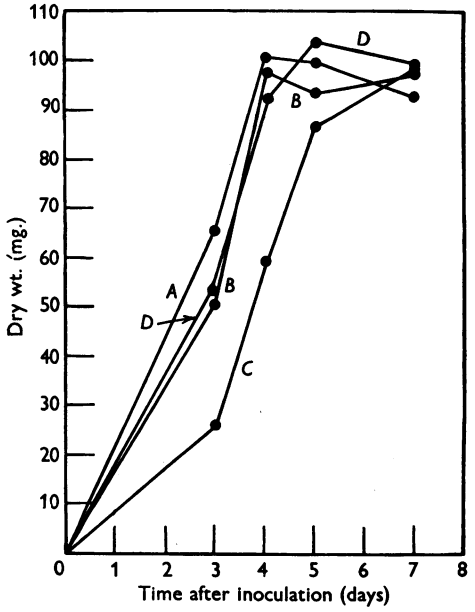

Fig. 3. The growth curves for *Phycomyces*, cultured on the standard medium (Garton *et al.* 1951) containing diphenylamine (M/6000) and adenylic acid (AMP) (M/3000) either singly or together. *A*, AMP alone; *B*, AMP + diphenylamine; *C*, diphenylamine alone; *D*, control (no AMP or diphenylamine).

In similar experiments to those just described it has been found that yeast adenylic acid (adenosine-3-phosphate) had no effect in counteracting diphenylamine inhibition.

Effect of riboflavin

The possibility that riboflavin would also counteract diphenylamine was examined. Control experiments using riboflavin alone showed that it has a very characteristic action on *Phycomyces*. The effect varies according to the age of the culture. Typical results for a growing culture (4 days) are given in Fig. 6 and for a fully developed culture (7 days) in Fig. 7. It will be seen that in young cultures (Fig. 6) riboflavin up to a concentration of 1:20000 stimulates growth (dry weight not lipid), lipogenesis and carotenogenesis until a concentration of 1/20000 is reached; thereafter a marked inhibition of all three processes occurs.

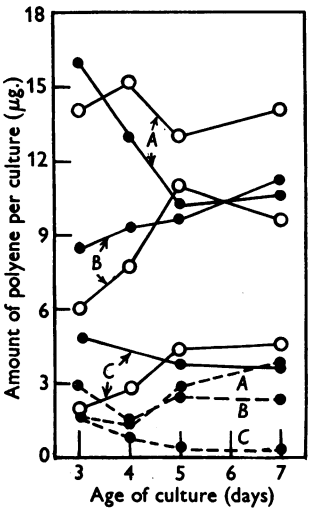

Fig. 4. The production of phytofluene, ζ-carotene and neurosporene by *Phycomyces* cultured on the standard medium (Garton *et al.* 1951) containing diphenylamine (M/6000) or adenosinemonophosphate (M/3000) either singly or together. ○—○, diphenylamine alone: *A*, phytofluene; *B*, ζ-carotene; *C*, neurosporene. ●- - ●, AMP alone; *A*, phytofluene; *B*, ζ-carotene; *C*, neurosporene. ●—●, AMP + diphenylamine; *A*, phytofluene; *B*, ζ-carotene; *C*, neurosporene. *Note.* The production of phytofluene, ζ-carotene and neurosporene on the standard medium (Garton *et al.* 1951) is not significantly different from that in the presence of AMP alone.

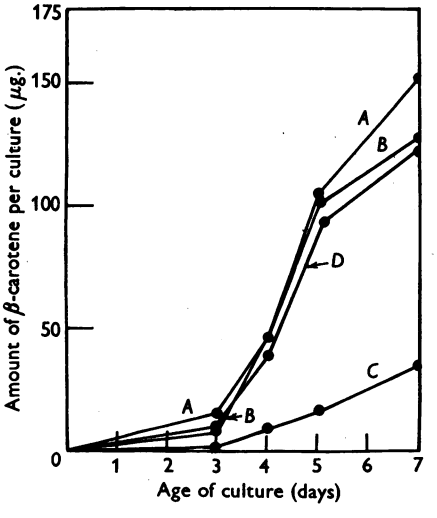

Fig. 5. The production of β-carotene by *Phycomyces* cultured on the standard medium containing diphenylamine (M/6000) or adenosinemonophosphate (M/3000), singly or together. *A*, diphenylamine + AMP; *B*, AMP alone; *C*, diphenylamine alone; *D*, control (no AMP or diphenylamine).

Table 3. *The lipid content of mycelia of* Phycomyces *grown on the standard medium (Garton* et al. 1951*) alone or containing diphenylamine* (M/6000) *and adenosinemonophosphate* (M/3000) *either alone or together*

(The values in mg. are lipid produced in 8 oz. medicine bottles containing 15 ml. of medium.)

Age of culture (days)	Control (mg.)	Control % of total dry wt.*	Diphenylamine (mg.)	Diphenylamine % of total dry wt.*	Adenosine-monophosphate (mg.)	Adenosine-monophosphate % of total dry wt.*	Adenosinemonophosphate + diphenylamine (mg.)	Adenosinemonophosphate + diphenylamine % of total dry wt.*
3	11·8	21·9	6·1	23·0	8·6	12·9	8·3	16·0
4	25·8	29·3	17·3	28·9	27·9	27·5	29·5	30·7
5	35·6	32·4	32·9	37·8	26·1	26·3	25·7	27·6
7	27·1	27·1	36·1	36·1	16·4	17·8	18·7	19·2

* The relevant dry weights are recorded in Fig. 3.

Table 4. *The effect of adenine and adenosine in counteracting the inhibition of carotenogenesis by diphenylamine*

(8-Day-old cultures grown on the standard medium. Amounts produced on 15 ml. of medium in 8 oz. medicine bottles.)

Substance added	Concn.	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Phytofluene (μg.)	Phytofluene (p.p.m.)	β-Carotene (μg.)	β-Carotene (p.p.m.)	ζ-Carotene (μg.)	ζ-Carotene (p.p.m.)	Neurosporene (μg.)	Neurosporene (p.p.m.)
Diphenylamine	M/6000	88	14·7	16·7	14	158	25	283	12	141	3·7	42
Diphenylamine + adenine	M/6000 + M/3000	88	16·2	18·4	19	218	23	264	19	214	5·7	65
Diphenylamine + adenosine	M/6000 + M/3000	90	14·7	16·3	19	209	40	445	21	236	6·7	74

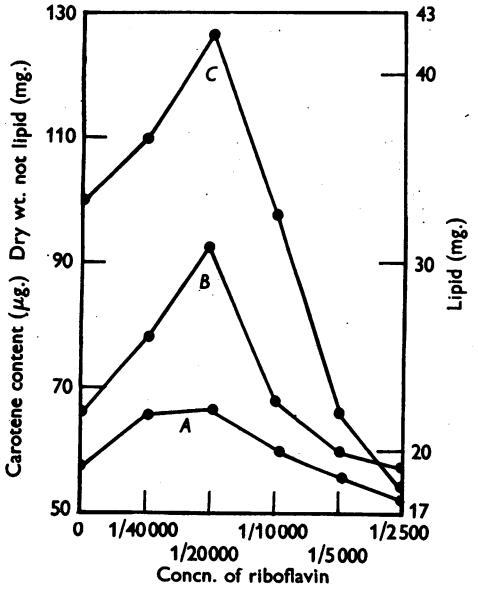

Fig. 6. The effect of varying concentration of riboflavin added to the standard medium (Garton *et al.* 1951) on growth, lipogenesis and carotenogenesis in young (4-day) cultures of *Phycomyces*. *A*, dry weight not lipid; *B*, lipid; *C*, β-carotene.

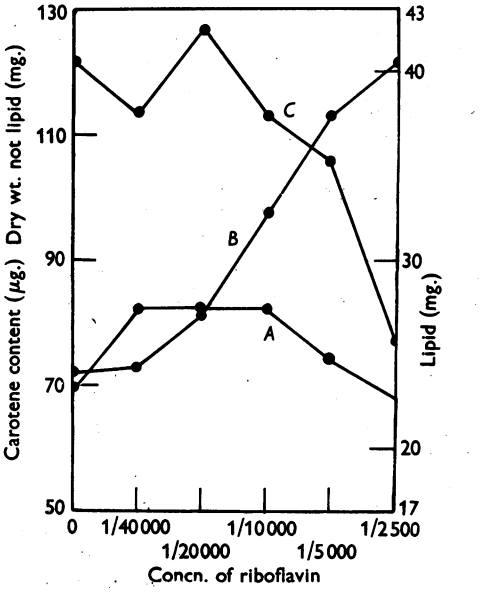

Fig. 7. The effect of varying amounts of riboflavin added to the standard medium (Garton *et al.* 1951) on growth, lipogenesis and carotenogenesis in fully grown (7-day) cultures of *Phycomyces*. *A*, dry weight not lipid; *B*, lipid; *C*, β-carotene.

Table 5. *The effect of riboflavin on polyene production in the presence and absence of diphenylamine*

(8-Day cultures in standard medium (Garton *et al.* 1951). 8 oz. medicine bottles containing 15 ml. of medium.)

Additions to medium	Concn.	Total dry wt. (mg.)	Lipid (mg.)	Phytofluene (μg.)	β-Carotene (μg.)	ζ-Carotene (μg.)	Neurosporene (μg.)
—	—	89	23·2	4·5	117	6·3	Trace
Riboflavin	1/40 000	101	23·0	—	106	—	—
Riboflavin	1/20 000	102	25·8	—	121	—	—
Riboflavin	1/10 000	110	31·0	—	107	—	—
Riboflavin	1/5000	106	36·1	—	107	—	—
Diphenylamine	1/35 000	72	17·9	86	12	26	9
Riboflavin + Diphenylamine	1/40 000 / 1/35 000	86	17·1	57	77	40	17
Riboflavin + Diphenylamine	1/20 000 / 1/35 000	87	18·1	51	114	35	33*
Riboflavin + Diphenylamine	1/10 000 / 1/35 000	89	23·4	34	107	27	11
Riboflavin + Diphenylamine	1/5000 / 1/35 000	102	30·0	73	107	47	21

* Doubtful value.

Table 6. *The dry weight, lipid and polyene content of mats of* Phycomyces *grown for 3–4 days on a standard medium (Garton* et al. 1951) *containing* 1/70000 *diphenylamine, washed thoroughly, transferred for 1 day to water, then to the various media indicated*

Medium on to which mats were transferred	Total dry wt. (mg.)	Lipid (mg.)	Phytofluene (μg.)	β-Carotene (μg.)	ζ-Carotene (μg.)	Neurosporene (μg.)
Water	47·5	10·5	22	116	17	10
Glucose 1% (w/v)	58·5	15·0	40	110	15	11
AMP M/3000	60·0	10·5	28	80	16	7
Riboflavin M/3000	54·5	9	21	52	14	7
Control (levels at transfer)	63·5	13	29	68	17	6

In fully grown cultures (Fig. 7) similar results are obtained for dry weight not lipid and for β-carotene. The stimulatory effect is, however, less marked, especially in the case of β-carotene, than in younger cultures, but the inhibitory action of higher concentrations of riboflavin was still very marked. In the case of lipogenesis, however, there is a continued stimulation with increasing concentrations of riboflavin up to the maximal concentration used in these experiments. As all the experiments just described were carried out in the light, the possibility existed that riboflavin had undergone some photochemical change involving the production of an inhibitory product. This possibility has been eliminated by carrying out experiments in the dark; these yielded results identical with those from experiments carried out in the light.

The effect of riboflavin in counteracting diphenylamine inhibition was therefore examined in 8-day cultures, using different concentrations of riboflavin. It will be seen (Table 5) that although in some cases riboflavin alone slightly stimulates carotene synthesis, this cannot account for the increased β-carotene production observed in cultures containing both riboflavin and diphenylamine compared with those containing diphenylamine alone. It must be concluded, therefore, that riboflavin counteracts the inhibitory action of diphenylamine. It will also be seen from Table 5 that riboflavin also tends slightly to decrease the synthesis of phytofluene and to increase that of ζ-carotene and neurosporene. The effect is much less marked on ζ-carotene and, in some experiments not recorded, was not apparent. The marked effect of riboflavin on lipogenesis in old *Phycomyces* (Table 5) is still apparent in diphenylamine cultures.

Attempts to demonstrate the conversion of more saturated polyenes into β-carotene in vivo

Mycelia of *Phycomyces* were grown on Petri dishes for 3–4 days in the presence of diphenylamine. They were then washed thoroughly, incubated 1 day in water and transferred to new media (Goodwin & Lijinsky, 1952). These media contained either 1% (w/v) glucose, riboflavin or AMP. The results of one experiment are given in Table 6 which shows that in no case do the data indicate any conversion of the more saturated polyenes into β-carotene. In all cases except the riboflavin medium there was some synthesis of β-carotene but no comparable reduction of the other components; there was actually some synthesis of phytofluene on the glucose medium. On riboflavin there was a slight decrease in all components.

DISCUSSION

Probably the most important point which emerges from the present study is that the synthesis of β-carotene and the more saturated polyenes (phytofluene will be used in the discussion as the typical example) almost certainly occurs by different routes. The three observations leading to this conclusion are (*a*) the rates of synthesis of the two groups are different, phytofluene being synthesized during the early stages of growth, whilst most β-carotene is formed only after growth has been completed; (*b*) the addition of either AMP or riboflavin to a medium containing diphenylamine results in normal synthesis of β-carotene without a concomitant decrease in phytofluene, which remains at the same elevated level as in 'diphenylamine only' cultures; and (*c*) it has never been possible on transferring mats containing large amounts of phytofluene to media not containing diphenylamine to observe a disappearance of phytofluene and the appearance of β-carotene.

This demonstration of the separate routes of synthesis of β-carotene and the phytofluene series in *Phycomyces* accords with the observations recently made suggesting their separate biosynthetic route in tomatoes (Goodwin & Jamikorn, 1952*b*).

When it was first noticed that diphenylamine inhibited synthesis of β-carotene in *Phycomyces* whilst stimulating that of phytofluene (Goodwin, 1952), it was suggested that although it could be concluded that intermediates in the synthesis of β-carotene (phytofluene, etc.) were accumulating, it could equally be claimed that the accumulation of these polyenes was due to the shunting of considerable amounts of a common intermediate, normally converted into β-carotene, into an alternative pathway leading to the production of the phytofluene group. It now appears that this is what happens in the presence of diphenylamine.

No other compound related in structure or properties has been found which has the same action as diphenylamine; in fact one, 2-naphthylamine, has been found to act in the opposite direction and to stimulate carotenogenesis.

The exact mode of action of diphenylamine in inhibiting carotenogenesis cannot be decided until much more is known concerning both the initial steps in carotenogenesis and in glycolysis in *Phycomyces*. Whatever the locus of action, it is obvious that the fungus can, from the point of view of growth and lipogenesis, by-pass it, for diphenylamine only slows up growth and lipid synthesis, it does not decrease the final amounts produced. Presumably, the mechanism concerned in the synthesis of β-carotene cannot by-pass this step. The most reasonable assumption is to consider that diphenylamine inhibits carotenogenesis by blocking the transfer of a high-energy phosphate to an acceptor. This would explain the action of AMP in terms of a high-energy phosphate acceptor. Riboflavin might then act by allowing the organism to by-pass the blocked stage rather than directly to overcome the inhibition. If this is so, then it is possible that the synthesis of β-carotene involves the reaction AMP $\rightleftharpoons$ ADP catalysed by an enzyme similar to myokinase. This enzyme, which could be only slightly involved in the general growth of the fungus, might be the *point d'appui* of diphenylamine in inhibiting carotenogenesis.

In all inhibition studies, the possibility always exists that the counteracting material functions by destroying the inhibitor rather than by successfully competing with it for a substrate or enzyme centre. In the present case, it must be accepted that neither riboflavin nor AMP destroys diphenylamine, because the increased production of the phytofluene group, which is a characteristic of 'diphenylamine cultures', still takes place in the presence of diphenylamine and either AMP or riboflavin, but not in the absence of diphenylamine.

The action of riboflavin on Phycomyces

All fungi so far examined are heterotrophic for riboflavin (see, for example, Burkholder, McVeigh & Moyer, 1944) and except for one case in which lipid synthesis was slightly stimulated in some strains of *Fusaria* (Nord, Fiore, Kreitman & Weiss, 1949) there has been no report of a fungus responding to riboflavin, although the concentrations used in previous experiments were lower than those used in the present investigation. It is possible that, using higher concentrations of riboflavin, observations similar to those reported here will be made on other fungal species.

The explanation of the inhibitory effects of riboflavin when used in higher concentrations is not yet apparent, but the stimulatory effects on lipogenesis in older cultures can probably be explained as follows: on our standard medium (Garton *et al.* 1951) *Phycomyces* is fully grown after 5 days; at this time a considerable amount of glucose remains in the medium and is dissimilated, a small fraction being converted into β-carotene (Goodwin & Willmer, 1952) but most being oxidized to carbon dioxide and water. It can only be assumed that in the presence of riboflavin, the products of glycolysis are canalized into lipid synthesis.

SUMMARY

1. The rate of production of the more saturated polyenes (phytofluene, etc.) is much more rapid in *Phycomyces* than is that of β-carotene. The former have reached their maximal values within 3–4 days of inoculation, whilst the latter is maximal only 5–7 days after inoculation.

2. The decreased synthesis of β-carotene and the increased synthesis of the phytofluene group which occurs in the presence of diphenylamine is directly proportional to the concentration of the inhibitor.

3. No compound of similar structure or oxidation-reduction potential has been found which has the same effect on carotenogenesis as diphenylamine. Phenol is also inactive. 2-Naphthylamine stimulates β-carotene synthesis.

4. Ferrous sulphate does not counteract the action of diphenylamine. Either AMP (muscle adenylic acid) or riboflavin returns the synthesis of β-carotene to normal in diphenylamine cultures, but neither lowers the synthesis of the more reduced polyenes to normal. Yeast adenylic acid is without effect.

5. AMP alone has no effect on the growth, lipid production or carotene production by *Phycomyces*.

6. In growing cultures, riboflavin, up to a concentration of 1/20000, stimulates growth, lipogenesis and carotenogenesis; at higher concentrations inhibition of the three processes occurs. In older cultures the same effect is observed with growth and carotenogenesis, but lipogenesis is stimulated at all concentrations of riboflavin tested.

7. Mats cultured in the presence of diphenylamine and containing large amounts of the phytofluene group do not convert these into β-carotene, when washed and transferred to non-diphenylamine media.

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Studies in Carotenogenesis

8. THE CAROTENOIDS PRESENT IN THE BASIDIOMYCETE *DACROMYCES STILLATUS*

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Although *Phycomyces blakesleeanus* is in many ways an excellent organism for the study of carotenogenesis (Garton, Goodwin & Lijinsky, 1951), it has certain disadvantages, chief of which is that it cannot be grown in shake cultures. Other carotenogenic fungi which have been examined recently, e.g. *Rhodotorula* spp. (Lederer, 1938; Bonner, Sandoval, Tang & Zechmeister, 1946) and *Neurospora* spp. (Haxo, 1949), are more suitable from this point of view, but themselves suffer from two disadvantages; (*a*) some of the constituent carotenoids are unstable and (*b*) it is extremely difficult to extract quantitatively the pigments, thus precluding the accurate routine examination of a large number of cultures.

A survey of the old observations of Zopf (1890) and Kohl (1902) concerning the occurrence of lipochromes in fungi suggested that *Dacromyces stillatus* might fulfil the present requirements. An investigation into the suitability of this organism was therefore begun. It soon became apparent, however, that it was not going to fulfil our requirements, mainly owing to its inability to grow quickly and reproducibly on fully defined media. A pre-