Evidence for a Flavoprotein Photoreceptor in Phycomyces

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SUMMARY

It is shown that the flavin inhibitors mepacrine and lumichrome have a
greater effect on the growth of illuminated than unilluminated static liquid
cultures of Phycomyces. The possible relevance of these findings to our
understanding of the mechanism of phototropism is discussed.

INTRODUCTION

The photoresponses of the sporangiophore of Phycomyces, especially phototropism, have been intensively studied and recently reviewed by Banbury (1959),
Reinert (1959) and Thimann & Curry (1960), but the possibility that the vegetative
mycelium of this fungus is also photosensitive has been neglected. This is unfortunate, since the demonstration of metabolic differences between illuminated and
unilluminated cultures might facilitate our understanding of sporangiophore
photosensitivity, and hence of the basis of phototropism. A possible method of
demonstrating such metabolic differences is the application of compounds likely to
inhibit the action of the photoreceptor substance. Such compounds should have
greater effects on illuminated than on unilluminated cultures. Several workers
(e.g. Reinert, 1952; Carlile, 1957; Delbrück & Shropshire, 1960) have suggested that
the sporangiophore photoreceptor is a flavoprotein. It was therefore decided to
investigate the effect of flavin inhibitors on the growth of cultures of Phycomyces
in light and in darkness. An alternative hypothesis is that of a carotenoid photo-
receptor (Curry & Gruen, 1959); therefore the effect of diphenylamine, which
inhibits carotenoid biosynthesis, was also examined.

METHODS

Preparation of inoculum. Stock cultures of a minus strain of Phycomyces blakes-
leeanus Burgeff were maintained on malt agar slopes at room temperature in
daylight. The concentration of spore suspensions prepared from such cultures
was adjusted so that one drop, as transferred to a microscope slide with an inocu-
lating loop, contained about 100 spores. The spore suspensions were heated for
30 min. at 50° to break dormancy, this procedure being necessary to obtain ger-
mination on defined media which lack hypoxanthine (Robbins, Kavanagh &
Kavanagh, 1942). Before using the suspensions it was necessary to shake them well,
since it was found that the Phycomyces spores sedimented rapidly. Experimental
cultures were inoculated with a single drop of spore suspension.

Medium and culture vessels. The following defined solution, devised after pre-
liminary nutritional experiments, gave excellent growth and was used throughout as

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the basal medium. Macronutrients (g./l.): D-glucose, 50; potassium citrate (K$_3$C$_6$H$_5$O$_7$.H$_2$O), 13; NH$_4$Cl, 3.5; KH$_2$PO$_4$, 1.5; MgSO$_4$.7H$_2$O, 0.5; glass-distilled water. Micronutrients (mg./l.): ferric citrate (FeC$_6$H$_5$O$_7$.5H$_2$O), 25; CaCl$_2$.6H$_2$O, 10; ZnSO$_4$.7H$_2$O, 10; MnSO$_4$.4H$_2$O, 3; Na$_2$MoO$_4$.2H$_2$O, 1.5; CuSO$_4$.5H$_2$O, 1; thiamine hydrochloride, 1. Fifty ml. portions of medium were placed in 500 ml. Erlenmeyer flasks, the necks of which were covered with 50 ml. beakers, and sterilized by steaming for 1 hr.

The thermolabile compound mepacrine dihydrochloride dihydrate (= mepacrine B.P. = atebriin) was used in some experiments. Stock solutions were sterilized by passage through a sintered glass filter, and added to media aseptically.

Cultural conditions and dry weights. The static liquid cultures were incubated in a growth cabinet in a thermoregulated darkroom at a temperature of 23 ± 1°. Continuous illumination at an intensity of about 100 foot-candles was provided by two 40 watt Mazda fluorescent 'daylight' tubes. Alternatively, to obtain darkness, cultures were wrapped in opaque black paper. After 9 days of incubation the mycelial mats were filtered off, washed, dried overnight at 80° and weighed. Replication was normally fivefold.

Chromatography and spectrophotometry of mepacrine, riboflavin and derivatives. The riboflavin photolysis product lumichrome was identified by its ultraviolet (u.v.) absorption curve (McNutt, 1954), and by its $R_f$ value on chromatography with solvent no. 1 of Kilgour, Felton & Huennekens (1957). In subsequent chromatographic studies on media and culture filtrates solvent 1 of Hardy, Holland & Nayler (1955) was used since this gave better separation of lumichrome and mepacrine, which sometimes were present simultaneously. Chromatograms were examined by means of a lamp emitting mainly u.v. radiation of wavelength less than 300 mµ; this results in riboflavin having a yellow, mepacrine a green, and lumichrome a blue fluorescence. Quantitative study of the photolytic conversion of riboflavin to lumichrome was based mainly on absorption measurements at 450 mµ.

RESULTS

Growth on basal medium

Daily observations and measurements of dry weight were made on a series of cultures on basal medium in light and in darkness. On inoculation the spores sank to the bottom of the flask where they germinated, and a lag phase of slow growth followed. After about 4 days floating colonies became established. This occurred more readily in darkness than in light; hence the resulting phase of rapid growth took place about one day later in illuminated than in unilluminated cultures. For example, in one experiment the average dry weight at 5 days of illuminated cultures was 119 mg. and of unilluminated cultures 376 mg.; at 6 days the corresponding figures were 361 and 687 mg. Maximum dry weights were attained in about 8 days, after which they remained roughly constant for several days, and then slowly declined. Maximum dry weights of illuminated cultures were usually slightly lower than those grown in darkness, the respective averages in one experiment, for example, being 882 and 918 mg. It is clear, therefore, that the classical growth parameters of lag time, growth rate and total growth (Monod, 1949) are not applicable to static liquid cultures of Phycomyces blakesleeanus. A fully valid approach to
the effect of inhibitors on Phycomyces growth would be to ascertain maximum dry weight and the time of its attainment for each treatment, but facilities were not available for carrying out experiments on the scale necessary to achieve this. The procedure adopted, that of obtaining dry weights at 9 days, compares total growth of control cultures with amount of growth achieved at that time by the experimental cultures. This is a sound practical procedure for ascertaining whether an inhibitor retards illuminated or unilluminated cultures more severely, but does not permit quantitative interpretation of the effects in terms of the usual growth parameters.

**Effect of a flavoprotein inhibitor, mepacrine**

The effect of the anti-malarial drug mepacrine upon the fungus was examined. Most workers regard mepacrine as a flavoprotein inhibitor, and although its specificity has been questioned by Hemker & Hülsmann (1960), it is undoubtedly effective against a number of well-authenticated flavoprotein electron-transfer enzymes, for example, cytochrome c reductase (Haas, 1944). The fungus was grown on basal medium to which a range of concentrations of mepacrine had been added. At a mepacrine concentration (0.18 mM) at which dry weights of illuminated cultures were decreased to 1% of the control value, the yield of cultures in darkness was decreased only to 91%. It is clear (Fig. 1) that mepacrine was more effective in inhibiting growth of illuminated than of unilluminated cultures.

To establish that under the conditions of this experiment mepacrine was stable in light and in darkness, uninoculated flasks of basal medium to which 0.15 mM mepacrine had been added were illuminated for 7 days, a similar set of flasks being maintained in darkness. Chromatographic and spectrophotometric examination at the end of this period showed that no change in mepacrine content had occurred.

**Effect of riboflavin**

It was hoped to prevent the mepacrine effects described above by means of the simultaneous presence of riboflavin; therefore it was desirable to investigate first the effect of riboflavin alone on the fungus. Cultures were grown in light and in darkness on basal medium to which 0, 0.1, 0.2, 0.5 and 1.0 mM riboflavin had been added. The dry weights obtained (Fig. 2) showed that in darkness the concentrations of riboflavin used had little or no effect on the growth of the fungus, but that in light, low concentrations (0.1 and 0.2 mM) of riboflavin caused severe depression of growth and high concentrations (0.5 and 1.0 mM riboflavin) less severe depression. Spectrophotometric and chromatographic examination of the culture filtrates showed that in the illuminated series of cultures, but not in the dark series, total or partial conversion of riboflavin to lumichrome had occurred with low (0.1 and 0.2 mM) and high (0.5 and 1.0 mM) initial concentrations of riboflavin, respectively. Since lumichrome can act as a competitive inhibitor of riboflavin (Mitchell & Houllahan, 1946) the effects obtained are explicable if a non-biological photolysis of riboflavin (Karrer et al. 1934) had occurred early in the growth of the cultures.

To investigate this possibility, flasks of uninoculated medium containing 0.1 and 1.0 mM riboflavin were illuminated, and a control set of flasks was maintained in darkness Both sets were examined chromatographically and spectrophotometrically at frequent intervals. At 9 days the riboflavin content of the unilluminated flasks remained unchanged. Almost complete conversion of riboflavin to
lumichrome had occurred in 2 days in flasks containing 0·1 mM riboflavin. In those flasks containing 1·0 mM riboflavin a 60% conversion of riboflavin to lumichrome had taken place in 9 days. Hence, illuminated cultures on a basal medium initially containing 0·1 mM riboflavin would be exposed subsequently to 0·1 mM lumichrome. Still higher concentrations of lumichrome would be experienced by those cultures on a medium initially containing 1·0 mM riboflavin, but here sufficient unchanged riboflavin would remain partially to counteract the effect of lumichrome. It is clear, therefore, that lumichrome has a strongly inhibitory effect on illuminated cultures, and that this effect can be partially annulled by riboflavin.

Flasks of basal medium with added riboflavin which had been treated as above were inoculated and transferred to darkness. Cultures on irradiated medium initially containing 0·1 mM riboflavin gave an average dry weight 46% of the untreated control; those on medium initially containing 1·0 mM riboflavin gave 76%. Thus, as would be expected, lumichrome was inhibitory to unilluminated cultures, but to a far lesser extent than to illuminated ones, and the effect could be annulled by riboflavin.

![Fig. 1](image1.png)  ![Fig. 2](image2.png)  ![Fig. 3](image3.png)

**Fig. 1.** Effect of mepacrine on the dry weight at 9 days of illuminated (○) and unilluminated (●) cultures of *Phycomyces blakesleeanus*.  
**Fig. 2.** Effect of riboflavin on the dry weight at 9 days of illuminated (○) and unilluminated (●) cultures.  
**Fig. 3.** Effect of diphenylamine on the dry weight at 9 days of illuminated (○) and unilluminated (●) cultures.

**Riboflavin annulment of the mepacrine effect**

Cultures were grown in darkness on medium containing 0·3 mM mepacrine, and in light on medium containing 0·15 mM mepacrine, these concentrations being selected as likely to give severe but not total inhibition of growth. To both sets of media riboflavin was added at concentrations of 0, 0·1, 0·2, 0·5 and 1·0 mM.

Control cultures on basal medium were also used. It will be noted (Table 1) that in both illuminated and unilluminated cultures mepacrine brought about inhibition of growth, and that this was largely prevented by the simultaneous presence of 1·0 mM riboflavin. Lower concentrations of riboflavin had a similar but smaller effect.

Uninoculated medium, containing 0·15 mM mepacrine and riboflavin at 0·1 and 1·0 mM, was illuminated for 9 days, similar medium being maintained in darkness.
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Chromatographic and spectrophotometric study showed that illumination brought about the conversion of riboflavin to lumichrome more slowly than in the absence of mepacrine, but that, also, part of the mepacrine was converted to a compound of lower $R_f$ value with blue fluorescence in u.v. radiation. In the unilluminated medium no breakdown of mepacrine or riboflavin occurred. Hence, whereas the riboflavin annulment of mepacrine inhibition of cultures in darkness can be accepted at its face value, the effect observed in illuminated cultures may well be due, in part at least, to the riboflavin-catalysed photoconversion of mepacrine into another compound, possibly inactive.

Table 1. The action of riboflavin in counteracting the mepacrine effect on the dry weights of 9-day cultures of Phycomyces blakesleeanus grown in light and in darkness

<table>
<thead>
<tr>
<th>Condition of growth</th>
<th>Additions to basal medium</th>
<th>Dry wt. (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Mepacrine</td>
</tr>
<tr>
<td>Illuminated</td>
<td>866</td>
<td>14</td>
</tr>
<tr>
<td>In darkness</td>
<td>928</td>
<td>208</td>
</tr>
</tbody>
</table>

Dry wt. as % dry wt. control

<table>
<thead>
<tr>
<th>Condition of growth</th>
<th>Dry wt. as % dry wt. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illuminated</td>
<td>100</td>
</tr>
<tr>
<td>In darkness</td>
<td>100</td>
</tr>
</tbody>
</table>

The effect of diphenylamine

The finding that mepacrine and lumichrome had much more marked effects on illuminated than on unilluminated cultures constitutes evidence favouring a flavoprotein photoreceptor; but this argument would lose much of its force if other toxic compounds were shown to behave similarly. For this reason a test on such a compound was considered desirable. Diphenylamine was selected, since it is known to inhibit strongly the formation of the more unsaturated carotenoids in Phycomyces (Goodwin, Jamikorn & Willmer, 1953), and many workers regard carotenoids as the probable photoreceptor in phototropism.

Diphenylamine was added to the basal medium at concentrations of 0.084, 0.125, 0.146 and 0.167 mM; the corresponding dry weights obtained are shown in Fig. 3. It will be seen that although a slightly greater effect was observed in cultures grown in light than in those grown in darkness, this was in no way comparable to the effect obtained with mepacrine and lumichrome. It seems likely that the impression of a slight differential effect on illuminated and unilluminated cultures is misleading. For example, at a diphenylamine concentration of 0.146 mM the ‘light’ yield was 110 mg, and the ‘dark’ yield, 206 mg. This compares fairly closely with the 5-day yield in control cultures, in which, as indicated earlier, the ‘light’ yield was 119 mg and the ‘dark’ yield 376 mg. So the apparent differential effect may well have resulted from a non-differential slowing down of the growth of both illuminated and unilluminated cultures.
The differential effect of mepacrine and lumichrome on the growth of Phycomyces blakesleeanus in light and in darkness suggests that there is some important metabolic difference between illuminated and unilluminated cultures. Since mepacrine and lumichrome are flavin inhibitors, it seems likely that in illuminated cultures there is active a flavoprotein system which is non-functional in darkness. Lenhoff, Nicholas & Kaplan (1956) showed that in Pseudomonas fluorescens a molybdo-flavoprotein and a mainly iron-dependent system constituted alternative terminal electron-transfer paths, and the subsequent work of Nicholas (1957) on Neurospora suggested that this is not a unique instance. Vernon (1959) demonstrated that flavin mononucleotide could mediate the photoreduction of cytochrome c with concurrent oxidation of reduced diphosphopyridine nucleotide, thus indicating the possibility that light might initiate the activity in vivo of an electron-transfer system which has a flavoprotein component. A similar finding is that of Lewis, Schiff & Epstein (1961) who showed that the photo-oxidation of cytochromes can be brought about by a flavoprotein from Euglena.

Hence it is possible that Phycomyces blakesleeanus has alternative electron-transfer paths, one fully functional only in darkness and another which requires light, the latter including as a component a flavoprotein photoreceptor, and being susceptible to poisoning with mepacrine or lumichrome. A difficulty of this hypothesis is that mepacrine and lumichrome, although suppressing the activity of the postulated 'light' electron-transfer path, do not interfere with the suppression by light of the alternative 'dark' path. This difficulty is not, however, insuperable. Mepacrine, and probably lumichrome, are essentially flavoprotein inhibitors, whereas free riboflavin, which is photolytically active, could be the photoreceptor involved in the suppression of the 'dark' path.

The hypothesis here outlined, that of a light-activated flavoprotein-containing electron-transfer path, and an alternative 'dark' path, could provide a basis for the interpretation of the photoresponses of the sporangiophore. The kinetic analysis of sporangiophore behaviour by Delbrück & Reichardt (1956), indicated that light brought about a fast growth-stimulating reaction and a slow adaptive growth-depressant reaction. Such reactions could well be the immediate stimulation of a flavoprotein electron-transfer path and the slower depression of an alternative path. Such a hypothesis, although highly provisional, could account for most of the reported behaviour of the sporangiophore in response to changes in light intensity.

Whereas remarkable advances have been achieved recently by an essentially biophysical approach to the behaviour of the Phycomyces sporangiophore, as is shown, for example, by the publications of Castle (1961), Curry & Gruen (1959), Delbrück & Shropshire (1960), Jaffe (1960) and Reichardt & Varjú (1958), our knowledge of the metabolic effects of light on this fungus has remained rudimentary. The required knowledge seems more likely to be obtained by biochemical studies of the effect of light on the vegetative mycelium than on the sporangiophore.

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