Presented:

~~Free~~ Symposium on Behavior
on Microorganisms
(no location cited) August, 1970
on Progress Rpt.

COO-908-37

(proceedings in press)

Phototaxis in *Euglena*: Biochemical Aspects*

MASTER

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1) Introduction

Phototaxis can be defined as a translational response of a freely moving organism to a light stimulus. In the alga *Euglena*, a sensory perception system is involved which is an easily-recognized structure consisting of an eyespot (or stigma) and the paraflagellar swelling located at the base of the flagellum (Tollin, 1969). These organelles, functioning as a photosensitive device, allow the cell to become oriented with respect to the direction of a light source and to maintain its swimming motion in that direction. The physical mechanism by which this is accomplished is the subject of another paper to be presented at this symposium (Diehn, 1970a) and thus will not be considered further here.

At the present stage of the investigation of phototaxis, there are two well-defined biochemical questions which can be asked and to which at least partial answers can be given. These are:

1) What is the energy source which permits the organism to become oriented in the light beam?

2) What is the identity of the pigment(s) responsible for photosensory perception (the paper by Diehn also describes experiments dealing with this question)?

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* Supported in part by the U. S. Atomic Energy Commission, Contract No. AT(11-1)908.

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The remainder of this paper will be devoted to reviewing the present state of knowledge concerning these aspects of the phototactic process in Euglena.

2) Energy Source for Phototactic Orientation

There are three obvious sources from which Euglena could derive the energy required for achieving and maintaining an orientation which results in directed motion. These are: the light stimulus itself (this would imply an energy conversion system, perhaps analogous to photosynthesis), oxidative metabolism (locomotion, i.e., flagellar beating, derives its energy from this process) and photosynthesis (this must be the ultimate supply under autotrophic growth, of course, but what is meant here is a direct coupling of the phototactic and photosynthetic systems).

The first of these possibilities, i.e., energy conversion involving the light stimulus, is the easiest to test. This has been done (Diehn and Tollin, 1966a) by determining the light intensity dependence of the phototactic response. Phototaxis was measured with a device, which we have called a phototaxigraph, which uses photoelectric recording to determine the rate of accumulation of cells in a small region of a cuvette in response to a stimulating light beam (Lindes et al., 1965). A typical response curve is shown in Fig. 1. Phototactic rates are calculated from the maximum slope of the curve. The distance from the baseline to the top of the curve is a measure of the extent of accumulation. Fig. 2 shows

the results of a determination of the light intensity dependence of the rate and extent of phototaxis in Euglena. If the energy for the response is derived from the stimulating light, one would expect to obtain linear relationships at low intensities which saturate at some higher intensity, similar to what is observed for the photosynthetic process. However, in the case of phototaxis, a logarithmic dependency is found. We have also done experiments using white light as the stimulus and have observed that the logarithmic relationship holds over a considerable range of intensities (up to $140 \text{ ergs/cm}^2\text{sec.}$, which was the highest intensity obtainable with our apparatus). Thus, the phototactic response in Euglena obeys the Weber-Fechner law, indicating that the stimulating light functions as a trigger which releases previously-stored metabolic energy.

The first indication that the photosynthetic apparatus might be involved in generating the energy required for phototaxis was obtained from experiments in which Euglena cultures were incubated in the dark and their phototactic ability measured (Diehn and Tollin, 1966a). Normally, the cultures were exposed to a light-dark cycle and under these conditions phototaxis was maintained over long periods of time (several weeks). However, in the dark, the phototactic response of the culture persisted for about 24 hours and then decayed, being almost completely gone in 72 hours. This approximates the time course for chloroplast destruction found by Schiff et al. (1965) in Euglena. Also of significance

is the fact that the dark incubation did not affect the motility of the culture (in these experiments, as well as in all of the following studies, the cultures were grown heterotrophically).

An additional experiment provided still further evidence for an interaction between phototaxis and photosynthesis (Diehn and Tollin, 1966a). A Euglena culture was grown completely in the dark following the initial inoculation (from a normally-grown culture). As expected, no chlorophyll formation or chloroplast development occurred (Wolken, 1961). Microscopic examination indicated normal eyespot pigmentation, however. The culture displayed no phototactic response, although again motility was apparently normal. When the organisms were subsequently placed in the light, it was found that chlorophyll formation and return of phototactic ability occurred simultaneously (Fig. 3).

A similar effect was observed using a furadantin-bleached Euglena culture which had neither chloroplast nor eyespot pigmentation (Diehn and Tollin, 1966b). These organisms, which were fully motile, did not respond phototactically to light stimulation.*

A more direct approach to this problem was pursued through the use of metabolic inhibitors (Diehn and Tollin, 1967). When substances such as cyanide ($5 \times 10^{-4} \text{M}$), azide (10^{-8}M)

* More precisely, the culture did not respond positively (i.e., accumulate in the light-stimulated region). The organisms did, however, show negative phototaxis (a light-induced depletion of cells within the stimulated zone).

and rotenone ($5 \times 10^{-7} \text{M}$), which are known inhibitors of respiratory electron transport, were added to Euglena cultures, both motility and phototaxis were reduced to 50% of the control in about 30 minutes. Similar results were obtained with uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol ($5 \times 10^{-5} \text{M}$) and dichlorophenol indophenol (DCPIP, 10^{-5}M). This latter substance was metabolized by the organisms within 24 hours, as evidenced by the disappearance of the blue color, and both motility and phototaxis were fully restored. These results indicate that motility is required for phototactic orientation, although it is not a sufficient property as shown by the experiments with achlorophyllous cultures cited above.

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) is well known as a specific inhibitor of photosynthetic electron transport (Vernon and Avron, 1965). At concentration levels as low as 10^{-7}M , it strongly inhibits phototaxis without any apparent effect on cell motility. The time course of the effect of DCMU is quite interesting (Fig. 4). Initially, a slight stimulation of the phototactic response is observed.* This is followed by inhibition and recovery. The rate at which the inhibitory effect is manifested depends on the DCMU concentration. The recovery effect is not due to depletion of

* This is somewhat variable. The reasons for this are not clear.

DCMU by metabolic processes, because it occurs even when DCMU-saturated culture is left in contact with crystalline material. It is also not due to the emergence of a DCMU-resistant strain, inasmuch as recovery can occur in much less than one generation time and, more importantly, DCMU prevents further growth of the culture. The most likely explanation of the recovery effect is that the organisms are somehow able to by-pass the DCMU-blocked step. Another of the selective inhibitors of photosynthesis, methyl octanoate (10^{-4} M) (Pedersen et al., 1966), gave results which were quite similar to those obtained with DCMU (Fig. 5).

The fact that phototactic inhibition by DCMU occurs in the absence of any appreciable effect on motility provides suggestive evidence for the functioning of the photosynthetic system* in supplying energy for phototaxis. As a further test of this concept, we utilized the fact that the addition of external electron donors, such as reduced DCPIP, can restore photophosphorylation activity to DCMU-inhibited photosynthetic systems (Jagendorf and Margulies, 1966). When ascorbate (10^{-4} M) and DCPIP (10^{-5} M) or ferrocyanide (10^{-3} M) were added to Euglena cultures which were also treated with DCMU or methyl octanoate, no inhibition of phototaxis resulted (Fig. 5).

* It is also possible, of course, that the phototactic energy source is actually not the photosynthetic apparatus but a separate system having the same inhibition spectrum as photosynthesis. However, Dichm (1970b) has observed that a Euglena mutant impaired in photosystem II is only weakly phototactic.

It is significant that phototactic ability is also sensitive to uncoupling of photophosphorylation. The substance carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CCP) (Bamberger et al., 1963), at a concentration of 10^{-6} M, strongly suppresses Euglena phototaxis (Figure 6). This points directly to the involvement of photosynthetically-generated high-energy compounds in providing energy for phototactic orientation.

The next stage in our investigation of this aspect of the problem was an attempt to directly evaluate the suggested correlation between photosynthetic ATP production and phototaxis by simultaneously measuring both responses in a single culture (Tollin and Robinson, 1970). In order to do this, we developed a procedure for determining the ATP content of whole Euglena cells using the firefly bioluminescence assay (Strehler, 1953). The experimental protocol was as follows. A 1 ml. aliquot of Euglena suspension was removed from the culture into a 2.5 ml. hypodermic syringe. This was illuminated with red light for a given length of time and then quickly injected into a small volume (0.5 ml) of boiling distilled water*, boiled for 1 min. and stored in an ice bath until the ATP assay was performed. An unilluminated sample was used as a control.

The effect of increasing illumination time on the ATP content of a normal Euglena culture is shown in Fig. 7. Transients, similar to those observed with O_2 evolution and chlorophyll fluorescence (French, 1963; Kautsky and Hirsch,

* It is important to do this quickly because of the apparent high ATPase activity present in Euglena.

1931), are found to occur during the first minute of illumination. After 5 min., the ATP level in the illuminated sample is invariably greater than in the dark control.

The effect of DCMU addition on phototaxis and photophosphorylation, measured on aliquots of the same culture, is shown in Fig. 8. The kinetics of inhibition of the two responses are quite similar.

We thought it might be of interest to ascertain whether the delay in the response to DCMU (~2 hours) is due to the time required for the inhibitor to enter the cell (or chloroplasts). Thus, we measured O_2 evolution rates and their response to DCMU addition. In Fig. 9 is shown the results of an experiment in which DCMU was quickly injected into a Euglena culture whose O_2 evolution was being monitored with an oxygen electrode. The initial downward slope of the curve represents dark respiratory O_2 uptake. As soon as the light is turned on, O_2 is evolved. Upon DCMU addition (arrow), inhibition of O_2 production occurs within 30 seconds. These results, considered in terms of the data shown in Fig. 8, seem to indicate the presence of an endogenous pool of electron donors in Euglena, which allows photophosphorylation to proceed in the presence of a photosystem II inhibitor such as DCMU until the pool is depleted.

A similar experiment to that of Fig. 8, using CCP as the inhibiting substance, is shown in Fig. 10. Again, the kinetics of inhibition of ATP formation and phototaxis are similar, although the time course is different from that

observed with DCMU. Particularly interesting is the fact that the CCP inhibition is manifested significantly more quickly than is the case with DCMU. This would be consistent with the suggestion of an electron donor pool, inasmuch as an uncoupling of phosphorylation caused by CCP would not be expected to allow such a pool to manifest itself as light-produced ATP.

Another way to test the existence of a relationship between ATP levels in Euglena and phototaxis would be to turn off the photosynthetic process, not with inhibitors, but with darkness. As was mentioned above, phototactic ability is maintained for about 24 hours under these conditions (Fig. 4). In Table I, the results of ATP determinations during the dark incubation are shown. Two points of interest emerge from these data. First, placing the organisms in darkness during the middle of a normal light period inhibits growth (as measured by turbidity). Second, ATP levels per cell in the dark culture actually increase over a 24 hour period (after 2-3 days, ATP levels decrease to quite low values). These results, along with the data shown in Fig. 4, suggest the presence of a control system which acts to maintain phototactic energy reserves (and ATP levels) during an extended period of little or no photosynthetic activity, perhaps at the expense of cell growth and division.

3) Identity of Pigments Involved in Photosensory Perception

As was pointed out above, one of the organelles implicated in phototactic light perception in Euglena is the eyespot

Table I.
Effect of Darkness on ATP Levels in Euglena

<u>Time (hrs.)</u>	<u>Normal</u>		<u>Dark</u>	
	<u>A_{800 nm}</u>	<u>ATP (μg/ml)</u>	<u>A_{800 nm}</u>	<u>ATP (μg/ml)</u>
0	0.26	0.35	0.26	0.30
2	0.27	0.27	0.27	0.36
4	0.33	0.30	0.28	0.40
6	0.37	0.30	0.28	0.38
24	0.43	0.53	0.30	0.54
26	0.45	0.75	0.30	0.55
28	0.50	0.81	0.30	0.61
30	0.53	0.82	0.30	0.64

or stigma. This structure consists of a cup-shaped cluster of bright orange-red globules located near the paraflagellar swelling, although physically separate from it. Most workers have assumed (Wolken, 1961) that the pigmentation was due to carotenoids, based partly on the appearance of the eyespot and partly on the action spectrum for the phototactic response.

We have taken a more direct approach to this question (Batra and Tollin, 1964) by isolating sufficient quantities of eyespot granules, using centrifugation techniques, to permit pigment extraction and identification. The absorption spectrum of a suspension of isolated eyespots is shown in Fig. 11, along with the spectrum of a hexane extract. The two spectra are in reasonable agreement (the eyespot spectrum is broadened and red-shifted, probably due to pigment-pigment and/or pigment-protein interactions) and strongly suggest the presence of carotenoids. This was confirmed by chromatographic analysis of the hexane extract. The pigment composition found is given in Table II. This distribution of carotenoids is quite different from that present in whole cell extracts (Krinsky and Goldsmith, 1960). No evidence was obtained for significant quantities of any other pigment in the eyespot preparation. It is of interest to note that studies of phototaxis using polarized light (Bound and Tollin, 1967) have provided evidence for orientation of the carotenoid molecules within the eyespot. This has proven useful in investigating certain aspects of pigment involvement in phototaxis, as will be discussed by Diehn (1970a) in this symposium.

Table 2

Absorption Maxima and Relative Amounts of the Carotenoids

Isolated from Eyespot Granules

The pigments are listed in the order in which they were eluted from the column.

Fraction	% (V/V) acetone in petroleum ether required to elute from column	Relative amount %	$\lambda_{\max}(\text{m}\mu)$		
			Petroleum ether	Ethanol	Chloroform
1. β -carotene	0	8	475, 447, 423	---	490-1,462,437
2. Cryptoxanthin	1.5	32	476, 448, 424	482, 452, 426	491-2,461,436
3. Unknown	4-5	9 [*] (?)	475(?), 453, 430	482-4(?), 458 432, 406(?)	490, 465, 439
4. Lutein	14-15	51	473, 446, 424	475, 447, 425	485-6,457,433

* This figure is based on the assumption that the extinction coefficient of this pigment is 1.4×10^5 , which represents a median value for carotenoid pigments.

The eyespot is believed to function merely as a light filter or shading device for the true photoreceptor, which is located within the paraflagellar swelling (Tollin, 1969). Thus, the primary phototactic light process does not occur within the eyespot and is not mediated by the eyespot carotenoids. This leaves open the question of the actual identity of the photoreceptor pigment. A phenomenon which we discovered while looking for photocontrol effects has given some insight into this problem (Tollin and Robinson, 1969). Pohl (1948), Bruce and Pittendrigh (1956) and ourselves (Diehn and Tollin, (1965a) have shown that omission of a single dark cycle causes a markedly reduced level of phototactic activity in Euglena grown on a light-dark cycle. Subsequent exposure to darkness restores the response (Fig. 12). If one takes a normally-grown culture at any time during the light cycle and irradiates it with high intensity light of wavelength less than 650 nm, a marked suppression of phototaxis occurs (Fig. 13). Microscopic examination of the culture immediately after such a treatment shows that the suppression is not due to a loss of motility. As in the continuous light experiment, placing an irradiated culture in the dark causes a return of phototactic ability. The time course of this effect is shown in Fig. 14. The dark return of activity is not susceptible to acceleration by irradiation at any wavelength in the visible or near infra-red region.

A measurement of the light intensity dependence of the suppression phenomenon (Fig. 15) shows that it is saturable, but that quite high intensities are required.

The action spectrum for photosuppression is shown in Fig. 16, along with the absorption spectrum of a typical flavoprotein, L-amino acid oxidase. The similarities are striking, suggesting that perhaps a flavin chromophore is involved.

Experiments in which respiratory O_2 uptake was monitored during blue light irradiation, or in which irradiation was carried out under N_2 , suggest that the respiratory system is probably not involved in photosuppression or in dark recovery. Furthermore, the fact that inhibition or cessation of photosynthesis requires periods of 30 minutes to several hours to become manifested in a suppression of phototaxis (see above), whereas blue light irradiation is effective in less than one minute after as little as 10 seconds of light, suggests that the photosynthetic system is also not mediating the photosuppression effect.

These phenomena caused by bright light irradiation are reminiscent of the bleaching and dark regeneration of visual pigment in higher organisms (Rushton, 1964). Thus, it is possible that we are observing the effect of photobleaching of the phototactic receptor pigment. If this is so, the implication is that this pigment is a riboflavin derivative. It is of interest that flavins can be reversibly photobleached in solution (Green and Tollin, 1968). The paper by Diehn in this symposium discusses other types of evidence supporting the role of flavin in phototactic light detection.

It is interesting to speculate about a possible physiological function for photosuppression as a control mechanism which reduces phototactic activity when the organisms have accumulated in a region of high light intensity. This would serve to decrease the likelihood that they would respond to stray light signals coming from other directions.

4) Conclusions

The experiments outlined in section (2) of this paper strongly suggest that the photosynthetic phosphorylation system supplies the energy required by Euglena for phototactic orientation. This raises an interesting problem, however, inasmuch as the organelles which mediate these two phenomena (chloroplast and eyespot/paraflagellar swelling) are physically separate in this organism. Thus, it is necessary to postulate a transport system which carries ATP (or some other high-energy compound derived from the photophosphorylation system) from the chloroplast lamellae to some specific site in the flagellar apparatus. The release and/or utilization of this energy pool must then be controlled by the tactic photoreceptor. It is interesting to note that the euglenoid flagellates are the only algae in which the eyespot is external to the chloroplast. This would imply that, if the basic mechanisms of phototactic orientation are the same, a direct photocontrol of photosynthetic phosphorylation may be occurring in non-euglenoid species. However, it would still be necessary to have a transport system to carry high-energy materials from the chloroplast to the locomotive organelle.

One of the fundamental questions still remaining to be answered about phototaxis is the nature of the linkage between light absorption and the change in flagellar motion which leads to a reorientation of the swimming pattern of the organism. The simplest hypothesis might be that a change in light intensity activates a "switch" which controls the flow of photosynthetically-derived ATP to the flagellum. It seems reasonable to suppose that a cell which is moving in a straight line path as a result of the rhythmic beating of its flagellum can be made to change its direction by providing the flagellum with a "pulse" of ATP. This would cause an interruption of the normal motion of this organelle and hence a deviation in swimming direction.

An alternative to this type of mechanism could be one in which a membrane depolarization occurs in the paraflagellar swelling as a result of a change in photon flux. If this were propagated along the flagellum (perhaps as an alteration of conformation), a change in its beating pattern might be induced. The energy requirement in such a system would be a consequence of the re-establishment of the initial state of polarization (and/or conformation).

Such ideas are, of course, highly speculative. They do, however, provide a physicochemical basis for the primary events in Euglena phototaxis which can perhaps be tested experimentally.

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Legends for Figures

Fig. 1: Typical Phototactic Accumulation Curve for Euglena.
Ordinate is proportional to number of cells.

Fig. 2: Light Intensity Dependence of Rate and Extent of
Phototaxis in Euglena. $\lambda = 500 \text{ nm}$. $I_0 = 0.5 \text{ erg/cm}^2 \text{ sec}$.

- Δ extent of accumulation
- $\circ$ rate

Fig. 3: Relation Between Phototaxis and Chlorophyll Development in Dark-Grown Euglena after Light Incubation.

Fig. 4: Effect of DCMU and Darkness on Phototactic Rates in Euglena.

- $\bullet$ darkness
- Δ 10^{-7} M DCMU
- $\circ$ $5 \times 10^{-7} \text{ M DCMU}$

Fig. 5: Effect of Methyl Octanoate on Phototactic Rates in Euglena.

- $\circ$ $10^{-4} \text{ M methyl octanoate}$
- Δ $10^{-4} \text{ M methyl octanoate plus } 10^{-5} \text{ M DCPIP}$
and $10^{-4} \text{ M sodium ascorbate}$.
- $\bullet$ $10^{-4} \text{ M methyl octanoate plus } 10^{-3} \text{ M ferrocyanide}$

Fig. 6: Effect of CCP on Phototactic Rates in Euglena.

Fig. 7: Effect of Time of Illumination with Red Light on ATP Levels in Euglena. Ordinate represents difference in ATP between illuminated and un-illuminated aliquots. Two separate experiments are shown.

Fig. 8: Effect of DCMU ($2 \times 10^{-6}M$) on Phototaxis and Photophosphorylation in Euglena. Ordinate represents ratio of responses of DCMU-treated culture to control culture. The results of two separate experiments are shown; comparisons should be made between solid curves and between dashed curves.

Fig. 9: Effect of DCMU Addition on Photosynthetic Oxygen Evolution in Euglena.

Fig. 10: Effect of CCP ($5 \times 10^{-6}M$) on Phototaxis and Photophosphorylation in Euglena. Ordinate represents ratio of responses of CCP-treated culture to control culture. The results of two separate phototaxis measurements are shown.

Fig. 11: Spectral Properties of Euglena Eyespots.

— suspension of isolated eyespot granules
(the small peak at 675 nm is due to a slight chloroplast contamination).
--- hexane extract

Fig. 12: Phototactic Responses of Euglena Under Various Conditions of Preillumination.

Fig. 13: Phototactic Response of Euglena Before and After Exposure to 30 min. of Blue Light (Corning filter CS 4-76).

Fig. 14: Dark Recovery of Phototaxis in Euglena after a 15 min. Irradiation with Blue Light (Corning filter CS 4-76).

Fig. 15: Light Intensity Dependence of Photosuppression of Phototaxis in Euglena. Ordinate represents ratio

of maximum slope of accumulation curve prior to irradiation to that subsequent to irradiation.

Fig. 16: Action Spectrum for Photosuppression of Phototaxis in Euglena. Interference filters (10 nm bandwidth) were used to obtain monochromatic light. Data are corrected for light intensity and are expressed on a quantum basis. Ordinate same as in Fig. 15.

FIG. 1

FIG. 2

Phototaxis

4-

3-

2-

1-

0

.2

.4

.6

.8

O.D. 665

1 day after removal from dark room

0

2

3

4

FIG. 1

Relative rate of phototaxis

Relative Rate of Phototaxis

FIG. 7

PHOTOFLUX OF ATP (DCMU/CONTROL)

FIG. 9

FIG. 10

FIG. 11

Phototaxis (Arbitrary Units)

Control (12 Hours Light & 12 Hours Dark)

24 Hours Light

24 Hours Light & 15 Minutes Dark

0 30 60 90 120 150

Time (sec.)

Phototaxis (Arbitrary Units)

Control (Unirradiated)

30 min. Blue-Green Light (CS-4-76)

0 30 60 90 120

Time (sec.)