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The Effect of Azide on Phototaxis in *Chlamydomonas reinhardi*

(motility)

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ABSTRACT **Phototaxis in *Chlamydomonas reinhardi* was specifically inhibited by azide. The effect of azide was rapid and reversible, and did not depend upon the intensity of actinic light. Under conditions of completely inhibited phototaxis, azide had no effect on the number of motile cells in the population or on the rate of motility. The effect was not related to changes in oxygen uptake or cellular ATP concentration. Apparently, a cellular component or process specifically involved in phototaxis is inactivated by azide.**

The stimulus-response system that mediates the tactic behavior of microorganisms may be thought of as consisting of components that function as stimulus receptor, a transmission system linked to the receptor that controls cell movement, and an effector apparatus for cell movement. The identity or mechanisms of these components is unknown in all but a few cases. Chemoreceptors for galactose and ribose in bacteria have been identified (1, 2), and in *Paramecium* the mechanism of transmission involves ion permeability changes analogous to those that occur in neurons (3).

In studies of the phototactic response of *Chlamydomonas reinhardi* it has been possible to assay separately phototaxis and random motility. It was found that motility requires either Ca^{2+} or Mg^{2+} and is constant throughout growth; in contrast, phototaxis specifically requires Ca^{2+} and a monovalent cation, such as K^+ or NH_4^+, and declines at the end of exponential growth (4). The requirements of motility reflect the requirements of the effector alone, whereas the additional requirements of phototaxis reflect the requirements for reception or transmission. A further characterization of reception or transmission was the finding that azide and antimycin A inhibited phototaxis but not motility (4).

In the present study the specificity of the inhibition of phototaxis by azide is delineated. The data confirm that the azide-sensitive site of phototaxis is not the effector involved in cell movement, and further show that azide has no effect on oxygen uptake and cellular ATP levels.

MATERIALS AND METHODS

Algal Strains and Culture Conditions. *Chlamydomonas reinhardi* strain 21 gr, originally obtained from R. Sager, was grown photosynthetically in minimal medium as described (4).

Phototaxis Assay. The assay measures the rate at which a population of cells accumulates in the actinically lighted section of a cuvette. The maximum rate of accumulation (ΔOD_{800}/min) divided by the concentration of cells (OD_{600}) is the phototaxis coefficient, a measure of phototaxis independent of cell density. The actinic light used in these studies was an Osram 150-W xenon lamp in an Oriel 6137 lamp housing (Oriel Corp. of America, Stamford, Conn.). Light below 350 nm or above 650 nm was excluded by filtering the light source with an Oriel C-722-3900 long-pass filter, a G-774-4450 band-pass filter, and a G-776-7100 infrared-absorbing filter. The resulting light had a maximum intensity at 445 nm, with a half-maximum band width of about 100 nm and an intensity of about 100 J/m² per sec at the phototaxis cuvette. The turbidity of the cells in the actinically lighted section of the cuvette was monitored with a Zeiss PMQ-2 spectrophotometer at 800 nm with an Oriel G-722-7800 long-pass filter in front of the photocell. Optical density was recorded as a function of time with a Zeiss TE converter and a Heath EU-20B recorder (Heath Co., Benton Harbor, Mich.). Other aspects of the assay have been described (4).

Motility Assays. Motility was characterized by two parameters: the velocity of individual cells and the proportion of motile cells in a population. To measure velocity, a drop of cells was placed on a slide, and a coverslip with spots of stopcock grease in the corners was pressed over the cells. The depth of the cell suspension was adjusted by pressing the coverslip down until most of the cells were in the same focal plane but not stuck to the glass. A 1-sec dark-field exposure of the field was made with white-light illumination. The negative was projected onto a board, and the lengths of the tracks were measured with a contour length-measuring device. All of the complete tracks in an exposure were measured; nonmotile cells were not included in calculating the mean velocity. Those cells that did not move during the exposure included those that were not motile and those that were stuck to the glass. The results of the velocity determinations are given ±1 SD. A micrograph of a hemocytometer was used as a length reference. A similar type of assay was used by Feinleib and Curry (5).

To measure the proportion of cells that were motile, a 1-sec bright-field exposure was made of a drop of cells in a hemocytometer. The illuminator was filtered with a red Klett no. 66 filter so that cells would not phototactically accumulate in the illuminated field. With these conditions, only those cells that did not move during the entire exposure period showed up on the film. Counts of nonmotile cells were made directly from the negative. The total cell population was determined by counting a sample of formaldehyde-killed cells with a hemocytometer.

Abbreviation: CAT buffer, 10 mM $CaCl_2$–10 mM NH_4Cl–10 mM Tris·HCl (pH 7.0).

Respiration Measurements. The rate of oxygen uptake in the dark was determined at 25° with a Gilson KM Oxygraph with a Clark type electrode (Gilson Medical Electronics, Inc., Middleton, Wis.). The cell suspension was bubbled with nitrogen to achieve an oxygen concentration of about 160 μM before the assay was begun. The electrode was calibrated with respect to air-saturated water, assuming that the concentration of O_2 was 258 μM (6). The response of the electrode to oxygen was not affected by 5 mM NaN_3.

ATP Assay. The firefly luciferin/luciferase assay was used essentially as described by Stanley and Williams (7). Cells (1.0 ml) at 1 to 2 $\times$ 10^6/ml in medium or CAT buffer (10 mM $CaCl_2$–10 mM NH_4Cl–10 mM Tris·HCl, pH 7.0) were added to 1.0 ml of hot absolute ethanol. The mixture was incubated at 75° for 10 min, cooled in ice, and centrifuged at 10,000 $\times$ g for 2 min at 2°. A portion of the supernatant (1.2 ml) was evaporated to dryness in a Buchler Rotary Evapo-Mix (Buchler Instruments, New York, N.Y.), and the residue was suspended in 0.6 ml of PM buffer (10 mM potassium phosphate–4 mM $MgSO_4$, pH 7.4) or CAT buffer and assayed for ATP. In some experiments the residues were stored at −20° for up to 12 hr before assay. The ATP assay was carried out at ambient temperature in a polyethylene scintillation vial containing the sample, 2.0 ml of a buffer solution containing 50 mM sodium arsenate, pH 7.4, and 20 mM $MgSO_4$, and sufficient PM buffer to give a total volume of 3.0 ml. The reaction was started by the addition of 25–100 μl of firefly extract, and the vial was placed on the conveyor belt of a Nuclear Chicago Unilux II liquid scintillation counter. With the machine set for duplicate counting, the sample was counted for 0.2 min and then for 0.1 min. This second period of counting, which commenced 30 sec after the addition of enzyme, was used for the determination of ATP concentration. Single photon events were counted by turning off the coincidence circuitry of the liquid scintillation counter. Background counts and enzyme blank together were equivalent to about 10 pmoles of ATP. This was usually less than 10% of the amount of ATP being assayed.

About 80% of the ATP was recovered in the ethanol extraction, and the amount of ATP extracted from cells by ethanol was similar to that extracted by sonication. There was no loss of ATP that had been added to cells before extraction in the absence or presence of 1 mM NaN_3. Less than 4% of the ethanol-extractable ATP was present in a cell-free filtrate of cells in medium in the absence or presence of 1 mM NaN_3. The results of replicate cell extractions agreed within ±5% of the mean, and the amount of ATP is reported ±1 SD.

Light Intensity. A YSI model 65A radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) was used for all intensity measurements. Light intensity was varied with Zeiss neutral gray filters.

Materials. Disodium ATP from equine muscle and desiccated firefly tails were obtained from Sigma Chemical Co., St. Louis, Mo. Ten firefly tails were ground in dry ice and then in 5.0 ml of PM buffer. The extract was centrifuged at 10,000 $\times$ g for 30 min at 2°, and the supernatant was filtered through a 0.45-μm pore size nitrocellulose filter. The resulting firefly extract was stored in an ice bath and used 1–4 days after preparation. All other chemicals and materials were from commercial sources.

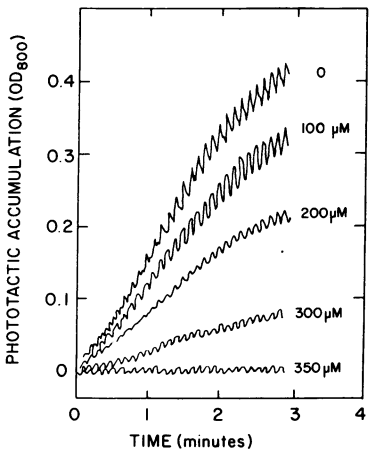

FIG. 1. Effect of azide on phototaxis. Cells (1 $\times$ 10^6/ml) were harvested by centrifugation, washed once with CAT buffer, and suspended at a final concentration of about 5 $\times$ 10^5 cells per ml (OD_{600} = 0.10) in CAT buffer. The cells were returned to the growth incubator for 90 min before they were used. Sodium azide in 1.0 ml of CAT buffer was added to 4.0 ml of cells, and the phototaxis assay was conducted immediately. The data shown are tracings of the results; the oscillation was caused by rotation of the cuvette.

RESULTS

Inhibition of Phototaxis by Azide. Azide inhibits the rate of phototactic accumulation of *C. reinhardi*. Half-maximum inhibition occurred when 200 μM NaN_3 was added, and the phototactic response was totally inhibited by 350 μM NaN_3 (Fig. 1).* The effect of azide was constant for a given culture of cells but was found to vary among different cultures. It did not depend on the concentration of cells in the range of 5 $\times$ 10^5 to 3 $\times$ 10^6 cells per ml. Excess Ca^{2+} and K^+, ions that are specifically required for phototaxis, did not affect the inhibition by azide, indicating that azide inhibition is not due to interactions between azide and these ions.

The time course of inhibition was studied, and it was found that under conditions of 50% inhibition by azide, the extent of inhibition was the same whether the cells were assayed 30 sec or up to 40 min after the addition of azide. Thus, the effect was rapid and stable. It was also rapidly reversible. Cells in the presence of 200 μM NaN_3 were diluted 4-fold with CAT buffer. Within 1.5 min the phototaxis coefficient increased from 0.2 to 1.2, the phototactic coefficient of cells tested in the presence of 50 μM NaN_3.

Increasing light intensity did not affect the inhibition by azide (Fig. 2). Phototaxis was assayed at a number of different actinic light intensities in the presence and absence of 250 μM NaN_3; in both the control and azide-inhibited cells, the response was saturated at 25–50% maximum light intensity. Double reciprocal plots of the phototactic coefficient versus the intensity of actinic light with and without azide did not intersect at the ordinate. Thus, the amount of inhibition appeared to be independent of light intensity.

* It is not clear whether azide affected the number of cells in the population that responded to light, or the rate at which all cells responded. Although the yield of phototactic cells was decreased by azide, this could be interpreted as either fewer cells responding to light, or as an increased number of cells swimming out of the actinic light path under steady-state conditions.

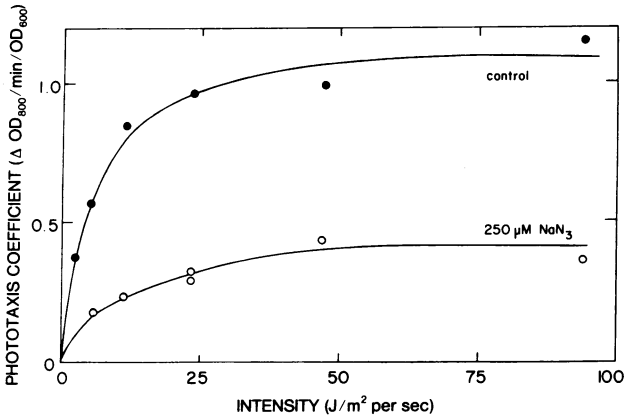

FIG. 2. Dependence of phototaxis on actinic light. Cells (1×10^6/ml) were assayed for phototaxis in the absence or presence of 250 μM sodium azide at the indicated intensity of actinic light. Phototaxis in this culture was totally inhibited by 600 μM azide.

Effect of Azide on Motility. In principle, the effect of azide on phototaxis could result from inhibition of any of the components of phototaxis, including the effector of cell movement, the receptor, or the transmitter systems. Initial microscopic examination of cells in which phototaxis had been inhibited by azide had suggested that cellular motility was relatively insensitive to azide (4). Measurements of the velocity of motile cells and the motile fraction of the population confirm that inhibition of phototaxis does not result from inhibition of motility.

In one assay the motility patterns of cells were examined in the absence and presence of 2 mM azide (Fig. 3). Under these conditions phototaxis was totally inhibited, but the velocity of the cells did not appear to have been changed, and there were no gross alterations in other aspects of motility, such as changes in mean free path, a situation that occurs in certain mutants with abnormal phototaxis (unpublished observations of R. Hirschberg and R. L. Stavis). By analysis of a series of photographs, it was found that cells had a mean velocity of 149 $\pm$ 43 μm/sec in the absence of azide and 139 $\pm$ 43 μm/sec in the presence of azide. The distributions of the velocities with and without azide were similar (Fig. 4). It is concluded from these data that cells that are motile in the presence of azide have a normal velocity and pattern of motility.

In a second assay the fraction of cells in the population that were motile was determined as a function of azide concentration (Table 1). The motility assay, carried out 3 min after the addition of azide, is compared to the phototaxis assays carried out 1 min after the addition of azide. The data demonstrate that at the time phototaxis is inhibited by azide, the proportion of motile cells is unchanged.

TABLE 1. *Effect of NaN_3 on phototaxis and percentage of motile cells*

NaN_3 concentration (μM)	Phototaxis coefficient	Motile cells (%)
0	1.1	74
100	0.5	82
200	0.1	78
300	0	62

Cells (2×10^6/ml) were harvested by centrifugation and suspended in CAT buffer at a final concentration of about 2×10^6 cells per ml. Phototaxis and percent motile cells were measured 1 min and 3 min, respectively, after the addition of sodium azide.

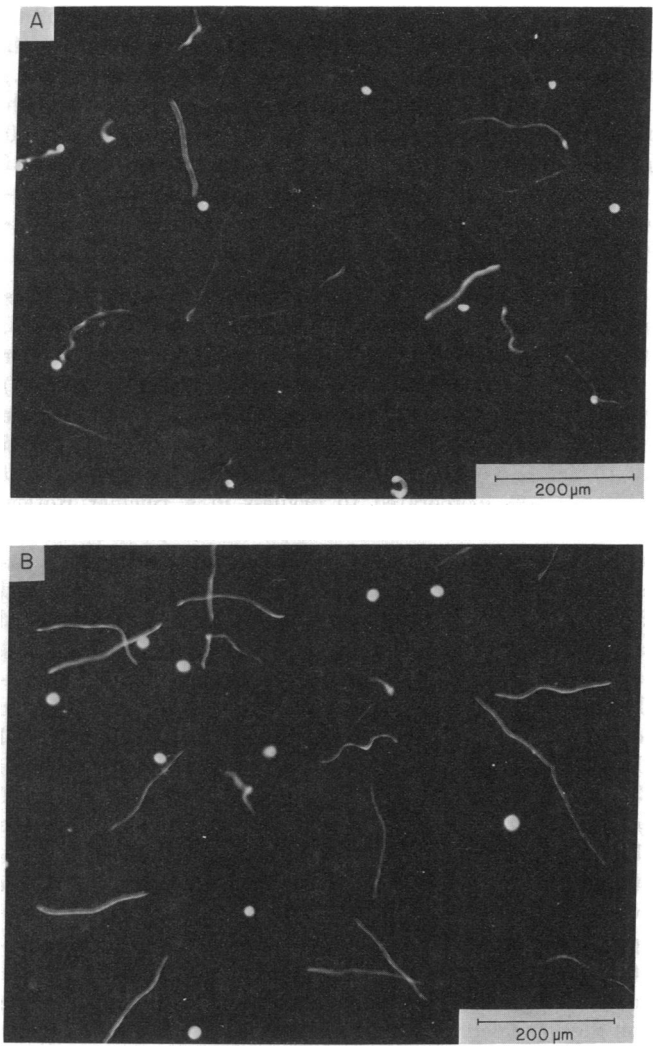

FIG. 3. Effect of azide on the velocity of motility. Cells (1×10^6/ml) were assayed for velocity as described in *Methods*. (*A*) Control, untreated cells. (*B*) Sodium azide was added to a final concentration of 2 mM, sufficient to totally inhibit phototaxis. The photograph shown is a velocity assay taken 16 min after the addition of azide.

Metabolic Effects of Azide. The effect of azide on cellular metabolism in many systems is attributable to its inhibitory effect on cytochrome oxidase and, consequently, on oxidative phosphorylation. In order to determine whether or not the effect of azide on phototaxis could be related to this mechanism of action, studies of oxygen uptake and ATP metabolism were performed. In these experiments the azide concentration was at least 2- to 5-fold greater than that required to inhibit phototaxis completely.

The rate of oxygen uptake with or without azide was 4.8 nmoles/min per 10^7 cells. This result indicates that the terminal oxidase is not inhibited by azide and is consistent with the observation that *Chlamydomonas* does not have a typical spectroscopically identifiable cytochrome oxidase (8). The amount of ATP in control cells was 98 $\pm$ 10 pmoles/10^6 cells, and did not change significantly up to 90 min after the addi-

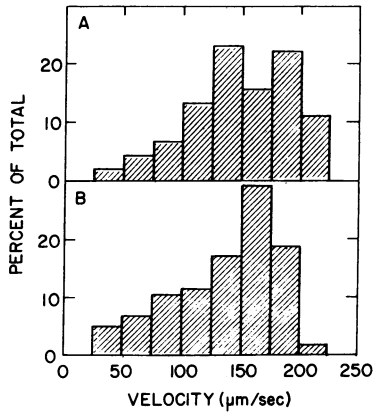

FIG. 4. Effect of azide on velocity. (*A*) Control: A total of 89 tracks were measured from the experiment described in Fig. 3*A*. (*B*) Azide: A total of 106 tracks were measured from the experiment described in Fig. 3*B*. The photographs were taken from 1 min 40 sec to 16 min after the addition of azide.

tion of azide. Assuming that *Chlamydomonas* is an 8-μm sphere, this corresponds to an intracellular concentration of about 0.4 mM. This experiment shows that the effect of azide on phototaxis is not due to a decrease in the amount of cellular ATP. It is nevertheless possible that azide acts by uncoupling oxidative phosphorylation, since ATP levels could be maintained by an increased rate of glycolysis. If this is the case, then mitochondrial function could still be involved in phototaxis.

DISCUSSION

The phototactic system may be formulated as a series of reactions beginning with the excitation of the photoreceptor and ending with the effector of the response:

$$h\nu + \text{photoreceptor} \rightarrow$$

$$\text{excited photoreceptor} \xrightarrow{\text{transmission}} \text{effector}.$$

The data indicate that azide acts rapidly and reversibly to inhibit a component or process involved in phototaxis. The insensitivity of motility to azide under conditions in which phototaxis is completely blocked suggests that the sensitive component is not part of the effector, but rather of the receptor or transmitter systems.

Azide apparently does not block excitation of the receptor by formation of a reversible photoreceptor–azide complex. If that were the case, the effect of azide should have been reversed by high light intensity, assuming inhibition of phototaxis by azide can be described in a manner analogous to competitive inhibition of an enzymatic reaction. The fact that this was not observed suggests that azide acts subsequent to the primary photoevent. One possibility, suggested to explain the partial inhibition of phototaxis by potassium iodide in *Euglena gracilis* (9), is that azide quenches the excited state of the photoreceptor.

Another possible mechanism for azide inhibition of phototaxis is suggested by studies of the depolarizing effect of azide on frog muscle (10, 11). In that system, it appears that electrically neutral hydrazoic acid enters the muscle cell and dissociates into the azide anion and a proton. The concentration of the azide anion inside and outside the cell and the potential across the cell membrane establish an electrochemical gradient for the azide anion, which tends to depolarize the cell. The extent of depolarization due to azide depends on the permeability of the membrane to the azide anion relative to its permeability to other ionic species (12). In *Chlamydomonas*, preliminary evidence based on the use of fluorescent cyanine dyes to detect changes in membrane potential (refs. 13–15, and unpublished results of P. J. Sims, C. -H. Wang, A. S. Waggoner, and J. F. Hoffman) is consistent with a depolarizing effect of azide (my unpublished results).

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