# NEGATIVE PHOTOTAXIS FROM BLUE LIGHT AND THE ROLE OF THIRD RHODOPSINLIKE PIGMENT IN *HALOBACTERIUM CUTIRUBRUM*

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ABSTRACT    Wild-type cells of *Halobacterium cutirubrum* show phototaxis. In negative phototaxis the cells are repelled by blue-near ultraviolet light, and in positive phototaxis the cells are attracted to green-red light. The extent of the responses are measured by monitoring the changes in the reversal frequency of the swimming direction of cells using a computer-linked automated method as described previously (Takahashi, T., and Y. Kobatake, 1982, *Cell. Struct. Funct.* , 7:183–192). When the intensity of the background light (illumination for the observation) was dramatically reduced, the sensitivity of the cells to the repellent light decreased markedly. This result has been previously explained by Bogomolni and Spudich (1982, *Proc. Natl. Acad. Sci. USA*, 79:6250–6254), who proposed that the photoreceptor for negative phototaxis is the long-lifetime intermediate in the photocycle of slow-rhodospin. The behavioral response in the negative phototaxis is dependent upon the intensity of the actinic light and the background light. This agrees quantitatively with our model based on the aforementioned hypothesis.

## INTRODUCTION

Several species of extremely halophilic bacteria have simple and unique energy transducing mechanisms (1, 2). In addition, they possess the ability to be attracted to long-wavelength ($\lambda$ > 530 nm) light and to avoid short-wavelength ($\lambda$ < 500 nm) light (3). The photobehavioral response was elicited by affecting the reversal frequency of the swimming direction (4).

Wild-type cells of *Halobacterium halobium* (*H. halobium*) contain three rhodopsinlike proteins: bacteriorhodopsin (bR), a light-driven proton pump, halorhodopsin (hR), a light-driven chloride pump, and the third rhodopsinlike pigment, slow rhodopsin (sR) (5–8). sR was found in bR$^-$ and hR$^-$ mutant strains selected by an excellent method (9), while sR's characteristic slow photocyle kinetics were reported independently (10). The spectrophotometric character of sR is described elsewhere (11–13). The mutant strains showed both positive and negative phototaxis (8) (in this report, we use the conventional term positive and negative phototaxis for the photobehavioral responses of the cells to attractant and repellent light).

Due to the slow photocylcing rate of sR, its intermediate accumulates in significant amounts under physiological conditions. Phototactic sensitivity of the mutant cells to both repellent and attractant light was almost the same as those of the wild type. Based on these findings, Bogomolni and Spudich (8) suggested that sR and its photointermediate are the photosensory pigments responsible for positive and negative phototaxis, respectively. Absorption spectrum of the photointermediate has a maximum at ~370 nm (8, 10) at which the negative phototaxis showed maximum sensitivity (3).

On the other hand, Schimz et al. (14, 15) suggested an additional retinal-containing protein, P565, for positive phototaxis because of the difference in the maximum wavelength between phototactic action spectrum and the absorption spectrum. They also suggested an independent pigment, P370, for negative phototaxis, because they observed the separation of negative and positive phototaxis during cell growth (16). In fact, no further evidence supports the idea that the photointermediate is the receptor for negative phototaxis (17).

The cell membrane of *Halobacterium cutirubrum* (*H. cutirubrum*) contains halorhodopsin and the pigment similar to sR (Watanabe, M., T. Takahashi, N. Kamo, and Y. Kobatake, unpublished results) in addition to bacteriorhodopsin. The bacterium also shows both positive and negative phototaxis, and we found that the negative phototaxis was inhibited when the intensity of background illumination was dramatically reduced. Here, we have measured the negative phototaxis of *H. cutirubrum* quantitatively as a function of light intensity, using the computerized, automated method described previously (18). The results are explained on the basis of photochemical equilibrium between sR and its photointermediate.

## MATERIALS AND METHOD

### Bacterial Strains and Growth Conditions

*H. cutirubrum* NRC 34001 (obtained from National Research Council, Ottawa, Canada) was selected for motility by the published method (4). The cells were grown in 10 ml pepton medium at 39°C. L-shaped test tubes and a rolling type shaker were used for gentle aeration. 3–4 d after 100 μl of cell suspension was inoculated, the growth was gradually shifted into stationary phase, and the culture reached a turbidity of ~0.7 at 660 nm. After ~1 d, 35–45 μl of the culture was transferred to 2.2 ml of tracking medium and incubated at 39°C for 20 min before measurement. The medium constituted nine parts of pepton growth medium and one part of 10 mM PIPES/NaOH buffer (pH 7.0) containing 10 mg/ml of bovine serum albumin.

### Automated Measurement of Reversal Frequency

A small drop of the bacteria in the tracking medium was put on a gap between a slide and a coverslip that was supported by two strips of Parafilm, and the slide was placed on a microscopic stage thermostatted at 39°C. Individual bacteria were tracked automatically and were subjected to actinic light for ~5 s at every 20–25 s. The process was continued for 20 min in each bacterial drop.

Automated method for counting the reversal frequency of the cells was essentially the same as described previously (18) except for the timing and the duration of actinic irradiation as illustrated in Fig. 1. These modification enabled real-time measurement of the reversal frequency. To measure negative phototaxis, the actinic light was turned on after the second frame in six consecutive digitized pictures was taken. Thereafter, the behaviors of the cells were recorded on four subsequent frames. Swimming tracks of the cells were extracted from the six pictures and counted by the computer during other 15–20 s.

The actinic light derived from 50-W high pressure mercury lamp was projected on the specimen through an incident-beam illuminator. The background light derived from a 12-V 100 W tungsten halogen lamp was passed through heat-absorbing and short cut-off (Y-55; Toshiba-Garasu Co., Tokyo, Japan) filters. Light intensities were measured at the position of the slide with a calibrated radiometer (model 65-A; Yellow Springs Instrument Co., Yellow Springs, OH), and adjusted with appropriate neutral density filters. For measurement under dim background light, a high sensitivity video camera equipped with a silicon-intensified target was used (CTC-9000; Ikegami Tsushinki Co., Tokyo, Japan).

### RESULTS

*H. cutirubrum* showed positive and negative phototaxis. The sensitivity for the attractant light at 560–600 nm was almost the same as that of *H. halobium* strain S9, and peak
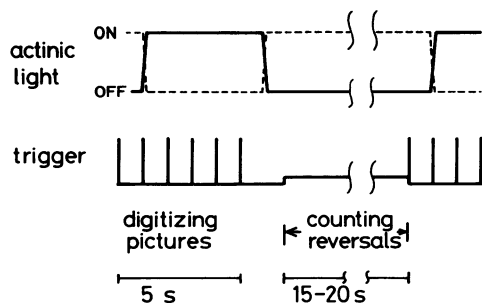


FIGURE 1 Timings for taking six consecutive pictures and for actinic irradiation. The solid line and broken line are for negative and positive phototaxis, respectively.

activity for repellent light was at wavelength <400 nm under strong background illumination (data not shown). Phototactic activity for both attractant light and repellent light developed after the cells had grown in the stationary phase.

### Dependence on Light Intensity

Fig. 2 shows the dependence of negative phototaxis at 380 nm on the intensity of background illumination. The response increased significantly along with increasing the intensity of the background light when the actinic light was applied at an intensity of $1.6 \times 10^{13}$ hν/mm²s.

The percentage of the bacteria reversed was plotted against the intensity of actinic light under strong and dim background light (Fig. 3). Sufficient response was observed even under dim background light when stronger actinic light was applied (see also upper curve in Fig. 2). This is not inconsistent with the idea that background illumination is necessary for eliciting negative phototaxis, if we consider that less but significant absorbance is expected at a lower wavelength such as 380 nm for the original pigment sR. There may be also another photoreceptor system that causes repellent response under dim background light, but whose contribution is very small at actinic intensities $<2 \times 10^{13}$ hν/mm²s.

### Background Action Spectrum

If the receptor for the negative phototaxis is a photointermediate of sR as suggested in reference 8, the action spectrum for the effect of background light should be similar to the absorption spectrum of sR. The experimental results is shown in Fig. 4. The result is consistent with the
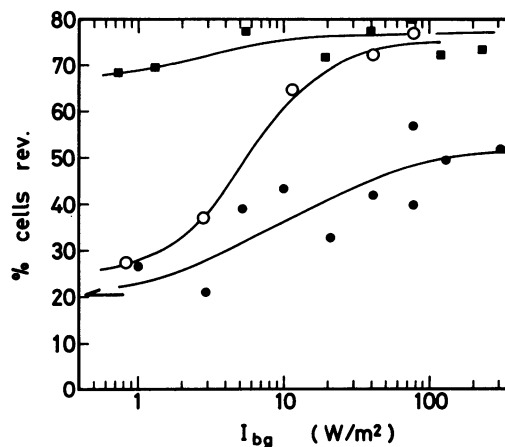


FIGURE 2 Dependence of negative phototaxis on the intensity of background illumination. The ordinate indicates the percentage of the reversed cells to those counted during 4 s after sudden exposure to actinic light. Each point represents the result obtained with 150–200 cells during a measurement (20 min). The arrow indicates the percent of spontaneously reversed cells, measured without actinic irradiation. Wavelength of the background light, λ > 550 nm. Intensities of actinic light (λ = 380 ± 10 nm) were: $6.4 \times 10^{13}$ hν/mm²s (■), $1.6 \times 10^{13}$ hν/mm²s (○), $4 \times 10^{12}$ hν/mm²s (●).
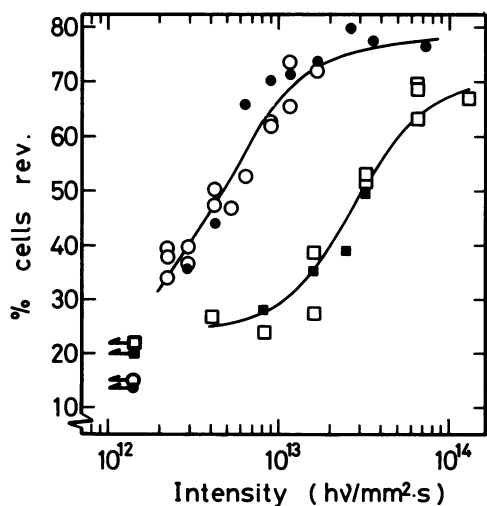
FIGURE 3 Dependence of negative phototaxis on the intensity of the actinic light. o ●, indicate results under strong background light ($\lambda > 550$ nm, 230 W/m²) of the two differenct cultures; □, ■ indicate results under dim background light ($\lambda > 550$ nm, 0.8 W/m²). The arrows indicate the percent of spontaneous reversals of the cells in the corresponding cultures.

absorption spectrum of sR, which showed broad maximum centered at 590 nm (12).

## Background-Light Effect on Positive Phototaxis

The signals from both repellent and attractant light compete and integrated inside the cell (4). This means that the opposite response is always suppressed whenever an apparent photobehavioral response (attracted to or repelled by light) is observed. Because strong background illumination causes a decrease in the sensitivity of positive phototaxis (18), it is also possible to explain the observed dependence of negative phototaxis on background illumination as a consequence of the inhibition of positive phototaxis by the background light.

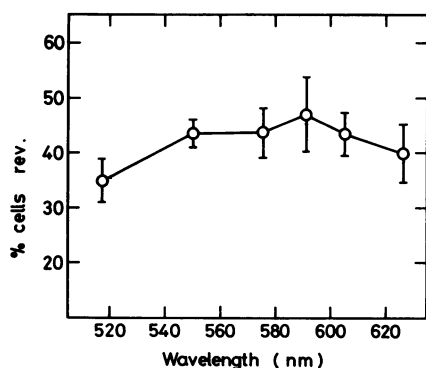To test this possibility, effects of background light on



FIGURE 4 Background action spectrum for negative phototaxis in H. cutirubrum. Average ± SE was obtained from the results of three to four experiments with 100–200 cells. Actinic light, $\lambda = 380 \pm 10$ nm, $9 \times 10^{12}$ hν/mm²s. The intensity of the background light was $9.2 \times 10^{12}$ hν/mm²s, which corresponds to 2.9–3.4 W/m².

positive and negative phototaxis were compared in Fig. 5. Relatively low intensity of actinic light was applied to emphasize the background light effect on positive phototaxis. If the observed dependence of negative phototaxis on background light was the consequence of the background effect on positive phototaxis, the threshold intensity of the background light for the effect should be nearly equal or higher in the effect on positive phototaxis. Furthermore, the extent of the background effect should be larger on positive phototaxis than on negative phototaxis. Judging from Fig. 5, we concluded that this is not the case. These findings are all in agreement with the idea that the photointermediate of sR, which is produced by the background illumination, serves as the photoreceptor for the negative phototaxis.

## DISCUSSION

As an index of the phototactic activity, we measured the fraction of the cells that reversed within 4 s after the onset of the light stimulus. Interpretation of the results may be dependent upon the relationship between this apparent response and the intrinsic excitability of those cells subjected to the stimulus. For example, one may ask whether the fraction of reversals corresponds to the amount of photoproduct that controls the bacterial behavior, or whether the saturation of the response reflects the intrinsic excitability or whether it is dependent on the method.

As for the latter question, we examined it by the experiment described below. First we adjusted the intensity of the actinic and the background light, as shown in the right-hand side of Figs. 2 or 3, where the response seemed almost saturated. When the observation period after the onset of the actinic light was shortened to 3 s so as to neglect a part of the reversed cells, the apparent response was decreased to almost one-half. Then the intensity of the actinic light is increased by factors of two and four. We
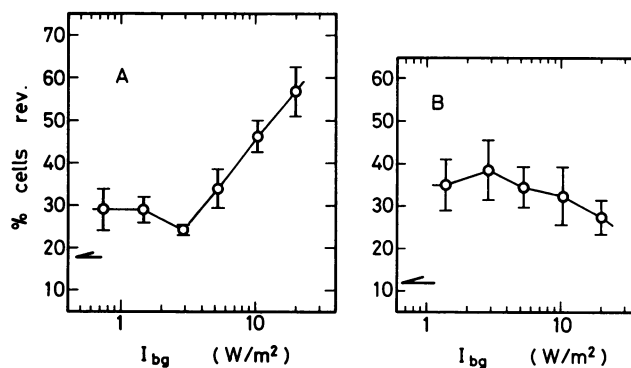


FIGURE 5 Effect of background light on the behavioral responses of both photosystems. The vertical bars represent the standard errors obtained by three to four measurements. 100–200 cells were counted in each period of measurement (15 min). The background light was monochromatic (636 ± 10 nm). (A) Dependence of negative phototaxis on the intensity of the background light. Actinic light, 380 ± 10 nm; 9 × 10¹² hν/mm² s. (B) Dependence of positive phototaxis on the intensity of the background light. Actinic light, 581 ± 7 nm; 4.1 × 10¹³ hν/mm²s.

observed that the percentage of the reversed cells remained almost unchanged. This indicates that the saturation is intrinsic.

## Kinetic Models For Negative Phototaxis

It is generally accepted that blue light accelerates the regeneration of sR from its photointermediate, $sR_{373}$ (8, 19). Therefore, it is necessary to explain the data shown above in connection with photoreaction cycle of sR if we adopted the hypothesis that the intermediate is the photoreceptor. First we will discuss the relationship between behavioral response and the possible alteration that occurred within the cell upon receiving the actinic light. We will start the discussion on the bases of the following assumptions. (a) The probability of the reversal of the swimming direction of a bacterium is proportional to certain alterations inside the cell, i.e., the amount of a substance that is either produced by chemical reaction or concentrated inside the cell by a transport mechanism, or by an ion-specific channel, which is stimulated by actinic light. The adaptation process, in which the reversal probability is returned to its prestimulus value, is thought to affect the amount of change as a function of time. (b) The bacteria that are reversed during a period (4 s) of observation cannot be reversed again within the same period. (c) A steady state value of the variables in the photocycle of sR is correlated to the phototaxis.

The second assumption is supported by our observation that during 5 s of the stimulus, no subsequent reversal was observed after the cell had reversed upon actinic irradiation. This holds even at the upper limit of the light intensity available.

The third is postulated for simplication. Because the bacterial behavior was observed for 4 s, the reversal frequency should reflect the result of the integration of the variable for this period. Therefore, strictly speaking, the steady state approximation holds only if the kinetic parameters of sR photocycle are large, i.e., the intensity of the background light and of the actinic light are high enough. Otherwise, it may cause overestimation of the reversal frequency at lower light intensity.

Using postulate $a$ and $b$, we can express the fraction of reversed bacteria after the sudden exposure to actinic light in a differential form

$$df/dt = a(1 - f)j, \tag{1}$$

where $f$ is the fraction of the bacteria reversed during time $t$, $a$ is proportionality constant, and $j$ is the amount that is proportional to the probability of the response. The amount $j$ can be written as a function of time and the intensity of both the actinic and the background light.

A simplified scheme of the photocycle of sR is illustrated in Fig. 6. If we assume the photointermediate, $sR_x$, which is generated upon absorption of the actinic light by the other photointermediate, $sR_{373}$, regenerates into sR
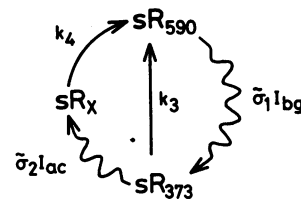


FIGURE 6 Simplified scheme of the photocycle of sR. Wavy lines indicate that the process is light dependent.

through a thermal process at the rate of $k_4$, then the steady state concentration of the photointermediate, $x = [sR_x]$, is given as follows

$$x = \frac{\tilde{\sigma}_1 I_{bg} \tilde{\sigma}_2 I_{ac}}{\tilde{\sigma}_2 I_{ac}(\tilde{\sigma}_1 I_{bg} + k_4) + k_4(\tilde{\sigma}_1 I_{bg} + k_3)}, \tag{2}$$

where $I_{ac}$ and $I_{bg}$ are intensities of the actinic and background light, respectively. The rate, $k_3$, of regeneration of sR from $sR_{373}$ through thermal process was already obtained spectrophotometrically by flash-photolysis experiment (8, 10, Watanabe, M., T. Takahashi, N. Kamo, and Y. Kobatake, unpublished results). The effective cross section (20) $\tilde{\sigma}_1$ and $\tilde{\sigma}_2$ for capture of the background and the actinic light is unknown but can be estimated from absorption data.

*Model 1.* One of the simplest assumption that combines the bacterial response with sR photocycle may be that $sR_x$ is the substance whose concentration is related linearly to the probability of reversals of bacteria: $j = bx$. Because the cells respond to a change in light intensity, the response should be a transient change in $j$. To express the adaptation process, we introduce the response decay term $k$ (20)

$$j = bxe^{-kt}. \tag{3}$$

Using this relation along with the initial condition $f = 0$ at $t = 0$, we obtain the solution of the differential Eq. 1,

$$\ln(1 - f) = abx\left(\frac{1}{k}e^{-kt} - \frac{1}{k}\right). \tag{4}$$

*Model 2.* The second possibility is that the production of the substance is coupled with the turnover of $sR_x$. A similar mechanism was discussed by Lipson and
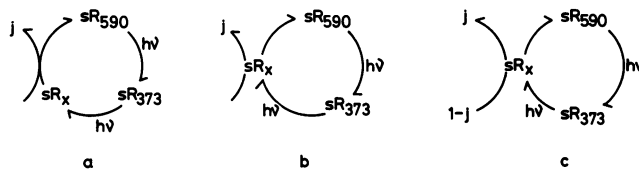


FIGURE 7 Temporal kinetic models for the interpretation of the observed data. The potential of photophobic response was attributed to the amount of the substance $j$. $a$, $b$, and $c$ correspond to Models 2, 3, and 4 in the text, respectively.

Presti for the analysis of the data in the light-induced absorbance change in lower plants (20). The expression is

$$dj/dt = k_4 x - kj \quad (5)$$

where $k$ is the rate constant for the decay of response. With the initial condition $j = 0$ at $t = 0$, the solution of Eq. 5 is

$$j = \frac{k_4 x}{k}(1 - e^{-kt}). \quad (6)$$

The differential equation that combines Eq. 6 and Eq. 1 has the following solution with the initial conditon $f = 0$ at $t = 0$

$$\ln(1 - f) = \frac{ak_4 x}{k}\left(-t - \frac{1}{k}e^{-kt} + \frac{1}{k}\right). \quad (7)$$

*Model 3.* In our third model, $sR_x$ is taken as if it is either an enzyme or a channel. If the rate of the production of the substance is higher than that of regeneration of $sR_x$, we can write,

$$dj/dt = bx - kj, \quad (8)$$

where $b$ and $k$ are constants. This equation is similar to Eq. 5. We obtain the solution

$$\ln(1 - f) = \frac{abx}{k}\left(-t - \frac{1}{k}e^{-kt} + \frac{1}{k}\right). \quad (9)$$

*Model 4.* In Model 3, production of the substance is independent of the concentration of the substance. If, on the other hand, the scarcity of the substrate was considered as shown in the scheme $c$ in Fig. 7, the rate of the production process may be written as

$$dj/dt = bx(1 - j). \quad (10)$$

Using Eq. 1, the fraction of the reversal can be expressed as,

$$\ln(1 - f) = a\left(-t - \frac{1}{bx}e^{-bxt} + \frac{1}{bx}\right). \quad (11)$$

Note that $f$, the fraction of the reversals of the cells, was not $r/100$, where $r$ was the percentage of the cells that
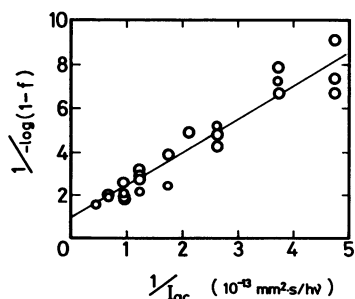


FIGURE 8  Double reciprocal plot of $-\log(1 - f)$ and the intensity of the actinic light under the strong background illumination.

reversed. Because measured reversals included spontaneous reversals, the fraction of the reversals is represented ideally, by the relation, $f = (r - s)/(100 - s)$, where $s$ is the percentage of the spontaneous reversals. Eqs. 4, 7, and 9 predict that if the logarithm of the unreversed fraction of the bacteria was plotted against the concentration of $sR_x$, the linear relation should be obtained.

When the background illumination is strong enough, i.e., $\tilde{\sigma}_1 I_{bg} \gg k_3, k_4$, Eq. 2 indicates that the fraction of $sR_x$ under steady state is given by $\tilde{\sigma}_2 I_{ac}/(\tilde{\sigma}_2 I_{ac} + k_4)$. Therefore,

$$\log(1 - f) = -2.303\, \alpha\, \frac{\tilde{\sigma}_2 I_{ac}}{\tilde{\sigma}_2 I_{ac} + k_4},$$

where $\alpha$ is

$$ab\left(\frac{1}{k}e^{-kt} - \frac{1}{k}\right), \quad ak_4\left(-t - \frac{1}{k}e^{-kt} + \frac{1}{k}\right)\bigg/ k,$$

or

$$ab\left(-t - \frac{1}{k}e^{-kt} + \frac{1}{k}\right)\bigg/ k$$

in Models 1, 2, or 3, respectively.

This equation indicates that the linear relation is obtained when $-1/\log(1 - f)$ is plotted vs. $1/I_{ac}$ as shown in Fig. 8. It appears in Fig. 8, that $\alpha$ is close to 1 and $k_4/\tilde{\sigma}_2$ is nearly $1.5 \times 10^{13}$ h$\nu$/mm$^2$s. If the quantum yield of the process is the maximum value, the capture cross section $\tilde{\sigma}_2$ is related to the molecular extinction coefficient as, $\sigma_2 = 0.38 \times 10^{-18} \epsilon$ mm$^2$ (21). The value $k_4$ is not yet known, but it must exceed 1, because we observed that the bacteria could repeatedly respond to pulses of actinic irradiation at the frequency of 1 Hz. When we take this into account, the
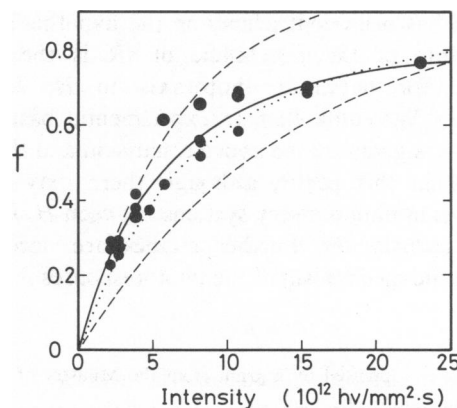


FIGURE 9  Plot of $f$ vs. the intensity of actinic light under the condition of strong background illumination. The solid line represents the theoretical curve based on the kinetic Model 4 with the following constants: $a = 0.54, b = 8, k_4 = 4.6$ s$^{-1}$, $t = 4$ s. The dotted line indicates the result with Model 4, when $k_4 = b = 4.6$ s$^{-1}$, $a = 0.75$. The broken lines indicate the results of simulation using Model 3: $a = 0.54, b = 8$ (*upper*); $a = 0.54, b = 3.5$ (*lower*). The molecular extinction coefficient of 53,000 was assumed for $sR_{373}$ at 380 nm.
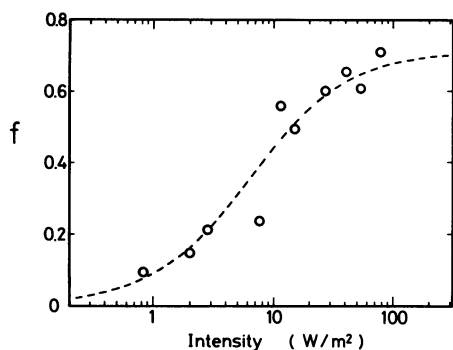
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FIGURE 10  Plot of $f$ vs. the intensity of background light. The intensity of actinic light was $1.6 \times 10^{13}$ h$\nu$/mm$^2$s. The broken line represents theoretical curve of Model 4. Parameters are: $a = 0.54$, $b = 8$, $\tilde{\sigma}_1 = 0.052$ m$^2$/Ws, $\tilde{\sigma}_2 = 2 \times 10^{-14}$ mm$^2$/h$\nu$, $k_3 = 0.9$ s$^{-1}$, $k_4 = 4.6$ s$^{-1}$.

molar extinction coefficient of sR$_{373}$ estimated from the plot should be extraordinarily large, i.e., 175,000 at least, if Models 1–3 are applied.

On the other hand, Model 4 fits the data well as shown in Figs. 9 (solid line) and 10, where the molecular extinction coefficient of sR$_{373}$ and that of sR$_x$ are assumed to be 53,000 and 46,000, respectively. If parameter $b$ equals $k_4$ in Model 4, the model turns out to be a modification of Model 2, where the depletion of substrate is taken account. The fit to the experimental result is shown in the dotted line in Fig. 9. Therefore, the data can be fit either by Model 4 or 2 with an additional term for substrate depletion.

These fittings to the data were made using kinetic parameters of sR. Another intermediate whose lifetime was shorter than 100 ms could not fit the data in Figs. 9 and 10. This excludes the possibility of the contribution of bR or hR to the negative phototaxis. Therefore, only sR$_{373}$ can be a photoreceptor molecule for negative phototaxis among the retinal proteins so far identified spectrophotometrically in halobacteria.

There has been controversy on the hypothesis that an intermediate of the photocycle of sR is the receptor molecule for negative phototaxis in H. halobium (8, 16, 17). We think that an experimental basis for the hypothesis is given by the above discussion and the results presented in this paper, although there may be some differences in photosensory systems between H. halobium and H. cutirubrum. Further studies are necessary to elucidate the mechanism of the photoresponses in halobacteria.

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