Photobehavior of *Halobacterium halobium:* sinusoidal stimulation and a suppression effect of responses to flashes

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ABSTRACT Sequences of reversals recorded by single-cell observation of *Halobacterium halobium* are analyzed. Autocorrelation functions of spontaneous and stimulated reversals are computed; the results show that the only periodicity present in our data is that of the stimulus. Several different patterns of light stimuli were used. Responses to repetitive linear ramps of different slopes and to sinusoidal lights with different mean values and/or modulation depths are reported, showing that the modulation depth is the stimulus parameter most effective in eliciting photoresponses. Responses to more complex stimuli obtained by superimposing flashes to sinusoidal stimuli are also reported; a suppression effect depending on the phase of the sinusoidal stimulation is shown in responses to complex stimuli. A model which accounts for this effect is proposed.

INTRODUCTION

The study of motor responses of *Halobacterium halobium* involves recording swimming direction reversals and looking for their correlation with the light stimuli. Reversals of swimming direction also occur spontaneously, in the absence of any stimulus. The different groups working on *H. halobium* obtain different histograms of the intervals between successive spontaneous reversals and suggest different interpretations. The hypotheses range from a stochastic mechanism (Mc Cain et al., 1986) to a deterministic and periodic one (Schimz and Hildebrand, 1985, 1986). A third mechanism, based on balancing two different substances and formally expressed by a system of two differential equations was also considered (Marwan and Oesterhelt, 1987)

H. halobium synthesizes several pigments; two of which have been identified as sensory pigments, SR-I and SR-II (Bogomolni and Spudich, 1982; Takahashi et al., 1985). SR-II mediates responses to blue light (Spudich et al., 1986; Sundberg et al., 1985) whereas SR-I causes responses to red light, but as it is photochromic, SR-I also mediates responses to blue. Responses due to the two pigments can be separated by using different strains and performing suitable tests by light stimuli (for instance if blue responses are produced only in a red background they are mediated by SR-I [Spudich and Bogomolni, 1984]).

Switching on blue light induces a reversal of motion, whereas turning on red light delays the following reversal. The opposite effects occur when the light is turned off: a step down in red light induces reversals and in blue light decreases the reversal frequency. A constant level of light does not change the frequency of the reversals. Immediately after turning on blue light, there is a reversal and then, in a few seconds, the microorganism adapts to the new level of light. *H. halobium* responds to abrupt changes of light level, but it was recently shown that also slow stimuli such as sinusoidal lights (Lucia et al., 1989) or saw-tooth stimuli (Schimz and Hildebrand, 1990) are effective in driving the reversals.

The photocycles of the sensory pigments have been studied in detail and experimental evidence for the existence of signaling states in the photocycles has been obtained; whereas little information exists on the chain of reactions which, starting from the absorption of light, control the motor responses. A methylation system is involved in sensory behavior of *H. halobium* (Schimz, 1982) and in particular, a reversibly methylated membrane protein is involved in sensory transduction by SR-I; moreover, transient changes in methyl turnover rates at the beginning and at the end of a photostimulation have been detected (Spudich et al., 1988, 1989).

The system of sensory transduction *H. halobium* can produce quite surprising effects: for instance, a red flash, which usually decreases the reversals frequency, elicits a reversal if it is delivered only a few seconds after turning off a constant red light (Mc Cain et al., 1986). Moreover, weak stimuli, when delivered early after a reversal, can produce the inverse effect of strong stimuli (Hildebrand and Schimz, 1987). Other results, obtained in experiments where a couple of flashes with a variable gap were used as a stimulus, show that a time integration of the stimulus occurs in *H. halobium* (Marwan and Oesterhelt, 1987).

The present paper contributes to the characterization and understanding of sensory transduction in *H. halo-*

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bium. Sequences of spontaneous reversals are analyzed here by using the autocorrelation function, which is a powerful mathematical tool to detect periodicity even in a very noisy temporal series. Responses to repetitive ramps and to a continuous sinusoidal stimulation are also reported for different values of the stimulus parameters; the purpose is to characterize the stimulus parameters most effective in eliciting photoresponses. Finally, we report on experiments where complex stimuli are used, such as sinusoidally modulated light with flashes superimposed. The results obtained in these last experiments are quite surprising and are a clear sign of the complexity and nonlinearity of sensory transduction in *H. halobium*.

METHODS

The experiments were carried out on the Flx 15 mutant (BR^-, HR^-, $SR\text{-}I^+$, $SR\text{-}II^+$). The cells were grown in standard conditions (Spudich and Spudich, 1982). Experiments were performed with the sample at room temperature or with the sample held at 37°C. The sample was observed in a dark field microscope by a videocamera. The light sources were quartz iodine lamps. The background light, also used for the observation, was obtained with a long pass filter, 570 nm cut wave length (Schott, Mainz), placed on the path of the microscope illumination, while the stimulating blue light, also reaching the sample through the microscope condenser, was obtained with a band pass filter centered at 400 nm (K40 Balzers, Liechtenstein, with half-width at half maximum of 25 nm) plus an infrared cutoff filter. The wave lengths were chosen to selectively affect the SR-I subsystem and we tested that no behavioral responses to blue light occurred with infrared background.

The single-cell observation method was used and the data reported in each figure were obtained from single cells. Each experiment was performed many times and, in agreement with previous observations (Mc Cain et al., 1987), the variability from a cell to another was very small.

The system used to obtain graded light stimuli of given shapes was described elsewhere (Lucia et al., 1989). The maximum light intensity falling on the sample $I_{\max}$ was equal to 0.6×10^{16} photons/cm$^2 \cdot$ s. Experiments with sinusoidal and ramp stimuli were performed. Ramp stimuli started from $I_{\min} = I_{\max}/10$ and reached $I_{\max}$; the rising time and the rest period interposed between consecutive ramps were controlled by an IBM PC. This computer was used to control the stimuli (ramps or sinusoids), and to record the times of the reversals as detected by the observer, who manually pushed a button whenever a reversal occurred.

SPONTANEOUS REVERSALS AND THE ENDOGENOUS OSCILLATOR

The idea that an endogenous oscillator drives the spontaneous reversals in *H. halobium* was suggested by Schimz and Hildebrand (1985), who obtained a very narrow distribution of the intervals between spontaneous reversals; on the other hand, very broad distributions were obtained in other laboratories.

In our conditions, spontaneous reversals are very irregular and occur rarely at room temperature. Holding the sample at 37°C increases the frequency of the reversals and makes their pattern more regular. A histogram of the intervals between spontaneous reversals from a sample maintained at 37°C is shown in Fig. 1 *a*.

To test the idea whether the oscillatory behavior underlies the pattern of the spontaneous reversals, we analyzed the data by using the autocorrelation function. For each single $f(t)$, the autocorrelation $R(\tau)$ is defined by:

$$R(\tau) = \int_0^T f(t) f(t + \tau)\, dt.$$

If $f(t)$ has a periodic component this is highlighted in $R(\tau)$. In our case $f(t)$ is a temporal sequence of discrete events (the reversals), so that $R(\tau)$ can be computed by simply counting the events with a distance τ, irrespective of the number of interposed events.

In Fig. 1 we report interval histograms (*left*) and autocorrelation functions (*right*) for some sequences of reversals. Data were obtained under steady conditions (Fig. 1 *a*), and during sinusoidal or ramp stimulation respectively (Fig. 1, *b* and *c*). During sinusoidal or ramp stimulation, the stimulus periodicity clearly appears, whereas in the series of spontaneous reversals no apparent periodicity is observed. However, the variability of the intervals, as shown by the intervals histograms, is about the same in both spontaneous and stimulated activity. Fig. 1 *d* shows the autocorrelation function for the same data as in Fig. 1 *c* on an expanded time scale. In this plot, which should make evident the periodicity, if any, inside each ramp, no periodicity is observed.

SINUSOIDAL STIMULATION: IDENTIFICATION OF THE IMPORTANT PARAMETERS

H. halobium responds to a continuous sinusoidal stimulation showing a phase preference (Lucia et al., 1989). For sufficiently slow stimuli some reversals occur in each cycle near the phase of the maximum positive slope. The number of reversals in each cycle is quite variable and at high frequencies of stimulation (for instance, for periods of 6–12 s) no more than one reversal occurs in a cycle and in some cycles no reversals occur. This is revealed by several peaks in the histogram of intervals (Fig. 2. *a*), while the phase preference of the reversals is shown by the cycle histogram, which is constructed by counting the number of reversals in the recorded sequence which occur at each given phase (Fig. 2 *b*).

A sinusoidal light stimulus:

$$I(t) = I_m(1 + M \sin 2\pi t/T),$$

is characterized by three parameters: the period T, the

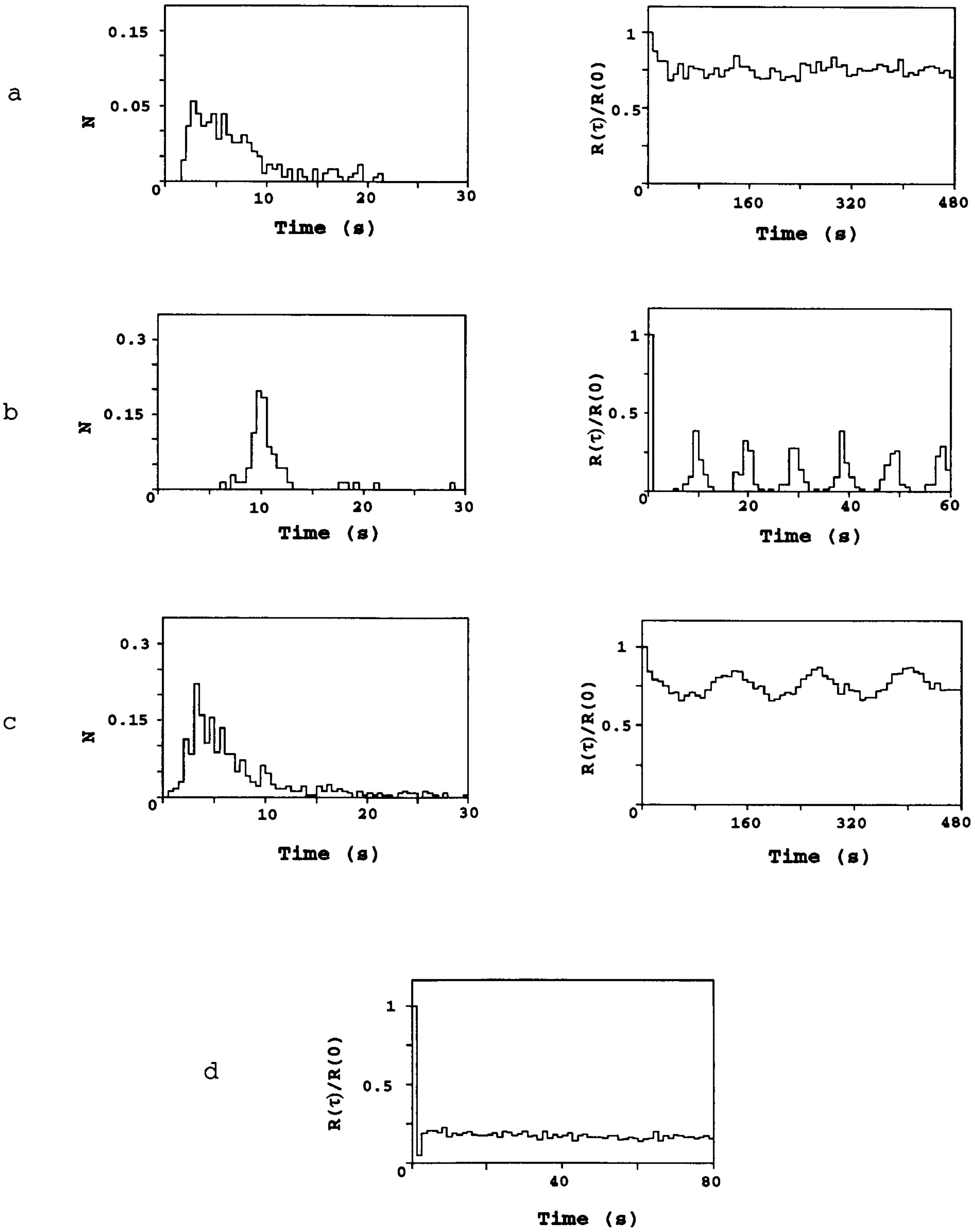

FIGURE 1 Interval histograms (*left*) and autocorrelation functions (*right*): (*a*) Spontaneous activity (240 reversals); (*b*) sinusoidal stimulus with a period of 10 s (71 reversals, 98 cycles); (*c*) ramp stimulus with a rising time of 80 s and 50 s of rest period (480 reversals, 28 cycles); (*d*) same data as in (*c*) on a different time scale. In (*b*) the organisms were at room temperature, in (*a*) and (*c*) at 37°C. *N* is the fractional number of reversal.

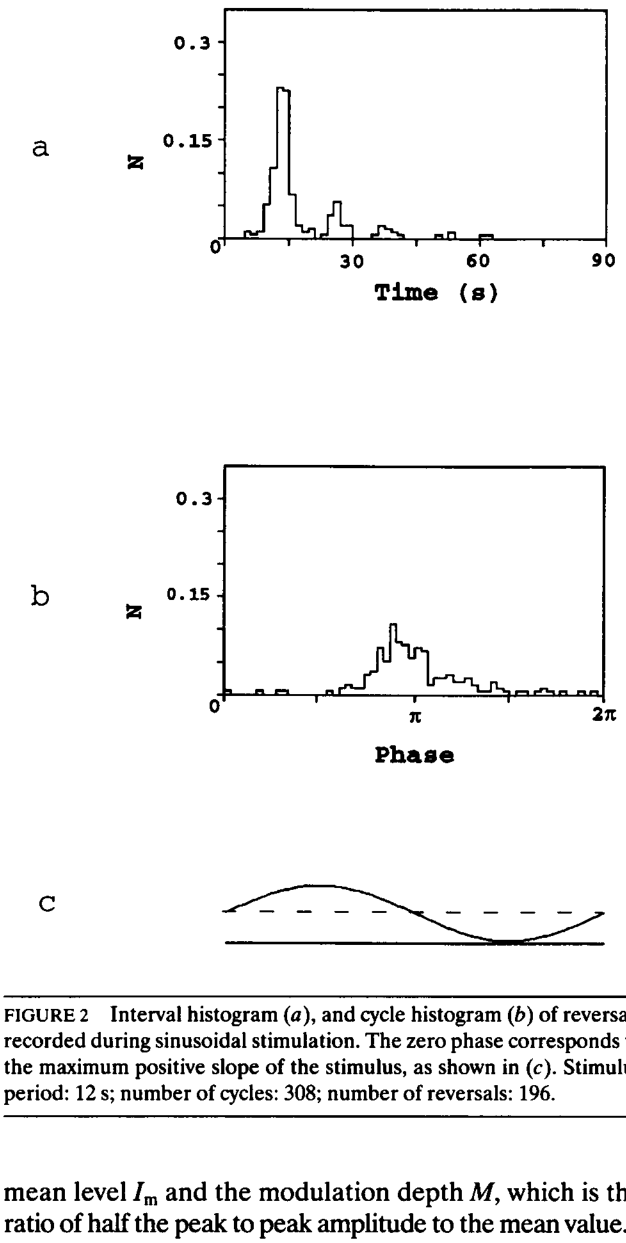

FIGURE 2 Interval histogram (*a*), and cycle histogram (*b*) of reversals recorded during sinusoidal stimulation. The zero phase corresponds to the maximum positive slope of the stimulus, as shown in (*c*). Stimulus period: 12 s; number of cycles: 308; number of reversals: 196.

mean level I_m and the modulation depth M, which is the ratio of half the peak to peak amplitude to the mean value.

To rank the importance of these parameters in driving the motor behavior of *H. halobium* we performed two different series of recordings. In the first, the mean level of light was reduced by interposing neutral density filters in the path of the light beam, thus keeping the modulation depth M constant. In the second series, the modulation depth was decreased by software, while the light level I_m was kept constant.

Fig. 3 shows the cycle histograms obtained in these experiments. The cycle histograms on the right were obtained by decreasing the light level and by keeping the modulation depth constant. In all these histograms there is a strong phase preference. This does not occur in the cycle histograms on the left side, which were obtained by decreasing the modulation depth and by keeping the mean light level constant. These results indicate that the most important stimulus parameter underlying the motor behavior of *H. halobium* is the modulation depth M. Moreover, in the cycle histogram reported in Fig. 3 *c* the phase of the response changes with the modulation depth. This is an indication of nonlinear processes in the sensory pathways.

In Fig. 4, the peak phase, measured in the cycle histograms by choosing the phase of maximum positive slope of the sinusoidal stimulus as zero, is plotted against the period of the stimulus. This plot was obtained by collecting data from different cells. For very slow stimuli (85 s period), reversals occur near the point of maximum slope. By decreasing the stimulus period the preferred phase shifts towards the maximum of the stimulus and by further decreasing the stimulus period it reaches the region of negative slopes. The continuous line in Fig. 6 is obtained by assuming a constant delay of reversals from phase zero. The agreement between computed curve and experimental points shows that a constant delay can account for the dependence of the phase on the stimulus period.

SINUSOIDAL STIMULATION: ANALYSIS OF CONSECUTIVE REVERSALS

The data obtained with sinusoidal stimuli were also used to check whether the phases of two consecutive reversals are correlated. The correlation can be highlighted by an *XY* plot reporting on the abscissa the phase Φ_i of the i^{th} reversal and on the ordinate the phase Φ_{i+1} of the $(i + 1)^{th}$ one. This is done in Fig. 5 *a* for a sinusoidal stimulus which elicits no more than one reversal for cycle (interval histogram and cycle histogram are reported in Fig. 2). Only points corresponding to reversals in consecutive cycles are reported in Fig. 5 *a*; the cloud of points so obtained is symmetrical around its center, what implies the absence of any correlation, and the value 0.08 of the correlation coefficient computed by linear regression confirms this conclusion.

The same set of data shows a correlation in the time intervals between consecutive reversals. The *XY* plots in Fig. 5, *b* and *c* report on the abscissa the interval t_i between i^{th} and $(i + 1)^{th}$ reversals and on the ordinate the successive one t_{i+1}. Fig. 5, *b* and *c* correspond to two different time scales. In Fig. 5 *b*, several clouds appear; this is because the reversals do not occur in every cycle and therefore, their time distances are approximately multiple of the stimulus period as shown by the histograms in Fig. 2. In Fig. 5 *c*, an enlarged time scale reveals the structure of the first cloud, which corresponds to reversals occurring in adjacent cycles. The elongated shape of the cloud indicates a correlation between

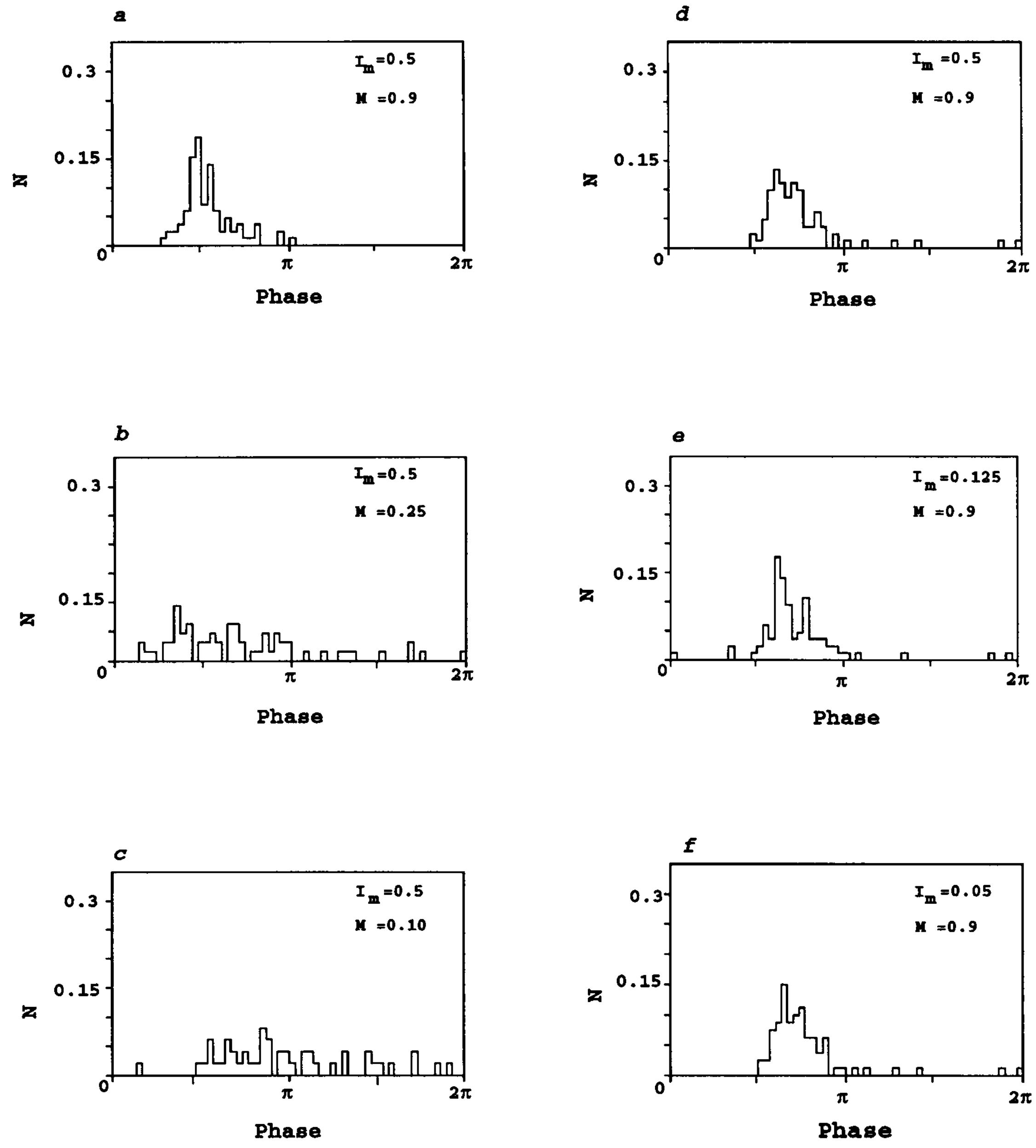

FIGURE 3 Cycle histograms of the reversals on sinusoidal light stimuli (stimulus period 12.5 s). Abscissa: phase of the stimulus. Ordinate: percentage of reversals occurring at that phase. The mean light level I_m is given as a fraction of I_{max} (0.6×10^{16} photons/cm^2 s). Number of reversals and number of averaged cycles are: in (*a*) 80, 113; in (*b*) 85, 119; in (*c*) 80, 113; in (*d*) 88, 105; in (*e*) 64, 113; in (*f*) 42, 107, respectively. Two different cells were used for the left and right side of the figure.

consecutive intervals (correlation coefficient equal to 0.44). This correlation is an obvious consequence of the absence of correlation between consecutive phases. In fact, if a reversal occurs a bit earlier in the cycle, it will have on average a shorter distance from the previous one and a longer distance from the following one, which qualitatively accounts for the shape of the cloud observed in Fig. 5 *c*.

An analysis like that of Fig. 5, *b* and *c* has been done by Schimz and Hildebrand (1989) on data obtained with exponentially decaying or increasing light stimuli. Their data become quite irregular for some particular slopes of the stimulus and in their *XY* plot of pairs of consecutive intervals a structured pattern appears which suggests the existence of a deterministic and chaotic process. In our data, obtained from a different strain under

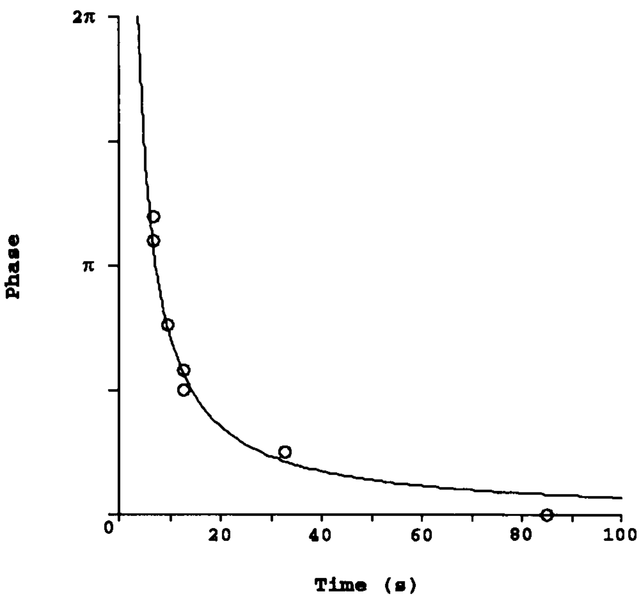

FIGURE 4 Phase of peak in the cycle histogram versus stimulus period. The continuous curve is obtained by assuming a constant delay of 3.5 s of the reversals from phase 0, where reversals occur for very slow stimuli. Data from different cells.

sinusoidal or linear ramp stimuli, we did not find evidence of an underlying chaotic dynamic system.

RESPONSES TO RAMPS OF LIGHT

The phases of the reversals on sinusoidal stimuli suggests that the first operation *H. halobium* performs on the stimulus is its time derivative. This is confirmed by Fig. 6 and 7 obtained by stimulating the cells with ramps of light; both figures concern bacteria at 37°C, but in the first one there are spontaneous reversals while in the second one, spontaneous reversals occur very rarely.

A typical ramp is shown in Fig. 6. Each run consists of the ramp and of an interposed rest interval; 28 cycles were averaged to obtain the cycle histogram of Fig. 6 *a*. Here and in Fig. 7 we report the fractional number of reversals in each bin versus the stimulus phase, defined as the ratio between time elapsed from the beginning of the ramp and total period of the stimulus (rising time plus rest interval). This density of reversals is almost constant inside the ramp. Fig. 6 *b*, obtained from the same data of Fig. 6 *a* by plotting t_{i+1} against Φ_i, shows that the interval variability in the ramp is lower than that of the spontaneous reversals. These results are quite different from those of Schimz and Hildebrand (1989),

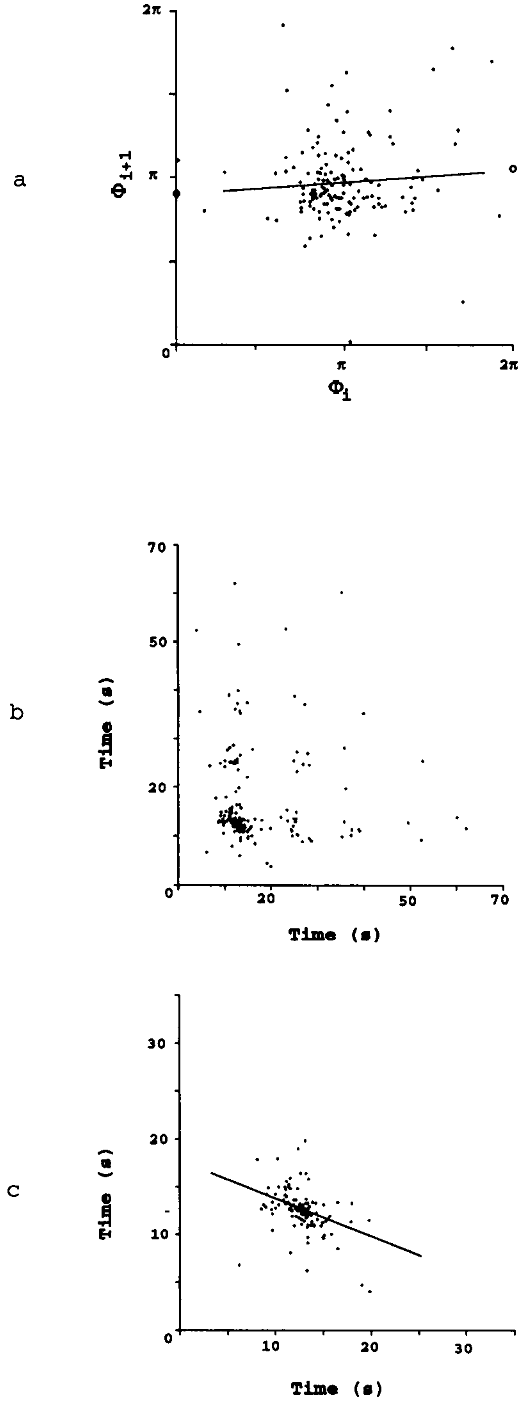

FIGURE 5 In (*a*) Φ_{i+1} against Φ_i for reversals occurring in consecutive cycles. In (*b*) interval t_{i+1} against interval t_i. In (*c*) the first cloud, which corresponds to reversals occurring in consecutive cycles, is shown on an expanded time scale. The stimulus period is 12 s.

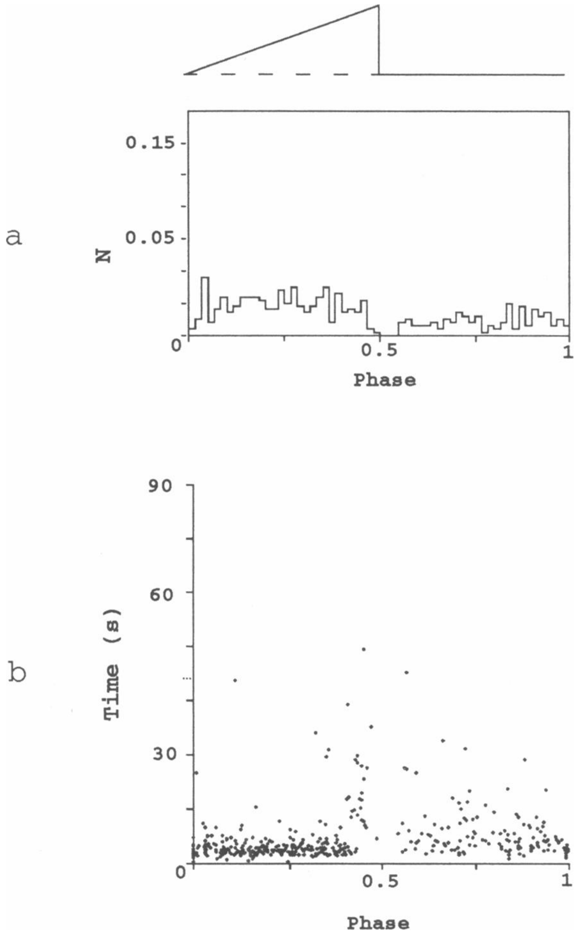

FIGURE 6 (*a*) Cycle histogram for a ramp stimulus; 28 cycles, 415 reversals. Note the presence of spontaneous reversals. (*b*) The interval between i^{th} and $(i + 1)^{th}$ reversal is reported against the phase of the i^{th} reversal. The variability of the intervals is lower during the ramp than in the rest period. On the top is shown the light stimulus. Rising time: 45 s, period: 85 s.

who reported a gradual decrease of the interval between following reversals during a linear ramp stimulus.

Fig. 7 reports on the left the interval histograms and on the right the cycle histograms for the same cell at three different slopes of the ramp stimulus. As in Fig. 6, the density of the reversals is almost constant in the stimulated part of the cycle and here the average frequency of the reversals increases with the slope of the ramp stimulus, as shown in Table 1.

RESPONSES TO COMPLEX STIMULI

We studied the responses to complex stimuli, obtained by superimposing blue flashes to a sinusoidally modulated blue light. Two light sources were used in this experiment and the superposition of the two beams was obtained by a beam splitter; flashes were delivered by opening a shutter placed in the path of the flash source.

TABLE 1

Rising time	dI/dt	Reversal frequency
(s)	(photons/$s^2 \cdot cm^2$)	(s^{-1})
20	3×10^{14}	0.20
40	1.5×10^{14}	0.14
80	0.7×10^{14}	0.09

Fig. 8 shows the cycle histograms of these responses. In Fig. 8, *a* and *b*, the period of the sinusoidal stimulus is 32 s. In Fig. 8 *a*, a flash was delivered at the phases shown by the arrows. The corresponding cycle histogram is practically the same of that obtained by only using the sinusoidal stimulus. In other words, in a large part of the cycle *H. halobium* does not respond to flashes. We call this effect the suppression effect. Flashes of this same energy are indeed always effective if carried out superimposed on a constant light level from zero to the maximum value of the sinusoidal stimulus. Fig. 8 *b* shows the cycle histogram obtained with flashes given at a phase near the point of maximum positive slope of the sinusoidal stimulus. A slight shift of the histogram towards the phase of the flash can be observed by comparing it with the previous one. Within a small phase range of the sinusoidal stimulus, flashes aid reversals, which otherwise would occur a bit later.

In Fig. 8, *c* and *d*, the period of the sinusoidal stimulus is 17 s. The histogram in Fig. 8 *d* (flash near the maximum slope of the sinusoidal stimulus) is slightly shifted from that of Fig. 8 *c*, obtained with flashes delivered near the minimum of the sinusoidal stimulus; this last histogram is equal to that without flashes. So, for both cases, the suppression effect occurs in the same phase range, independently of the period of the stimulus, showing that the phase of the stimulus is the determining parameter.

DISCUSSION

First, our data show that the spontaneous reversals do not exhibit any periodicity. Although it is possible that the same system exhibits different behaviors depending on the experimental conditions and on the values of the controlling parameters, we believe that the autocorrelation function we computed is the appropriate test for detecting an underlying periodicity.

A second conclusion is that the effective stimulus for

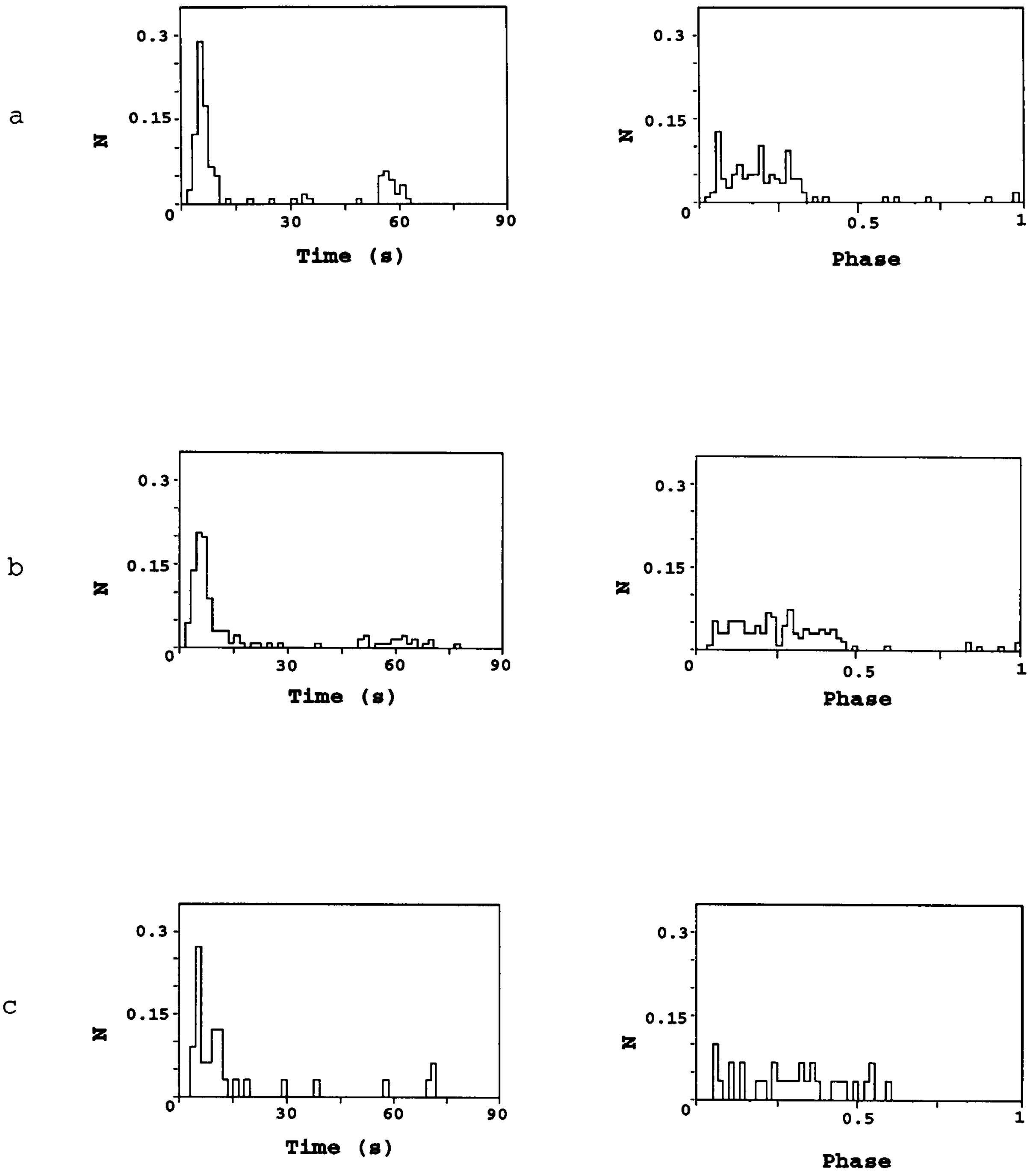

FIGURE 7 Cycle histograms (*right*) and interval histograms for the same individual cell at three different slopes of the ramp stimulus. (*a*) Rising time of 20 s, 123 reversals, 30 cycles; (*b*) rising time of 40 s, 138 reversals, 24 cycles; (*c*) rising time of 80 s, 35 reversals, 5 cycles. The rest time interposed between consecutive ramps is 50 s.

H. halobium is the time derivative of the stimulus signal. On the other hand, the results of experiments with pairs of flashes (Marwan and Oesterhelt, 1987), indicate that an integration of the stimulus takes place in the sensory transduction process; in their interpretation an unknown substance *Y*, which decays with its own time constant, is formed upon light absorption and a reversal occurs whenever *Y* reaches a threshold value. Moreover,

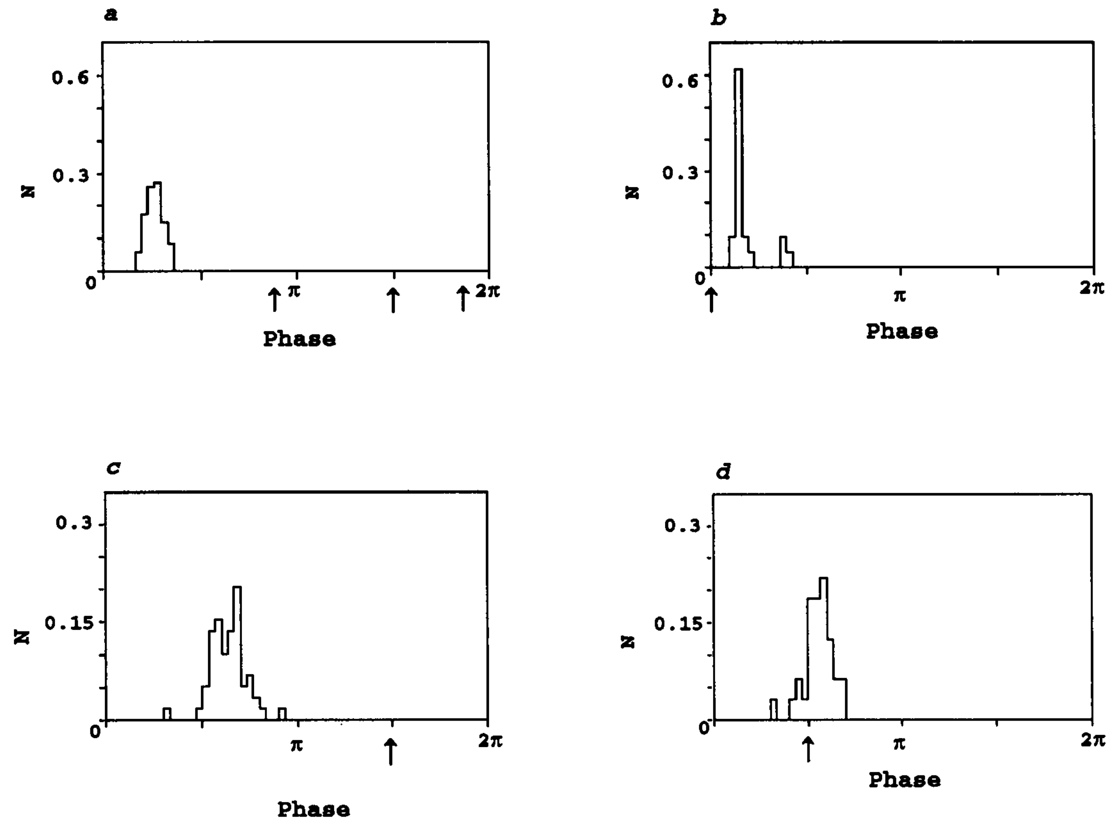

FIGURE 8 Cycle histograms obtained with flashes superimposed on sinusoidal stimuli with modulation depth equal to 0.9. The arrows show the phases at which flashes were delivered. In (*a* and *b*) the stimulus period was 33 s, and in (*c* and *d*) was 17 s. Data from two different cells. In (*a*) 80 reversals are collected by averaging 82 cycles of the sinusoidal stimulus; in (*b*) 21 reversals on 18 cycles. The second peak of the cycle histogram in (*b*) is due to three couples of reversals in the same cycle. In (*c*) 60 reversals on 67 cycles, in (*d*) 38 reversals on 41 cycles, are collected. Note the slight shift between histograms (*a* and *b*) and between histograms (*c* and *d*).

Y is set to a starting value at each reversal. In spite of the results obtained with sinusoidal stimuli (no correlation between the phases of reversals occurring in consecutive cycles), this interpretation looks logical and resetting to a starting value must be assumed to account for the responses to ramp stimuli.

However, our data show that the effectiveness of a stimulus to induce reversals depends on its time derivative. The scheme proposed by Marwan and Oesterhelt (1987) cannot account for the dependence of the responses on the time derivative of the stimulus or for the responses to complex stimuli. We suggest the following scheme to account for the data: upon light absorption the activated pigment can come back to its original form, or it can produce, before proceeding in the photocycle, an excitatory substance A or an inhibitory substance B (Mc Cain et al., 1987). The increase in time of A and B should be different, and the increase of B should occur later than that of A, so that the difference between A and B mimics the time derivative of the light stimulus. The excess of A over B is the effective stimulus, and the accumulation of A up to the threshold value determines the occurrence of a reversal. A and B could be thought of as having a great probability of combining together, so A could only exist in a free form if it exceeds B, as it occurs during the rising phase of a sinusoidal stimulus. Whereas during the falling, phase B exceeds A; so the free A goes to zero, introducing in the model a nonlinear feature which makes it possible to account for the lack of correlation between the phases of reversals occurring in consecutive cycles during sinusoidal stimulation. And we can imagine that during the falling phase of a sinusoidal stimulation, while A is near zero, B accumulates; so the system becomes insensitive to other stimuli, as it happens in the experiments with complex stimuli.

Schematically, the most important points of the proposed scheme are: the activation of the pigment and its photocycle, the activation of a transducer T (which could be the 94 kDa methyl-accepting membrane protein) and as the last step the production of the two antagonist species A and B.

Expressing these concepts more precisely, light absorption is supposed to start the following reactions:

$$S^*_{r610} + T \rightarrow T^* \rightarrow \text{other products}; \quad (1)$$

$$T^* + \text{substrate } 1 \rightarrow A \rightarrow \text{other products}; \quad (2)$$

$$T^* + \text{substrate } 2 \rightarrow B_1 \rightarrow B \rightarrow \text{other products}; \quad (3)$$

$$A + B \rightarrow AB \rightarrow \text{other products}. \quad (4)$$

Each of the products T^*, A, B, and AB is assumed to decay spontaneously. The two steps in reaction 3 and the intermediate B_1 are included to produce the delay in apparence of B compared to A. Reactions 2, 3, and 4 account for the dependence of the responses on the time derivative of the input signal, for the absence of correlation between phases of reversals occurring in consecutive cycles and for the responses to complex stimuli (Figs. 5 and 8). The curves in Fig. 9 were obtained by numerical solution of the system of differential equations reported below, which corresponds to the reactions 2, 3, and 4:

$$dA/dt = k_A T^*(t) - k_1 A - k_C AB;$$

$$dB_1/dt = k_A T^*(t) - k_1 B_1;$$

$$dB/dt = k_1 B_1 - k_2 B - k_C AB.$$

The computation was made by assuming a given time course for $T^*(t)$ (Fig. 9, *left*), and shows that such a scheme is suitable to account for some of the observed effects. In fact, the time course of A is almost independent of flashes delivered at phases where the A value is low (see Fig. 9, *b* and *c*), whereas the same flashes produce a large transient, when delivered on a constant light level (Fig. 9 *a*). This accounts for the suppression effect we observed and for the same reason for the absence of correlation between the phases of reversals occurring in successive cycles during periodic stimulation (Fig. 5).

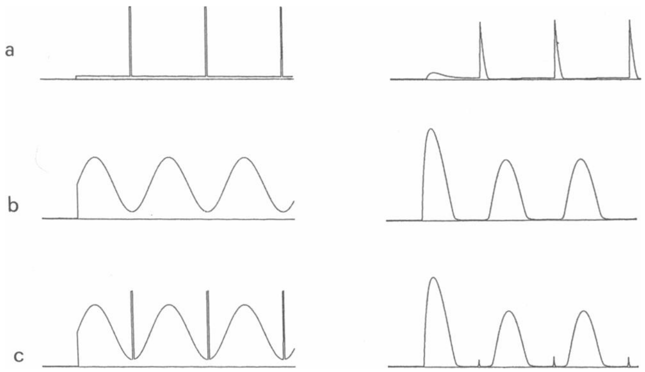

FIGURE 9 Time trend of the A species of the model for (*a*) repetitive flashes, (*b*) sinusoidal modulation, (*c*) sinusoidal modulation with superimposed flashes. Substance A reaches very low values. On the left side the time course of the "stimulus" $T^*(t)$ is shown. Model parameters: $k_A = k_1 = k_2 = 1\ s^{-1}$, $k_C = 0.1\ mol^{-1}s^{-1}$. Stimulus period: 10 s.

However, a complete model should account for the inverse effect of stimuli delivered in paticular conditions (Hildebrand and Schimz, 1987; Spudich et al., 1988) and for the dependence of the responses to sinusoidal stimuli on the modulation depth. Another effect to be accounted for is the methylation and demethylation occurring at the onset and offset of a light step (Spudich et al., 1989). The scheme could account for this last point by assuming that: (*a*) the light absorption produces, with different times, both the activation and the methylation of T; (*b*) the demethylation system is activated by both A and B. In fact, A or B is present at the beginning and at end of a light step, and so could account for methyl release.

Clearly the model parameters should be determined and the model should be tested by comparing it quantitatively with experimental data; work is in progress on this line. In its present formulation, the structure of this series of reactions makes it possible to account for the suppression of responses to flashes at unfavorable phases of sinusoidal stimuli and for the absence of correlation between the phases of consecutive reversals during sinusoidal stimulation.

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