QUANTUM EFFICIENCY OF LIGHT-DRIVEN PROTON EXTRUSION IN *HALOBACTERIUM HALOBIUM*

pH DEPENDENCE

MICHEL RENARD AND MICHEL DELMELLE, *Institut de Physique, Université de Liège, 4000 Sart Tilman, Belgium*

ABSTRACT The quantum yield for light-induced proton extrusion in *Halobacterium halobium* cells pretreated with an ATPase inhibitor was measured between pH 5 and 9 using two separate spectrophotometric techniques. The transmittance of the cell suspension was measured with a spectrometer equipped with "end-on" photomultipliers, whereas the reflectance was measured using a light-integrating sphere. The potentialities of the two techniques are critically compared. These measurements are used to evaluate the intensities of light absorbed by the cells. Since the initial rates of proton release into the extracellular medium were simultaneously measured, the quantum yield values [$QY(H^+)$] could be determined. The results obtained with the two techniques are in reasonable agreement. $QY(H^+)$ is 0.64 at pH 5.9 and decreases gradually to 0.28 at alkaline pH values.

INTRODUCTION

Under oxygen limiting conditions, *Halobacterium halobium* synthesizes the purple membrane which constitutes a specialized domain within the cell membrane (for a review, see reference 1). It is well established that bacteriorhodopsin (BR), the purple membrane protein, acts as a light-driven proton pump, ejecting protons from the cell interior into the extracellular medium and generating an electrochemical proton gradient (1–3). The light-induced proton extrusion has been described by several authors (2–6), but few studies were devoted to a determination of the quantum yield of the process [$QY(H^+)$] (7, 8). Such a determination implies the simultaneous measurements of the number of protons pumped per second [$n(H^+)$] for a given flux of photons absorbed [$n(\Phi)$]. Severe difficulties are encountered in the measurement of $n(H^+)$. They relate namely to the definition of the resting state of the cells and to the complexity of the light-induced pH changes since, in general, the proton efflux is tightly coupled to a proton influx. In anaerobic conditions, several influx pathways have been investigated: they are associated with passive leakage, ATP synthesis, and ion exchange processes (3, 6, 9).

In addition to the difficulties mentioned above, physical problems are also encountered in the measurement of $n(\Phi)$. The cells act as light-scattering centers so that the classical Beer-Lambert law cannot be applied to deduce the amount of light absorbed by the BR molecules. The present study emphasizes this latter point. $n(\Phi)$ was measured in two ways: experiments in the transmission mode were carried out with a spectrometer equipped with "end-on" photomultipliers, whereas measurements in the reflection mode were performed with a spectrometer equipped with an integrating sphere accessory. The potentialities of the

two techniques are critically compared. The corresponding quantum yields were determined in the particular case of suspensions of cells pretreated with the ATPase inhibitor, *N,N'*-dicyclohexylcarbodiimide (DCCD).

The influence of the extracellular pH on $QY(H^+)$ is examined in the second part of the paper. Surprisingly, to the best of our knowledge, this had not been studied systematically in the past. $QY(H^+)$ was measured in the 5 to 9 pH range and important variations were observed. Results are discussed in conjunction with what is presently known concerning the photochemical quantum yield of BR.

MATERIAL AND METHODS

Strain, Culture Conditions, and Sample Preparation

H. halobium strain R_1M_1, a mutant lacking gas vacuoles and bacterioruberin, was grown in shaking cultures under limited aeration at 40°C as described by Oesterhelt and Stoeckenius (10). Cells were harvested by centrifugation (10 min, 12,000 *g*, 4°C) ~90 h after the end of the exponential growth phase. Cells from 20 ml of culture medium were resuspended in 4.5 ml of basal salt solution containing 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, and 2 g KCl per liter. The pH of the suspension was adjusted to the desired initial value by addition of NaOH or HCl in 4 M NaCl solution. The protein concentration, determined by the Lowry method (11), was usually 1.5 mg/ml. To inhibit the membrane-bound ATPase, the cells were incubated in anaerobic conditions in the presence of 1 mM *N,N'*-dicyclohexylcarbodiimide (DCCD) for 3–5 h before the start of the measurements since, in our hands, the light-induced pH changes were maximum after such an incubation period.

Spectrophotometric Measurements

Two double-beam spectrophotometers were used: a Shimadzu MPS-50L equipped with "end-on" photomultipliers (Shimadzu Seisakusho Ltd., Kyoto, Japan) and a Cary 14 equipped with an integrating sphere (accessory 1411750) (Varian Associates, Palo Alto, Calif.). A light-integrating box coated with MgO was placed in the reference compartment of the latter spectrometer. The baseline was adjusted with a cylindrical cuvette filled with $MgCO_3$ powder. For the Shimadzu spectrometer, the baseline was adjusted with rectangular cuvettes filled with basal salt solution. The BR spectrum was obtained by subtracting the spectrum of the bleached suspension from the corresponding spectrum of the unbleached suspension. Bleaching was achieved after 8 h of illumination (lamp HBO 500, OSRAM GmbH, Berlin, West Germany; OG530 Schott filter; Schott Inc., N.Y.) in presence of 0.3 M hydroxylamine (12).

pH Measurements, Illumination Conditions, and Actinometry

The light-induced pH changes occurring in the extracellular medium were monitored electrometrically with a glass electrode (Beckman Instruments, Inc., Fullerton, Calif.; model Futura, 5 mm) attached to a Beckman 3500 digital pH meter. The pH meter was connected to a Varian G14 strip chart recorder. The buffer capacity of a suspension was determined by adding small aliquots of HCl in 4 M NaCl solution. Anaerobic conditions were maintained by passing nitrogen over the surface of the suspension. The cuvettes were sealed with parafilm except for small holes for the pH electrode and for the agitation glass rod. Sample illumination was performed with an OSRAM HBO 500 lamp. Light was filtered either through an interference Schott filter (SKF 22, λ_{max} = 575 nm, bandwidth 25 nm) or through a cut-off Schott filter (OG530). The light source was 25 cm away from the sample. Experiments were performed at 22°C. Since the first illumination of a suspension produced different pH variations from those obtained in subsequent illuminations, the suspensions were pre-illuminated during a period of 5 min followed by 10 min dark before the start of the recordings. Two experimental set-ups were used: the sample was illuminated either in the Shimadzu cuvette holder or in the integrating sphere. The incident light intensity was measured either with a Kipp and Zonen thermopile (Kipp and Zonen, Delft, The Netherlands) or by ferrioxalate chemical actinometry (13). Both methods yielded similar results. In the

case of the actinometric technique, since the quantum yield for Fe^{2+} production varies significantly in the 550–610 nm region, the incident light intensity was calibrated at 445 nm with an interference filter. The intensity in the 550–610 nm region was evaluated on the basis of the lamp emission spectrum, knowing the absolute intensity at 445 nm. Chemical actinometry yielded more precise results than thermopile measurements in reflectance geometry due to the geometrical arrangement of the integrating sphere.

RESULTS

Measurement of n(Φ)

Fig. 1 shows the visible spectra of a suspension of bacteria (1.5 mg of protein/ml) recorded in the transmission (T) and in the reflection (R) modes. The spectra were measured before (u) and after (b) bleaching of the suspension in the presence of 0.3 M hydroxylamine. The 570-nm peak of the unbleached suspension is more intense in the transmission mode and accordingly the reduction of intensity due to bleaching is also more pronounced. Moreover, at 420 nm the transmission spectrum presents a shoulder which is less pronounced in the reflection spectrum. Strictly speaking, these spectra are not readily comparable since the optical paths differ in the two measurements (Fig. 2). In fact, light scattering renders the definition of the light path imprecise. Hence, the applicability of the Beer-Lambert law becomes questionable and the determination of $n(\Phi)$ raises a serious problem. Classically, the absorbance of a solution is defined by the logarithm of the ratio between the incident and the parallel transmitted light. With light-scattering material, the concept of attenuance must be introduced, since both absorption and scattering are responsible for the attenuation of the

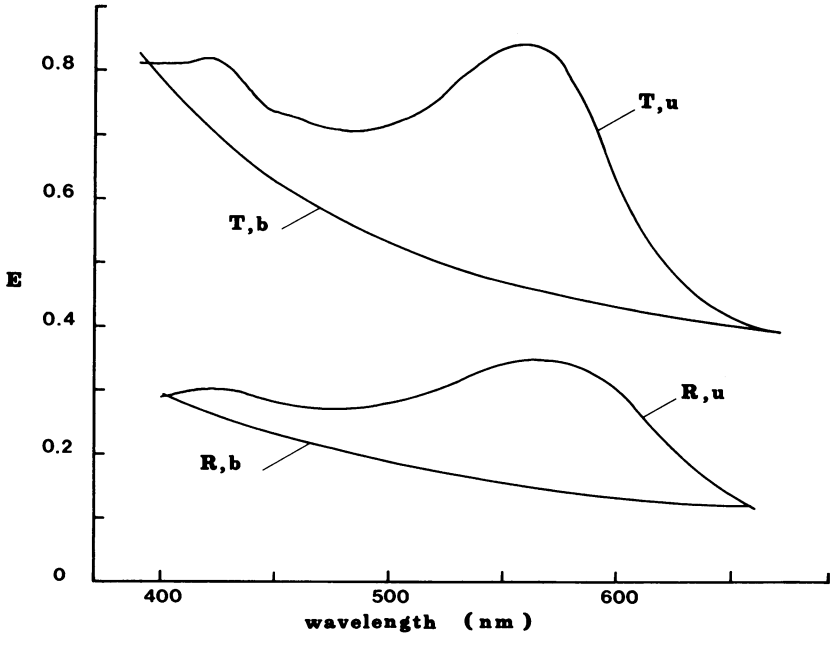

FIGURE 1 Attenuation spectra (see Eq. 4) of *H. halobium* (R_1M_1 strain) in basal salt suspension (1.5 mg protein/ml). The spectra were recorded in the transmission (T) and reflection (R) modes before (u) and after (b) extensive bleaching of the pigment.

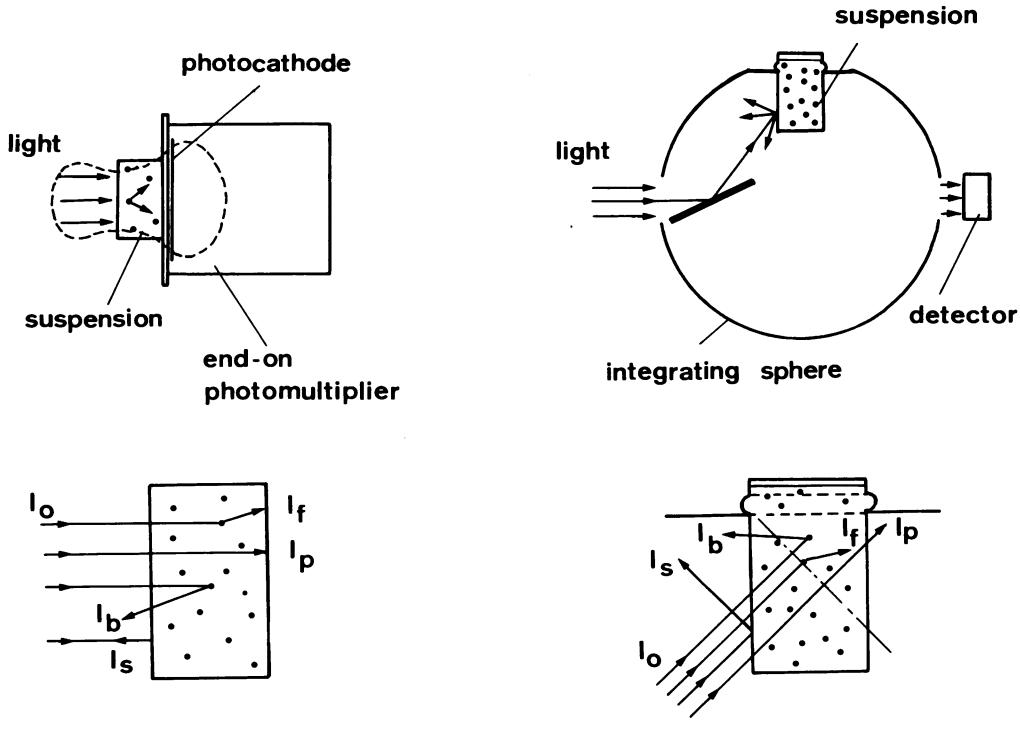

FIGURE 2 Diagram of the transmittance (left) and reflectance (right) detection systems. (*Upper left*) The "end-on" photomultiplier placed against the cuvette collects the parallel transmitted light (I_p) and a large fraction of the diffused transmitted light (I_f). The broken curve describes the intensity distribution of the diffused light (I_d) as a function of the direction (reference 16). (*Lower left*) Light path in the cell suspension and definition of the various components. I_0 = incident, I_s = specularly reflected, I_b = diffused in the backward direction (reflected), and I_f = diffused in the forward direction (transmitted). The total diffused light $I_d = I_b + I_f$. (*Upper right*) Diagram of the diffuse reflectance accessory. The detector of the integrating sphere collects the reflected light (I_b) and the fractions of I_f and I_p which do not leak through the sample port. (*Lower right*) The symbols are defined as in the transmission mode. The incident beam is not perpendicular to the cuvette wall. Hence I_s ceases to be negligible.

parallel light beam (14). We shall examine the conditions under which $n(\Phi)$ may be evaluated from attenuance measurements.

IN THE TRANSMISSION MODE The incident light (I_0) can be decomposed into the absorbed (I_a), the reflected (I_r), and the transmitted (I_t) components (14):

$$I_0 = I_a + I_r + I_t. \tag{1}$$

Reflectance is defined by I_r/I_0 and transmittance by I_t/I_0. Actually, I_t represents the sum of the parallel transmitted light (I_p) and of the diffused transmitted light (I_f). I_r also includes two components: the specularly reflected light (I_s) and the diffused reflected light (I_b). Eq. 1 can thus be rewritten:

$$I_0 = I_a + I_s + I_b + I_p + I_f. \tag{2}$$

Eq. 2 can be used to define various kinds of attenuance which enable the transmittance to be

estimated depending upon the experimental conditions (14). Our goal is, however, slightly different since we merely seek to derive a relationship between I_a and I_0. To do so, we found it useful to decompose the total diffused light (I_d) into the detected (I_m) and the undetected (I_n) components. Obviously,

$$I_d = I_f + I_b = I_m + I_n. \tag{3}$$

The amount of light which reaches the photomultiplier is thus $I_m + I_p$, and hence the measured attenuance can be written:

$$E_t = \log (I_0/I_p + I_m). \tag{4}$$

Under our experimental conditions, two approximations can be made: (*a*) the light reflected on the surface of the cuvette (I_s) is negligible since I_0 is normal to this surface (13). (*b*) $I_p/I_0 < 0.01$. The justification for this approximation is based on the following observation: in a conventional spectrophotometer, the distance from the cuvette to the photomultiplier is large ($d > 10$ cm). Consequently, the diffused light which is detected by the photomultiplier is negligible since the corresponding solid angle is very small ($I_m/I_d < 1\%$). However, under those conditions, the attenuance of the suspension is larger than 2. It follows from Eq. 4 that the parallel light transmitted through the suspension represents $<1\%$ of I_0.

Using these approximations, the attenuance of the unbleached suspension (E_t^u) can be written:

$$E_t^u = \log (I_0/I_m^u) = \log (I_0/I_0 - I_a - I_n^u). \tag{5}$$

After bleaching, $I_a = 0$ for R_1M_1 bacteria and the corresponding attenuance is given by:

$$E_t^b = \log (I_0/I_m^b) = \log (I_0/I_0 - I_n^b). \tag{6}$$

At any wavelength of the BR absorption band, the difference in attenuance due to bleaching is thus:

$$\Delta E_t = E_t^u - E_t^b = \log (I_0 - I_n^b/I_0 - I_a - I_n^u). \tag{7}$$

Note that although the amounts of light detected are different for the unbleached and bleached samples ($I_m^u \neq I_m^b$), it is reasonable to assume that the fraction of the diffused light detected remains constant:

$$I_m^u/I_d^u = I_m^b/I_d^b = \alpha. \tag{8}$$

Indeed, α depends only on the geometry and size of the scattering particles (15). Since bacteria are large ($\simeq 5$ μm) with respect to the wavelength of illumination ($\simeq 0.5$ μm), the scattering is anisotropic (16). Fig. 2 gives the relative intensities of the diffused light as a function of the direction. Under our experimental conditions, I_m represents the light diffused within a cone of about 120° aperture. Taking this estimation into account, α could be evaluated by integration of the curve shown in Fig. 2 ($\alpha = 0.65$).

Dividing the second term of Eq. 7 by I_0 and using the definition of α and Eq. 3, ΔE_t can be written:

$$\Delta E_t = \log \frac{1 - (1 - \alpha)\,(I_d^b/I_0)}{1 - (I_a/I_0) - (1 - \alpha)\,(I_d^u/I_0).} \tag{9}$$

Since $I_d^b = I_m^b/\alpha$ and $I_d^u = I_m^u/\alpha$, it follows from Eqs. 5 and 6 that:

$$\Delta E_t = \log \frac{1 - [(1 - \alpha)/\alpha] \times 10^{-E_t^b}}{1 - [(1 - \alpha)/\alpha] \times 10^{-E_t^u} - I_a/I_0}. \tag{10}$$

After transformation, a simple relationship between I_a and I_0 is obtained:

$$I_a = I_0 \times (1 - 10^{-\Delta E_t}). \tag{11}$$

IN THE REFLECTION MODE The geometry of the diffuse reflectance accessory is also shown in Fig. 2. A theoretical analysis of the integrating sphere has been performed by Jacquez and Kuppenheim (17) and O'Brien (18). The theory which yields a relationship between the incident light (I_0), the detected light (I_m), and the reflectance of the sample, is strictly valid when the surface of the sample lies on a continuation of the sphere wall and for a sample reflecting the light in a perfectly diffuse manner. Our system differs greatly from such an ideal case: namely, (*a*) the sample is contained in a cylindrical cuvette; (*b*) the scattering does not obey the Rayleigh law because of the size of the particles; and (*c*) a fraction of I_0 leaks out of the sphere through the sample port.

In any case, an accurate determination of the reflectance would be very complex, requiring an extensive theoretical analysis. As emphasized above, we only intend to derive a relationship

TABLE I
NUMBER OF PHOTONS ABSORBED PER SECOND BY THE CELL SUSPENSION [$n(\Phi)$]

Mode	λ	$I_i(\lambda)$	$\theta(\lambda)$	$I_0(\lambda)$	$\Delta E(\lambda)$	$I_a(\lambda)$
	(nm)	*(arb. units)*		*(× 10^{-14} photons/s)*		*(× 10^{-14} photons/s)*
Transmittance	550–560	25	0.07	17.9	0.375	10.4
	560–570	9	0.35	30.9	0.375	17.9
	570–580	72	0.35	253.0	0.355	141.3
	580–590	40	0.18	70.5	0.310	36.0
	590–600	8	0.09	7.2	0.240	3.1
	600–610	4.5	0.03	1.4	0.175	0.5
						$n(\Phi) = 2.09 \times 10^{16}$ photons/s
Reflectance	520–530	4	0.25	1.5	0.150	0.4
	530–540	6	0.61	5.7	0.165	1.8
	540–550	95	0.77	116.4	0.175	38.6
	550–560	25	0.82	33.4	0.190	11.8
	560–570	9	0.86	12.8	0.195	4.6
	570–580	72	0.88	106.9	0.195	38.7
	580–590	40	0.89	61.1	0.185	21.2
	590–600	8	0.90	12.6	0.165	4.0
	600–610	4.5	0.90	7.2	0.145	2.0
	610–620	4	0.91	6.6	0.115	1.5
	620–630	4	0.91	6.7	0.080	1.1
	630–640	4	0.91	6.8	0.045	0.7
	640–650	4	0.91	6.9	0.020	0.3
	650–660	4	0.91	7.0	0.010	0.2
						$n(\Phi) = 1.27 \times 10^{16}$ photons/s

$I_i(\lambda)$, relative intensities of the lamp over 10 nm intervals. $\theta(\lambda)$, mean transmittances of the filter. $I_0(\lambda)$, relative incident intensities. $\Delta E(\lambda)$, differences in attenuance due to bleaching (see Eq. 7). $I_a(\lambda)$, light intensities absorbed by the cell suspension.

between I_a and I_0. In fact, a direct transposition of the analysis performed in the transmission mode is feasible, provided that: (*a*) the specularly reflected light (I_s) is no longer negligible, since I_0 is not perpendicular to the surface of the cuvette. However, after reflection, I_s will reach the sphere wall and this amount of light will eventually become part of the diffused light I_d. (*b*) I_p, the parallel transmitted light, is again negligible, for reasons similar to those developed in the transmission mode treatment. (*c*) The undetected diffused light (I_n) represents the amount of light lost through the sample and the entrance ports as well as the light absorbed by the sphere wall. The fraction of the diffused light that remains undetected ($1 - \alpha$) is assumed to be constant for the unbleached and bleached samples. The justification for this approximation has also been given above. However, α is more difficult to evaluate for the integrating sphere.

It follows from an analysis similar to the one carried out in the transmission mode that if ΔE_r represents the difference in attenuance due to bleaching, the amount of light absorbed by the BR molecules is expressed by:

$$I_a = I_0 \times (1 - 10^{-\Delta E_r}). \tag{12}$$

INTEGRATION Eqs. 11 and 12 have to be integrated over the wavelength domain of illumination. In the transmission mode, the experiments were carried out with an interference filter (λ_{max} = 575 nm) and the integration domain extends from 550 to 610 nm, which are the cut-off wavelengths of the filter. Since

$$I_0(\lambda) = I_i(\lambda) \times \theta(\lambda), \tag{13}$$

where $I_i(\lambda)$ is the spectral intensity of the lamp and $\theta(\lambda)$ the transmission spectrum of the filter, it follows that:

$$n(\Phi) = \int_{550}^{610} I_a(\lambda)d\lambda = \int_{550}^{610} I_i(\lambda) \times \theta(\lambda) \times (1 - 10^{-\Delta E_r})\, d\lambda. \tag{14}$$

The results leading to the determination of $n(\Phi)$ are given in Table I. The wavelength domain to consider is defined by the first column. The second column gives the relative intensities of the lamp [$I_i(\lambda)$] over 10-nm intervals. These values were obtained by integration of the lamp spectrum. The mean transmittance of the filter [$\theta(\lambda)$] was measured over these intervals (column 3) and the relative incident intensities [$I_0(\lambda)$] were evaluated according to Eq. 13. Since the light flux which reached the sample was measured either with the thermopile or by chemical actinometry, the relative incident intensities were directly convertible into absolute values (column 4). The mean values of ΔE_t over these intervals (column 5) are then used to compute the absorbed intensities $I_a(\lambda)$ (column 6). Finally, $n(\Phi)$ is obtained by summation of the $I_a(\lambda)$ values. In every experiment it was ensured that the light intensity was sufficient for producing a measurable pH change. For this reason, the measurements in the reflection mode had to be carried out with an OG 530 cut-off filter instead of the interference filter. Under such conditions, the integration has to be carried out between 520 nm, the cut-off wavelength of the filter, and 660 nm, the tail of the BR absorption band ($\Delta E_{660} < 0.01$). This procedure is very similar to that followed in the transmission mode. Results of a typical experiment are also given in Table I. Obviously, the results obtained with the two techniques are not directly comparable, since the optical systems are very different.

Determination of $n(H^+)$

The number of protons extruded per time unit from the illuminated cells [$n(H^+)$] must be measured in a geometry which is identical to that used for the determination of $n(\Phi)$. Hence, two sets of experiments had to be carried out: with the transmission and with the reflection geometries, respectively. Fig. 3 presents some typical results obtained in the transmission mode. These results concern freshly harvested cells maintained anaerobically for 3 h in the dark but preilluminated for 5 min and kept 10 more min in darkness before the start of the recordings. The dashed trace describes the pH change induced within the extracellular fluid by a continuous illumination. At the onset of the illumination, a transient alkalinization occurs which raises the pH from an initial value of 6.8 to 7.0. The transient is followed by a net acidification. These pH changes were reversed when the light was shut off. This response compares favorably to other ones previously described (2, 4). Clearly, the transient phenomenon makes the determination of $QY(H^+)$ very difficult. Bogomolni et al. (4) have demonstrated that the presence of an ATPase inhibitor such as DCCD abolishes the transient. The solid trace in Fig. 3 was recorded from a suspension of cells incubated with this chemical at a concentration of 1 mM. All the $QY(H^+)$ values reported in the present paper were obtained with this kind of preparation. It can be seen in Fig. 4 *A* that, in these conditions, the light-induced acidification follows a first-order kinetics.

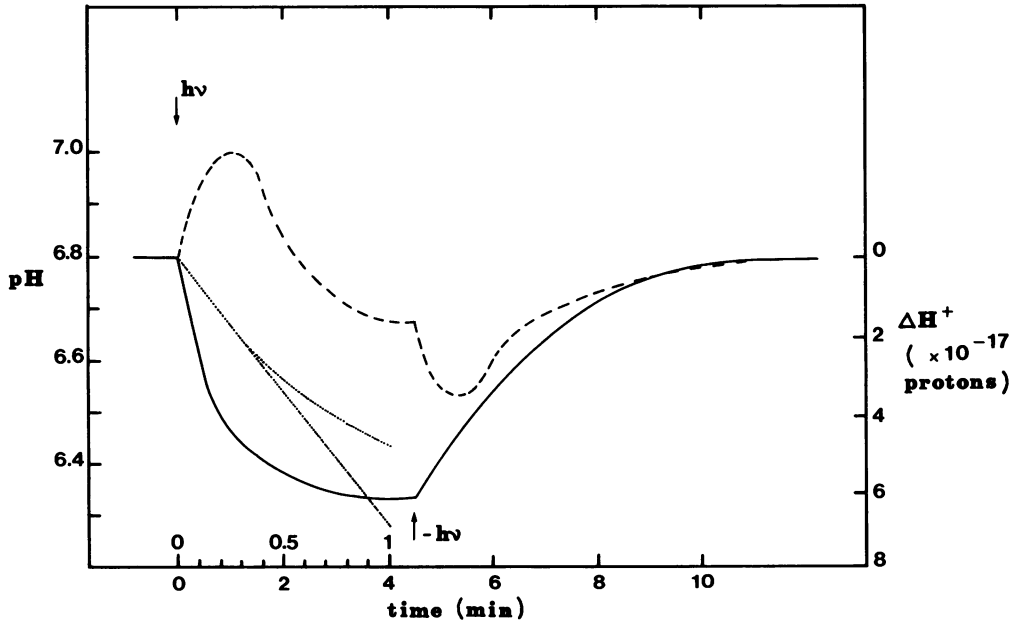

FIGURE 3 Light-induced pH changes in anaerobic suspensions of *H. halobium* cells (R_1M_1). Responses of an untreated suspension (----) and of a suspension treated with 1 mM DCCD (—). Illumination is continuous: light is turned on at time zero and lasts for 4.5 min (monochromatic light λ_{max} = 575 nm, intensity = 3.8 × 10^{16} photons/s, transmittance geometry). The early pH changes (dotted curve) are presented on a time-expanded plot (time scale from 0 to 1 min). The right-side ordinate converts these early pH changes into the number of H^+ pumped, taking into account the buffer capacity of the suspension. The dotted line is the tangent to the curve and gives the initial rate of H^+ pumping.

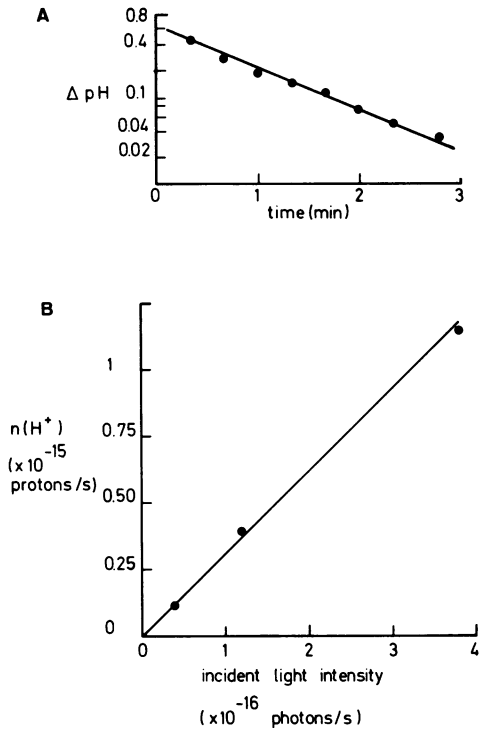

FIGURE 4 (*A*) Semilogarithmic plot of the light-induced pH changes occurring in an anaerobic suspension of R_1M_1 cells as a function of illumination duration. ΔpH is the difference between the actual and the asymptotic pH values. The experimental conditions are the same as in Fig. 3. (*B*). Relationship between the initial rate of light-induced proton extrusion and the intensity of the incident light. The neutral density filters used reduced I_0 by 0.5 and 1.0 log unit, respectively. Experimental conditions are the same as in Fig. 3.

The sustained pH value reached after several min of illumination (pH = 6.37 in Fig. 3) expresses a steady state between the inflow and the outflow of protons. The initial pH variations were recorded on a faster time scale and a higher sensitivity (dotted curve). The tangent to the curve (dotted line) gives the rate of proton extrusion [$n(H^+)$], while the difference between the curve and its tangent is associated with the H^+ inflow. After an illumination period of 30 s, the pH has decreased by 0.05 unit. To convert these variations into the number of protons pumped (ΔH^+), the buffer capacity of the medium had to be taken into account (see Material and Methods). The corrected results are expressed on the right-side ordinate of Fig. 3.

Fig. 4 *B* demonstrates that the number of protons pumped per second [$n(H^+)$] is linearly related to the intensity of the incident light. These data were obtained by inserting various neutral filters within the illumination beam.

Experiments similar to those described above were also performed using the integrating sphere. Table II shows that the QY(H^+) values obtained with the transmission and reflection techniques are in good agreement.

TABLE II
QUANTUM YIELD QY(H^+)*

Mode	$n(\Phi)$	$n(H^+)$‡	QY(H^+)
	($\times 10^{-16}$ photons/s)	($\times 10^{-15}$ protons/s)	
Transmittance	2.16§	1.15	0.53
	2.09‖	1.15	0.55
Reflectance	1.27§	0.655	0.52
	1.30‖	0.655	0.50

*The pH of the cell suspension is 6.8.
‡The buffer capacity of the suspension is determined by addition of 50-μl aliquots of HCl pH 2.35 in 4 M NaCl.
§The incident light intensity I_0 is measured by ferrioxalate chemical actinometry.
‖I_0 is determined with a thermopile.

Influence of the Initial pH Value on QY(H^+)

Fig. 5 shows the dependence of QY(H^+) upon the extracellular pH of the dark-adapted suspension. Results obtained with the two techniques are combined in the figure. At pH lower than 6.6, QY(H^+) is large, the maximum value being 0.64. By contrast, at alkaline pHs, the QY(H^+) values are systematically small. The destruction of the bacteria cannot be invoked to explain these variations, since some determinations were carried out with the same preparation, first at high pH and afterwards at lower pH values: systematically, the results corresponding to the acid pHs were in the 0.60 to 0.64 range.

The evaluation of the quantum yields implies the simultaneous measurements of the buffer capacities of the suspensions. This parameter was measured from pH 5.1 to 8.8. The results

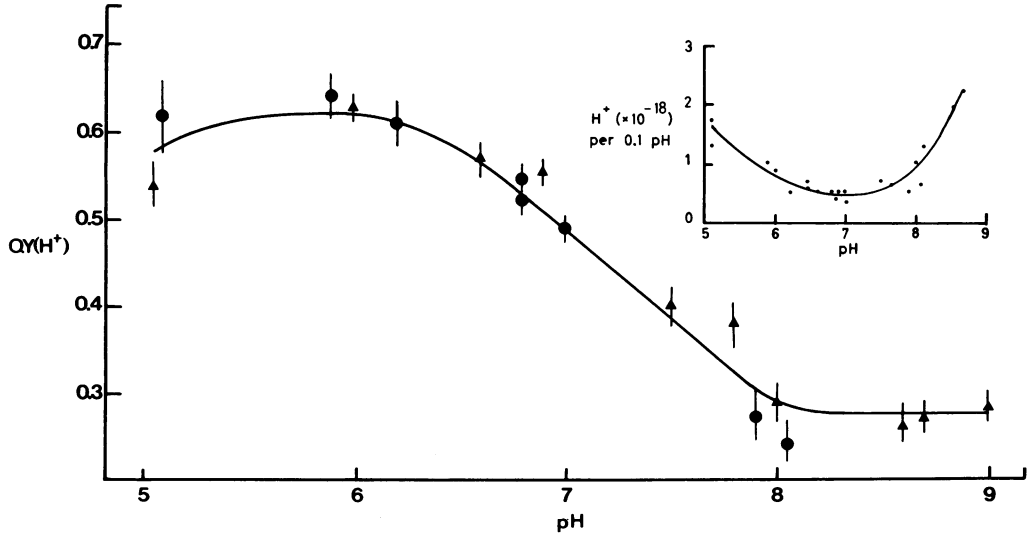

FIGURE 5 pH dependence of proton pumping quantum efficiency in *H. halobium*. Cells were in basal salt suspension incubated anaerobically with 1 mM DCCD. The results were obtained in transmittance (▲) and reflectance (●) modes, respectively. Each point represents the mean value of at least three measurements. Inset: relationship between the buffer capacity of the suspensions and the extracellular pH. The ordinate gives the number of H^+ necessary to produce a pH shift of 0.1 unit.

are shown in the inset of the figure. From pH 6.0 to 8.0, the buffer capacity remains virtually constant, but increases sharply outside this range.

DISCUSSION

This paper emphasizes the physical problems encountered in the determination of $QY(H^+)$. They originate from the limitations of the spectrophotometric techniques once they are applied to the study of light-scattering material. The framework of a general discussion of this question has been provided by Shibata (14, 19), Jacquez and Kuppenheim (17), O'Brien (18), and Butler (20). However, in the context of the determination of $QY(H^+)$ in *H. halobium,* this question had not been examined in detail. Our analysis emphasizes the difficulties associated with the determination of the absolute values of transmittance and reflectance. In the latter case, systematic errors due to multiple quantum hits and to imperfect reflectivity of the sphere wall should be considered. Note that the integrating sphere used in this work does not differ markedly from that used by some other investigators (7, 20). In our experimental system, the amount of diffused light which remains undetected (I_n) is simply slightly larger than in an Ulbricht sphere due to the small loss occurring through the sample port. Our analysis shows that the determination of the amount of light absorbed by the BR molecules [$n(\Phi)$] implies only the measurement of an attenuance difference (ΔE) provided that some simple assumptions are made. They can be summarized as follows: (*a*) the amount of parallel light transmitted through the samples is negligible; and (*b*) the fraction of the diffused light that is undetected remains constant whatever the bleaching state of the preparation. These assumptions seem to be justified in our experimental conditions. From a practical point of view, we tend to favor the transmission technique since its sensitivity is good, the theoretical analysis is straightforward, and the experimental approach is simple. The results obtained with the two techniques are in reasonable agreement, although the spectrophotometric methods as well as the illumination conditions (mono- and polychromatic) were very different. This seems to guarantee their reliability.

On the other hand, it must be emphasized that our determinations of $QY(H^+)$ concern a particular physiological situation, since all the measurements were performed with DCCD-treated cells. The preparations were kept under well-controlled experimental conditions (anaerobic incubation, pre-illumination), but the "resting state" of the cells remains physiologically poorly defined (1). The good reproductibility observed from one experiment to another demonstrates, however, that the preparations were in a steady state.

Few evaluations of quantum efficiency have been reported in the literature. The experiments of Hartman et al. (7) concerned R_1 and R_1M_1 cells incubated at pH 6.6 with phloretin and illuminated with monochromatic light in an Ulbricht integrating sphere. The $QY(H^+)$ values range from 0.4 to 0.6. On the other hand, Bogomolni reported a value of 0.6 to 0.7 (21, 22). More recent results from the same group were obtained at pH 5.0 or in presence of uncouplers, nigericin, or DCCD: the values range from 0.45 to 0.63 (8). Our low pH results are in good agreement with the largest of those reported values. Also note that our observations concerning the pH dependence of the buffer capacity agree with the results reported by Danon and Caplan (6).

Stoeckenius et al. (1) have emphasized the difficulties associated with an accurate determination of $QY(H^+)$ in living cells. They relate to the pre-existing proton gradient and to

the presence of leaky cells. In addition, several assumptions are implied, namely that no permeability change occurs upon illumination and that the blocking of ATPase does not affect the purple membrane (1). Concerning the first of these points, Michel and Oesterhelt (23) have shown that, in presence of DCCD, the transmembrane pH gradient of dark adapted cells remains virtually constant (0.4 pH unit) between pH 6.0 and 8.0. Since the potential gradient $\Delta\psi$ is also constant in these conditions, it follows that $\Delta\mu_{H^+}$ is pH-independent (23). Such a conclusion is important with regard to our observations, since it tends to demonstrate that the pH dependence of $QY(H^+)$ does not result from a variation of $\Delta\mu_{H^+}$. Actually, this pH dependence is not entirely unexpected: Bogomolni has suggested that the pH might act as a regulator of the proton pumping rate (22), and has observed that the BR photoreaction cycle is much slower at pH 8.5 than at pH 6.5 in intact bacteria (4). This finding could explain our observations.

Because of the difficulties encountered with intact cells in the $QY(H^+)$ determination, experiments with several models were undertaken by different groups. Stoeckenius et al. (1) reported a $QY(H^+)$ of 0.64 to 0.67 for envelope vesicles in KCl solution. On the other hand, Kanner and Racker (24) found that the number of H^+ translocated in illuminated vesicles is about six times larger at acid than at neutral pHs. Similarly, Eisenbach et al. (25) investigated subbacterial particles and found that the pH changes associated with the net H^+ efflux increase considerably with decreasing pHs. Identical results were obtained with proteoliposomes (25). On the contrary, with this latter model, Racker and Stoeckenius (3) found that the absolute values of H^+ uptake show little variations between pH 5.2 and 7.4. Finally, in the case of the simple model consisting of purple membrane suspension, Ort and Parson (26) measured a quantum efficiency of 0.43 at high ionic strength. This value was found to be pH independent between 6.0 and 8.75. This observation contradicts the results of Garty et al. (27) as well as those of Oesterhelt and Hess (28).

Whatever may be the explanation of this discrepancy, we may nevertheless conclude that, in general, our results agree reasonably well with the data previously published. The pH influence on $QY(H^+)$ has not yet been reported in the case of intact cells, but similar trends have been observed with several model systems (24, 25, 27, 28).

It is also worth discussing our findings in relation to the photochemical quantum yield of the BR. An early result of Oesterhelt and Hess (28) gave a value of 0.79 in the case of ether-treated purple membranes suspended in basal salts. However, more recent determinations from different groups yielded another value: a quantum efficiency of 0.3 has been obtained under various experimental conditions (29–32). If this value remains unchanged in vivo and is pH independent, our results would imply that at low pHs, two H^+ are released in the extracellular fluid per photochemical cycle, whereas at high pHs, only one H^+ would be extruded. Actually, it has been suggested recently (8, 26, 33) that two H^+ might indeed be pumped per photochemical cycle at high ionic strength. This conclusion would be very important in our understanding of the pumping mechanism of the BR. Experiments are currently underway in our laboratory to investigate this latter possibility.

We are grateful to Dr. N. A. Dencher (Biozentrum, Basel, Switzerland) for his generous gift of the R_1 and R_1M_1 strains. We wish to thank Professor A. Van de Vorst for his interest in this work.

Dr. Renard would like to thank Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture for financial support.

Received for publication 3 April 1980 and in revised form 11 August 1980.

Note added in proof: During the review of this manuscript, two important papers were published. Govindjee et al. (1980, *Biophys. J.*, **30**:231–242) measured the quantum yield [QY(H^+)] in purple membrane sheets. The values reported range from 0.64 to 0.70. They also measured the ratio between the number of protons pumped and the amount of intermediate M412 formed. At high ionic strength, this ratio is 1.8. On the other hand, Bogomolni et al. (1980, *Biochemistry,* **19**:2152–2159) provided a detailed study of the measurement of QY(H^+) in intact R_1 cells and in cell envelopes. The QY(H^+) values range from 0.4 to 0.7. From these results, the authors suggest that more than one proton is pumped during the BR photocycle.

REFERENCES

1. STOECKENIUS, W., R. H. LOZIER, and R. A. BOGOMOLNI. 1979. Bacteriorhodopsin and the purple membrane of Halobacteria. *Biochim. Biophys. Acta.* **505**:215–278.
2. OESTERHELT, D., and W. STOECKENIUS. 1973. Functions of a new photoreceptor membrane. *Proc. Natl. Acad. Sci. U.S.A.* **70**:2853–2857.
3. RACKER, E., and W. STOECKENIUS. 1974. Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. *J. Biol. Chem.* **249**:662–663.
4. BOGOMOLNI, R. A., R. A. BAKER, R. H. LOZIER, and W. STOECKENIUS. 1976. Light-driven proton translocations in *Halobacterium halobium. Biochim. Biophys. Acta.* **440**:68–88.
5. BAKKER, E. P., H. ROTTENBERG, and S. R. CAPLAN. 1976. An estimation of the light-induced electrochemical potential difference of protons across the membrane of *Halobacterium halobium. Biochim. Biophys. Acta.* **440**:557–572.
6. DANON, A., and S. R. CAPLAN. 1976. Stimulation of ATP synthesis in *Halobacterium halobium* R_1 by light-induced or artificially created proton electrochemical potential gradients across the cell membrane. *Biochim. Biophys. Acta.* **423**:133–140.
7. HARTMANN, R., H.-D. SICKINGER, and D. OESTERHELT. 1977. Quantitative aspects of energy conversion in Halobacteria. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **82**:1–6.
8. BOGOMOLNI, R. A., R. H. LOZIER, G. SIVORINOVSKY, and W. STOECKENIUS. 1979. Is more than one proton pumped in the bacteriorhodopsin photocycle? *Biophys. J.* **25**:318*a*. (Abstr.)
9. LANYI, J. K., and R. E. MACDONALD. 1977. Light-dependent cation gradients and electrical potential in *Halobacterium halobium* cell envelope vesicles. *Fed. Proc.* **36**:1824–1827.
10. OESTERHELT, D. and W. STOECKENIUS. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol.* **31A**:667–678.
11. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
12. BECHER, B., and J. Y. CASSIM. 1977. Effect of bleaching and regeneration on the purple membrane structure of *Halobacterium halobium. Biophys. J.* **19**:285–297.
13. HATCHARD, C. G., and C. A. PARKER. 1956. A new sensitive chemical actinometer. II. Potassium ferrioxalate as a standard chemical actinometer. *Proc. R. Soc. Lond. Ser. A. Math. Phys. Sci.* **235**:518–536.
14. SHIBATA, K. 1959. Spectrophotometry of translucent biological materials. Opal glass transmission method. *Meth. Biochem. Anal.* **7**:77–109.
15. TANFORD, C. 1961. Physical Chemistry of Macromolecules. John Wiley & Sons, Inc., New York, London. 275–316.
16. VAN HOLDE, K. E. 1971. Physical Biochemistry. Prentice-Hall Inc., Englewood Cliffs, New Jersey. 180–201.
17. JACQUEZ, J. A., and H. F. KUPPENHEIM. 1955. Theory of the integrating sphere. *J. Opt. Soc. Am.* **45**:460–470.
18. O'BRIEN, P. F. 1956. Network representation of the integrating sphere. *J. Opt. Soc. Am.* **46**:343–345.
19. SHIBATA, K. 1962. Spectrophotometry of opaque biological materials. Reflection methods. *Meth. Biochem. Anal.* **9**:217–234.
20. BUTLER, W. L. 1964. Absorption spectroscopy *in vivo:* theory and application. *Annu. Rev. Plant Physiol.* **15**:451–470.
21. BOGOMOLNI, R. A., R. H. LOZIER, and W. STOECKENIUS. 1976. Light energy transduction in *Halobacterium halobium. Biophys. J.* **16**:21*a*. (Abstr.)
22. BOGOMOLNI, R. A. 1977. Light energy conservation processes in *Halobacterium halobium* cells. *Fed. Proc.* **36**:1833–1839.
23. MICHEL, H., and D. OESTERHELT. 1976. Light-induced changes of the pH gradient and the membrane potential in *H. halobium. FEBS (Fed. Eur. Biochem. Soc.) Lett.* **65**:175–178.

24. KANNER, B. I., and E. RACKER. 1975. Light-dependent proton and rubidium translocation in membrane vesicles from *Halobacterium halobium. Biochem. Biophys. Res. Commun.* **64**:1054–1061.
25. EISENBACH, M., H. GARTY, E. P. BAKKER, G. KLEMPERER, H. ROTTENBERG, and S. R. CAPLAN. 1978. Kinetic analysis of light-induced pH changes in bacteriorhodopsin-containing particles from *Halobacterium halobium. Biochemistry* **17**:4691–4698.
26. ORT, D. R., and W. PARSON. 1979. The quantum yield of flash-induced proton release by bacteriorhodopsin-containing membrane fragments. *Biophys. J.* **25**:341–353.
27. GARTY, H., G. KLEMPERER, M. EISENBACH, and S. R. CAPLAN. 1977. The direction of light-induced pH changes in purple membrane suspensions. Influence of pH and temperature. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **81**:238–241.
28. OESTERHELT, D., and B. HESS. 1973. Reversible photolysis of the purple complex in the purple membrane of *Halobacterium halobium. Eur. J. Biochem.* **37**:316–326.
29. GOLDSCHMIDT, C. R., M. OTTOLENGHI, and R. KORENSTEIN. 1976. On the primary quantum yields in the bacteriorhodopsin photocycle. *Biophys. J.* **16**:839–843.
30. GOLDSCHMIDT, C. R., O. KALISKY, T. ROSENFELD, and M. OTTOLENGHI. 1977. The quantum efficiency of the bacteriorhodopsin photocycle. *Biophys. J.* **17**:179–183.
31. BECHER, B., and T. G. EBREY. 1977. The quantum of efficiency for the photochemical conversion of the purple membrane protein. *Biophys. J.* **17**:185–191.
32. HURLEY, J. B., and T. G. EBREY. 1978. Energy transfer in the purple membrane of *Halobacterium halobium. Biophys. J.* **22**:49–66.
33. HESS, B., and D. KUSCHMITZ. 1979. Establishments of electrochemical gradients: general views and experiments on purple membrane. *In* Frontiers of Biological Energetics. P. L. Dutton, J. Leigh, and A. Scarpa, editors. Academic Press, Inc., New York. 257–264.