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Proton translocation in intact cells of the photosynthetic bacterium *Rhodopseudomonas capsulata*

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(1) Cresol red and phenol red are found to be rapid and reliable indicators of external pH in intact cell suspensions of *Rhodopseudomonas capsulata*. (2) Light-induced pH changes measured with the indicators in 10 mM Na_2SO_4 are complex. A rapid acidification (phase A, $t_{1/2} \approx 30$ ms, 1 H^+ per 29 bacteriochlorophyll) was followed by a lag (phase B, approx. 300 ms duration) and then an extensive and slow acidification (phase C, duration, approx. 2 s, 1 H^+ per 0.8 bacteriochlorophyll). The pH reached a minimum after 2.5 s (phase D) and subsequently began to increase (phase E). (3) Phase A appears to be a component of outwardly directed, electrogenic proton translocation coupled directly to electron transport. The effects of antimycin A, venturicidin and an uncoupling agent, and the kinetics of the change during and after single and double light pulses, are fully consistent with this view. (4) The origin of the apparent lag, phase B is unknown, but it may be unrelated to chemiosmotic proton translocation. Events occurring during phase B distort observations on the kinetics of the proton-translocating activity of the bacterial membrane when recorded on a longer time scale. (5) Phase C does not coincide with a membrane-potential-generating component (judged by the kinetics of the electrochromic band shift) and is probably the result of electron-transport-driven, electrically neutral H^+ efflux accompanying the efflux of anions from, or uptake of cations by the cell. Because it is so extensive, phase C dominates the response of a glass electrode to pH during the first few seconds of illumination. (6) Observations on H^+ efflux measured with glass electrodes following respiratory pulses in *Escherichia coli* (Gould, J.M. and Cramer, W.A. (1977) J. Biol. Chem. 252, 5875–5882) and in *Paracoccus denitrificans* (Hitchens, G.D. and Kell, D.B. (1984) Biochim. Biophys. Acta 766, 222–232) which were thought to be inconsistent with the chemiosmotic coupling hypothesis, are confirmed in *Rhodopseudomonas capsulata*. These observations are discussed with reference to the processes observed on a more rapid time-scale with pH indicators.

Introduction

The measurement of pH changes in weakly buffered, intact cell suspensions of a variety of species of bacteria has provided evidence in favour of the chemiosmotic hypothesis of energy transduction [1–3]. Generally the medium becomes more acidic when electron transport is initiated by the introduction of an appropriate energy source. This suggests that there may be an obligate coupling between outward proton translocation and electron flow. It is usual to carry out such experiments in the presence of counter ions, ostensibly to increase the natural backlash of the membrane and to permit the full expression of the proton-

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Abbreviation: BTPP, butyltriphenylphosphonium.

translocation process [1]. Indeed, the stimulation of the external pH change by counter ions may be evidence of the primary, electrogenic nature of the electron-transport-linked proton translocator. Without special treatments, most intact bacteria are insensitive to uniporter ionophores like valinomycin and, as an alternative, it has become common practice to employ rather high concentrations of intrinsically membrane-permeable ions such as SCN^- as the electrophoretic agents [2,3]. However, it has been noted that SCN^- is chaotropic and may distort the natural properties of the bacterial membranes in other ways [4]. In the absence of artificial, added co-ions, the pH changes displayed by freshly harvested bacterial cell suspensions are complex. In a recent series of experiments by two independent research groups, it has been found that measurements of the pH of bacterial suspensions with a glass electrode in response to O_2 pulses in the absence of added counter ions are not easily reconciled with the concept of an electron-transport-driven electrogenic proton pump [5–9]. It was proposed that the data were more consistent with models of energy coupling in which H^+ proceeds directly from the electron trnasport complexes to the ATP synthase without first coming into equilibrium with H^+ in the bulk aqueous phase: there may be a barrier between the protons translocated across the membrane and the external medium. There were three findings which were said to be contrary to the predictions of the chemiosmotic hypothesis. (1) The $t_{1/2}$ of H^+ appearance in the external medium, measured with a glass electrode was substantially greater than the period of the respiratory pulse measured with an oxygen electrode [5,9]. (2) A decrease in the size of the respiratory pulse did not lead to an increase in the calculated H^+/O ratio in the manner expected if respiratory electron flow were limited by the build-up of membrane potential ($\Delta\psi$) [5,9]. Similarly, two consecutive, small oxygen pulses a few seconds apart yielded equal increments in the acidification of the external medium [5]. It was reasoned that if the failure to observe full H^+/O ratios in the absence of added counter ions was a consequence of a limitation by $\Delta\psi$ on electron transport, then the second pulse should have elicited a smaller decrease in pH. (3) Low concentrations of uncoupling agent were found to stimulate the release of H^+ produced by the respiratory pulse [7]. In the chemiosmotic hypothesis uncoupling agents are believed to operate as proton conductors, serving to dissipate the proton electrochemical potential gradient. Hence the effect of uncouplers in the classical oxygen pulse experiment [1,10] was to reduce the extent of efflux by accelerating the rate of proton return across the membrane. A stimulatory effect of uncoupler on respiratory-dependent H^+ efflux [11] and on light-induced H^+ efflux [12–14] in intact photosynthetic bacteria has also been observed.

The rationale behind the work described below was to investigate in more detail the apparent discrepancy between the rates of proton translocation and electron transport in intact bacteria and the problem relating to the control of H^+ efflux by $\Delta\psi$. We have used the photosynthetic bacterium *Rhodopseudomonas capsulata* in which external pH changes can be driven either by light or by respiration. We have attempted to use indicator dyes to measure external pH changes in the bacterial suspending medium with a much faster response time than with the glass electrode. In a recent report we described a period of rapid H^+ release following flash light activation of intact bacterial cells [15]. The kinetics of the reaction were consistent with hypotheses which predict tight-coupling between electron transport and proton translocation across the membrane between bulk aqueous phases. Suitable controls now show that the dyes cresol red and phenol red do not bind significantly to the bacterial cells and respond satisfactorily to the external pH as judged by the effect of added buffer. We find a rapid phase of H^+ release at the onset of continuous illumination which may reasonably be compared with the rate of photosynthetic electron transport. Subsequent pH changes in the external medium are highly complex, indicative of contributions from other protolytic reactions in and across the bacterial membrane. Recently, the factors responsible for the burst of light-induced H^+ uptake by chromatophore membranes were systematically described within the framework of the chemiosmotic hypothesis [16]. Because of the additional complexities observed in intact cells, this kind of analysis is not sufficient to explain completely the light-driven H^+ efflux from intact cells.

Methods

Rps. capsulata strain N22 was grown photosynthetically under anaerobic conditions [17]. The cells were harvested, washed by centrifugation and resuspended in 10 mM sodium phosphate pH 7.0. The bacteriochlorophyll concentration was assayed by extraction in acetone: methanol [18].

Light-induced absorbance changes were recorded in unstirred 2.5 ml samples at room temperature under anaerobic conditions using a specially constructed argon gas train [19] in a single-beam spectrophotometer equipped with a 90°C 250 W quartz-halogen lamp filtered through Wratten 88A gelatin filter. The photomultiplier signal was stored digitally and averaged in a microcomputer. The experimental media are given in the figure legends. Because of small time-dependent changes in the cresol red signal over a period of 1–2 h in the measuring cuvette, the following procedure was adopted to examine systematically the effects of inhibitors and other reagents. After an initial preincubation under argon for 30 min, the light-induced absorbance changes were recorded in the absence of inhibitor or uncoupler (usually an average of four illumination periods separated by a dark time of 2 min). The cresol red changes were measured first, followed by the carotenoid band shift kinetics. Then, either the inhibitor in methanolic solution or the equivalent volume (a few microlitres) of pure methanol, was added under anaerobic conditions and mixed by tilting the cuvette. A further dark, anaerobic incubation period of 5 min was followed by the measurement of light-induced absorbance changes. In this procedure the measured changes were indistinguishable before and after the addition of methanol. Control experiments in buffer showed that the inhibitor and uncoupler solutions did not significantly alter the isosbestic wavelength used for recording the cresol red absorbance change.

Light-induced and respiration-dependent pH changes were measured in magnetically stirred 12 ml samples with a glass electrode and a remote calomel electrode separated from the observation chamber by way of a ceramic plug and salt bridge. The medium was flushed continuously with argon throughout the experiment. The cell suspension was incubated anaerobically at 30°C for approx. 30 min in the observation chamber before commencing the experiment.

Results

Indicator dyes as suitable probes for external pH changes in suspensions of intact bacteria

In earlier experiments with chromatophores, criteria were established to show that some pH indicator dyes can be used reliably to measure the external pH of the suspension [20]. We have applied the same criteria to freshly harvested and washed intact cells of *Rps. capsulata*. The indicators cresol red and phenol red, which cover a useful pH range in the region of 7–8, were found to meet these criteria. First it was shown that the indicators do not bind significantly to the cells. The data are shown in Table I. All of the dye, whether it was cresol red or phenol red, was recovered in the supernatant. Secondly, it was shown in simple titration with a glass electrode that the pK_a of neither cresol red nor phenol red was shifted significantly (viz. less than 0.1 pH units) in the presence of bacteria. This demonstrates that there was no preferential binding of either the

TABLE I

NO DETECTABLE BINDING OF CRESOL RED OR PHENOL RED TO INTACT CELLS OF *RPS. CAPSULATA*

Harvested and washed intact cells were incubated aerobically at room temperature in the dark for 30 min in 5 ml 10 mM Na_2SO_4, 100 μM indicator at pH 7.8 at the bacteriochlorophyll concentration shown. The cells were sedimented by centrifugation. An aliquot of the supernatant was diluted into 100 mM glycylglycine (pH 9.5) and the absorbance was read at 560 nm.

Bacteriochlorophyll concentration (μM)	Absorbance at 560 nm	
	cresol red	phenol red
0	0.36	0.37
0	0.36	0.36
2	0.36	0.37
2	0.37	0.38
5	0.36	0.36
5	0.37	0.37
10	0.36	0.37
10	0.36	0.38
50	0.36	0.38
50	0.36	0.36

protonated or deprotonated forms of the indicator to the bacterial cells.

Finally, in each set of experiments (see control data in Figs. 1 and 4) it was checked that added hydrophilic buffer quenched completely the light-induced absorbance changes attributed to the pH indicator. Generally 10 mM phosphate was used to buffer the cresol red absorbance changes, but 10 mM tricine was equally effective. In contrast, simply raising the ionic strength from 10 mM to 20 mM Na_2SO_4 had no effect on the signal.

Kinetics of H^+ ejection by intact cells of Rps. capsulata

587.5 nm is an isosbestic wavelength for light-induced absorbance changes in intact cells of *Rps. capsulata* strain N22, at least for the first few seconds of illumination. This wavelength is sufficiently close to the absorbance maximum of the alkaline form of cresol red and phenol red to be used for measurements of light-induced pH changes. Concentrations of indicator up to 100 μM were without significant effect on the light-induced electrochromic absorbance changes of endogenous carotenoid pigments in buffered cell suspensions (not shown) and this was taken as evidence against any destructive effect of the probe.

Upon illumination of an intact cell suspension of *Rps. capsulata* at pH 7.8 in a Na_2SO_4 medium in the presence of cresol red (Fig. 1 and 2) there was a decrease in absorbance at 587.5 nm, which was entirely eliminated in the presence of 10 mM phosphate buffer. We attribute this absorbance decrease to a fall in the external pH of the suspension. The kinetics of the absorbance change were complex. During the first 50 ms of illumination there was an immediate and rapid decrease in pH (phase A: $t_{1/2}$, approx. 30 ms). This phase of the cresol red change coincided with the electrochromic absorbance change of the carotenoids (shown for comparison in Fig. 1b). The average extent of phase A for a number of preparations of bacteria was equivalent to approx. 1 H^+ per 29 bacteriochlorophyll. Following phase A there was a lag (phase B) which persisted for about 300 ms. The pH then began to decrease once more (phase

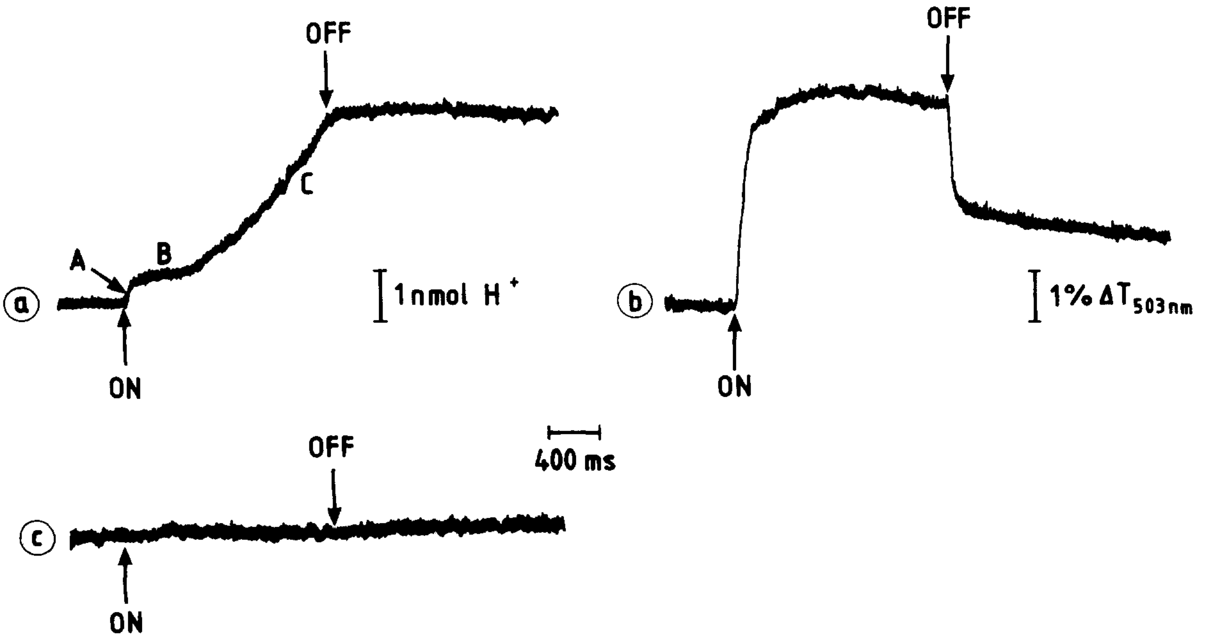

Fig. 1. Light-induced carotenoid absorbance changes and H^+ release from anaerobic intact cells of *Rps. capsulata* measureed with cresol red. 10 μM bacteriochlorophyll, 10 mM Na_2SO_4, 100 μM cresol red (pH 7.8). (a) Absorbance changes at 587.5 nm. The light was switched on and off at the time indicated by the arrows. Average of four recordings, 2 min apart. The letters A, B, C correspond to phases of H^+ release as described in the text. The trace was calibrated by the addition of HCl. (b) Absorbance changes at 503 nm in the same sample. Average of four recordings. (c) As in (a), but in the presence of 10 mM phosphate.

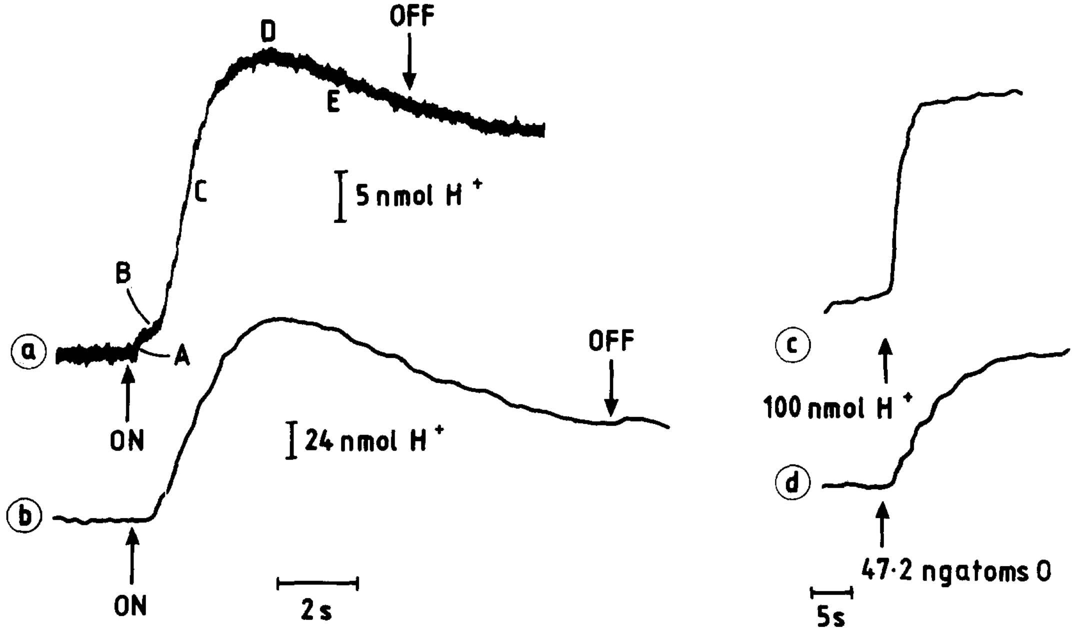

Fig. 2. Light-induced and oxygen-induced pH changes in intact cells of *Rps. capsulata* recorded on a slow time-scale with cresol red and with a glass electrode. (a) Cresol red recording; conditions as in Fig. 1 (a) (amount of bacteriochlorophyll, 25 nmol). (b) Light-induced pH changes measured with a glass electrode in 10 mM Na_2SO_4 – see Methods (amount of bacteriochlorophyll, 240 nmol). (c) Response of the glass electrode to an addition of dilute HCl. (d) H^+ efflux from intact cells measured with an electrode following a burst of respiratory activity in the dark. The oxygen was added as an air-saturated solution of 10 mM Na_2SO_4. Recordings (c) and (d) were performed on the same bacterial suspension. The time-scale bar on the left refers to figures (a) and (b); that on the right to (c) and (d).

C), reaching a minimum (phase D) about 2500 ms after the light was switched on (see Fig. 2). Thereafter the direction of the pH change reversed (phase E) and the alkalinisation of the medium continued for a least several more seconds (Fig. 2). The kinetics of generation of $\Delta\psi$, measured in parallel in buffered suspensions, did not show the complexities observed in the cresol red data. As noted above there was a kinetic relationship between phase A and the electrochromic absorbance change but thereafter $\Delta\psi$ remained fairly constant throughout the period during which the medium pH was undergoing the reactions described by phases B and C, implying that these phases are electrically neutral. On a longer time scale the electrochromic absorbance change partially declined and this may be related to an increased activity of the ATP synthase [21].

Each of these features was consistently observed in all our intact cell samples, which had been grown, harvested and washed under standard conditions. However, there was some preparation-dependent variability in the kinetics and extents of each phase. Extents and rates also varied slightly on the same sample of cells over a period of several hours when maintained at room temperature under argon in the dark. The reason for this variability has not been investigated although other authors have noted analogous effects with glass electrode experiments on chemosynthetic bacteria [1]. After long illumination periods (approx. 1 s) the signal did not completely reverse on the time scale in which we could accurately measure (see below). However, the signal-to-noise ratio was sufficient to show that the pattern of proton efflux during four illumination periods separated by 2 min dark time was similar ($t_{1/2}$ values and extents agreed within 10%). Consequently, the data shown in Fig. 1 were averaged for four sweeps. In other experiments we have averaged for eight sweeps and obtained similar results. Similar features to those shown in Figs. 1 and 2 with cresol red were also observed with phenol red at pH 7.4. Again the light induced absorbance changes were fully sensitive to added buffer. Phases A–E with comparable extents and kinetics were identified with the alternative pH indicator dye.

Our glass electrode system was too slow to detect phase A of the light-induced pH change. The time taken to reach half-maximal response to an addition of HCl was approx. 1 s (Fig. 2c). The glass electrode did, however, respond to the light-induced decrease in pH in the bacterial suspension after a lag which might have been the result of both phase B and of the limited instrumental response time (Fig. 2b). Phases D and E were also observed with the glass electrode. A strict quantitative comparison of the glass electrode and cresol red data shown in Fig. 2. is not possible because of the different experimental conditions demanded by the two procedures and because of the time-dependent effects described above. Within the technical resolution of the data the traces shown in Fig. 2a and b are adjudged to be a response to the same phenomenon viz. the external pH of the bacterial suspension.

Cells of *Rps. capsulata* grown anaerobically under photoheterotrophic conditions develop a competent respiratory chain. The addition of a small quantity of oxygen to an anaerobic suspension of intact cells led to acidification of the medium measured with the pH electrode (Fig. 2d). The kinetics of the pH change that we observed were rather similar to those described for chemosynthetic bacteria [1,5,9]. The risetime of the pH change resulting from the oxygen pulse had a $t_{1/2}$ of about 5 s and was preceded by a short lag. The relaxation of the pH change was very slow ($t_{1/2} > 1$ min).

Relaxation rate of the light-induced pH change of intact cells of Rps. capsulata upon darkening

The rate of relaxation of the light-induced pH change depended on the illumination time. When the light was extinguished after 1500 ms of illumination, i.e., during the period that we have categorised as phase C, the pH continued to fall for a few milliseconds and then flattened off (Fig. 1a). Relaxation to the original, preillumination base-line was extremely slow. This is probably equivalent to the slow relaxation of the pH change measured with the glass electrode after an oxygen pulse (Fig. 2d) which has also been noted in other organisms [1,5,9]. Similarly, when the light was switched off after even longer illumination periods (e.g., Figs. 2a and 2b, measured by pH indicator and glass electrode, respectively) then the rate of decay in the dark was unmeasurably slow.

According to the chemiosmotic hypothesis, the synthesis of ATP is driven by an inward current of protons across the cytoplasmic membrane. At the instant at which the photosyntheitc light is switched off, the outward, electron transport driven proton translocation should promptly cease and, provided that no other vectorial or scalar protolytic reactions mask the reaction, we should expect to observe a return of H^+ into the bacteria. Fig. 3a shows that when the actinic light was extinguished shortly after the initial period of proton production (phase A), then there was indeed a reuptake of H^+. Significantly, there also appeared to be a correlation between the decay of the $\Delta\psi$-indicating carotenoid band shift and the cresol red absorbance change under these circumstances (Fig. 3a and b). A semi-log plot of the decays up to 3 s after the illumination period is hown in Fig. 3e. When the illumination period was extended to include the apparent lag (phase B) or phase C (see above) then the rapid re-uptake of H^+ was no longer evident (Fig. 2); relaxation of the cresol red change was very slow. The electrochromic absorbance changes, which did not share the complexities of the cresol red change on this time scale, did reveal a rapid component in the relaxation upon darkening the suspension. It is suggested that the rapid re-uptake of H^+ still occurs after long illumination periods (> 100 ms), but that it is masked by either scalar or electrically neutral protolytic reactions.

The results of glass electrode measurements of pH changes associated with chemoheterotrophic bacteria in response to two consecutive oxygen pulses, have been said to be inconsistent with the tenets of the chemiosmotic hypothesis [5]. Fig. 3 shows the results of double pulse experiments with cells of *Rps. capsulata* in which two short illumination periods were separated by a dark period. In both Fig. 3a and 3c each illumination period lasted 50 ms but the intervening dark period was 2000 ms and 100 ms, respectively. Corresponding re-

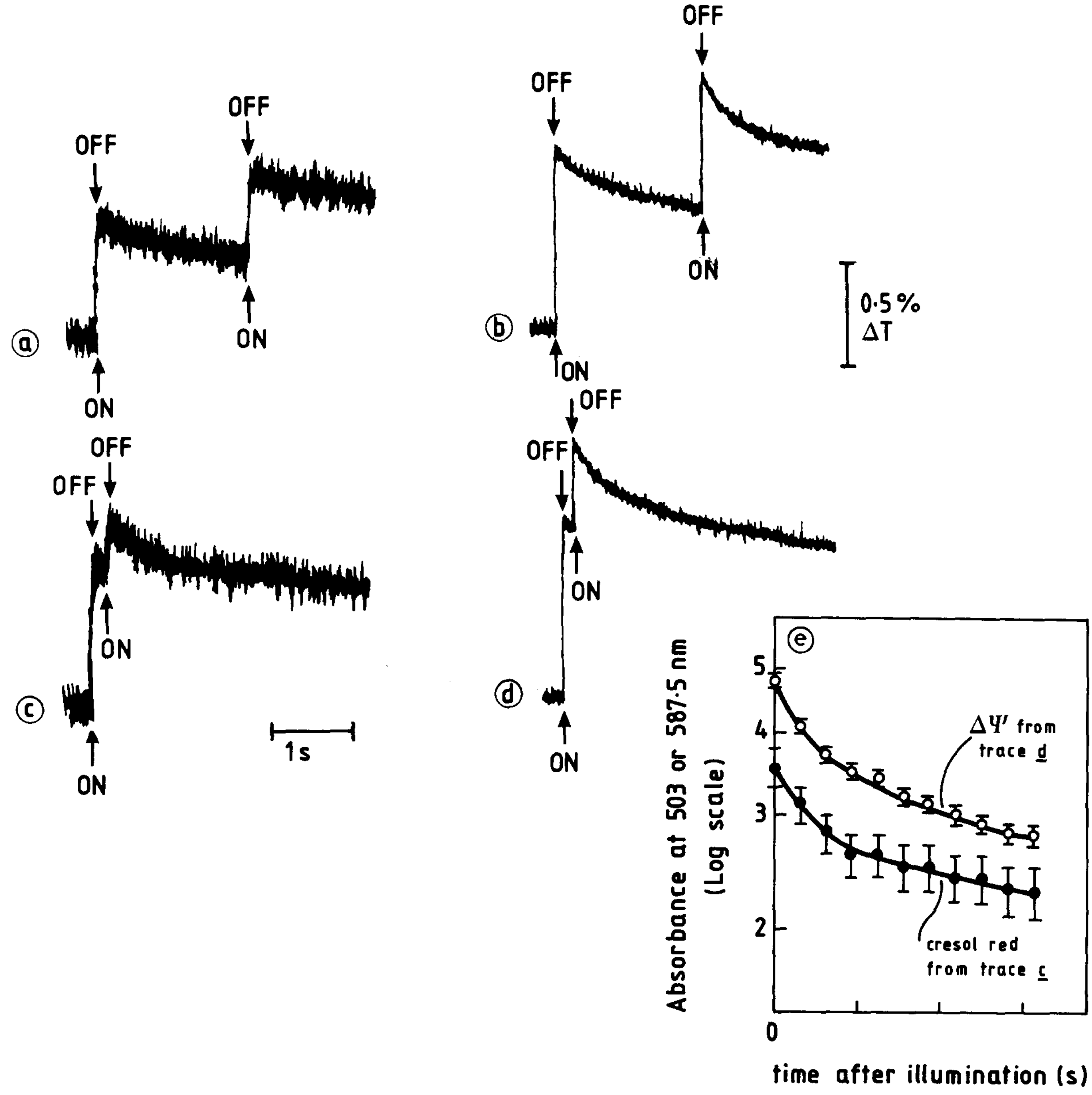

Fig. 3. Relaxation of the cresol red and carotenoid absorbance changes upon darkening after a short period of illumination and the effect of a second light period. Conditions as in Fig. 1a but note the difference in time scale. The traces are an average of eight recordings, 2 min apart. The illumination period (arrows) were all 50 ms duration. (a) and (c) cresol red absorbance change; (b) and (d) carotenoid absorbance change. In (a) and (b) the time between the illumination periods was 2000 ms and in (c) and (d) 100 ms. The cresol red change was not calibrated, but all absorbance changes relate to the scale bar (shown in % ΔT) on the right hand side of the figure. (e) Shows a semi-log plot of the decay after the second illumination period from traces (c) and (d). The error bars show approximate limits of the noise on the trace.

cordings of the carotenoid absorbance changes are shown in Fig. 3b and d, respectively. When the time between the two illumination periods was long and the cresol red signal and carotenoid band shift were allowed partly to decay, then the extent of the light-induced changes elicited by the second light period approached those elicited by the first (Fig. 3a and b). However, when the intervening dark period was insufficient to permit a substantial decay (100 ms), then the cresol red change and carotenoid absorbance change were much smaller after the second than after the first illumination period (Fig. 3c and d). In contrast to glass electrode measurements of oxygen-dependent pH changes on a much longer time scale, the results of these double-light pulse experiments fit very simply within the framework of the chemiosmotic hypothesis (see Discussion).

The effect of electron-transport inhibitor, uncoupling agent and energy-transfer inhibitor on light-induced pH changes in intact cells

Intact cells of *Rps. capsulata* are remarkable for their sensitivity to specific inhibitors of electron-transport phosphorylation. Antimycin A, an inhibitor of the cytochrome b/c_1 complex, venturicidin, an inhibitor of the ATP synthase and a range of uncoupling agents, have been shown to be effective at concentrations which are only slightly higher than those necessary in membrane vesicles [17,22]. Fig. 4 shows the results of a series of experiments in which these reagents were used to characterise the light-induced pH changes measured with cresol red. In separate experiments (data not shown) it was established that lower concentrations of inhibitors affected the cresol red response in a manner which was consistent with their specific site of action.

2.5 μM antimycin led to strong inhibition (40%) of phase A and virtually complete inhibition of subsequent pH changes (Fig. 4g). 5 μM FCCP completely abolished the light-induced cresol red absorbance change (Fig. 4e). 0.5 μM uncoupler led to 40% inhibition of phase A and 75% of phase C (Fig. 4d). 5 μg/ml venturicidin gave rise to marked stimulation of phases A and C (Fig. 4b).

Stimulation of light-induced pH changes with permeant ions

Unfortunately, like other bacteria, intact cells of *Rps. capsulata* are only sensitive to high concentrations of valinomycin and even then only after long incubation periods [1]. Consequently, we have not used this reagent in the present study. Thiocyanate is commonly used as a counter ion to maintain electrical neutrality in oxygen pulse determinations of the H^+/O ratio of chemoheterotrophs. The addition of 20 mM NaSCN to a suspension of *Rps. capsulata* intact cells in a medium of 10 mM Na_2SO_4 using a similar procedure to that described for the inhibitors, led to a pronounced stimulation of the light-induced cresol red absorbance change. Phases A and C were stimulated in both rate and extent (Fig. 5). This concentration of SCN^- had a marked effect on the development of the membrane potential. A rapid rise in $\Delta\psi$ at the onset of illumination, 75% of that seen in the absence of SCN^-, was still evident. The subsequent rise in $\Delta\psi$ was much slower and reached a maximum, 22% less than in the control. Thereafter, $\Delta\psi$ declined until the light was extinguished.

Fig. 5 also shows the results of experiments in which [butyltriphenylphosphonium]$^+$ was used as a permeable ion. As did SCN^-, $BTPP^+$ caused an increase in the extent of light-induced H^+ release during phase A. This was evident even at very low concentrations of $BTPP^+$. The phosphonium cation abolished the lag period phase B. The effect of $BTPP^+$ on the extent of phase C was concentration dependent; 30 μM gave rise to slight inhibition and 200 μM to stimulation. As explained in an earlier report, in which we assessed $BTPP^+$ as a distribution probe for the determination of membrane potential, concentrations in the region of 30 μM had no significant effect on the extent of the $\Delta\psi$-indicating carotenoid band shift in steady-state light [17]. However as shown in Figure 5h 30 μM $BTPP^+$ delayed the establishment of the steady-state by a few hundred milliseconds. Higher concentrations of $BTPP^+$, in the region of 200 μM, led to a permanent collapse of $\Delta\psi$.

The effect of low concentrations of uncoupling agent on dark- and light-induced values of $\Delta\psi$

The stimulatory effect of very low concentrations of uncoupling agent on proton efflux has been demonstrated in several species of bacteria [7,12–14]. Gould [7] has suggested that the uncou-

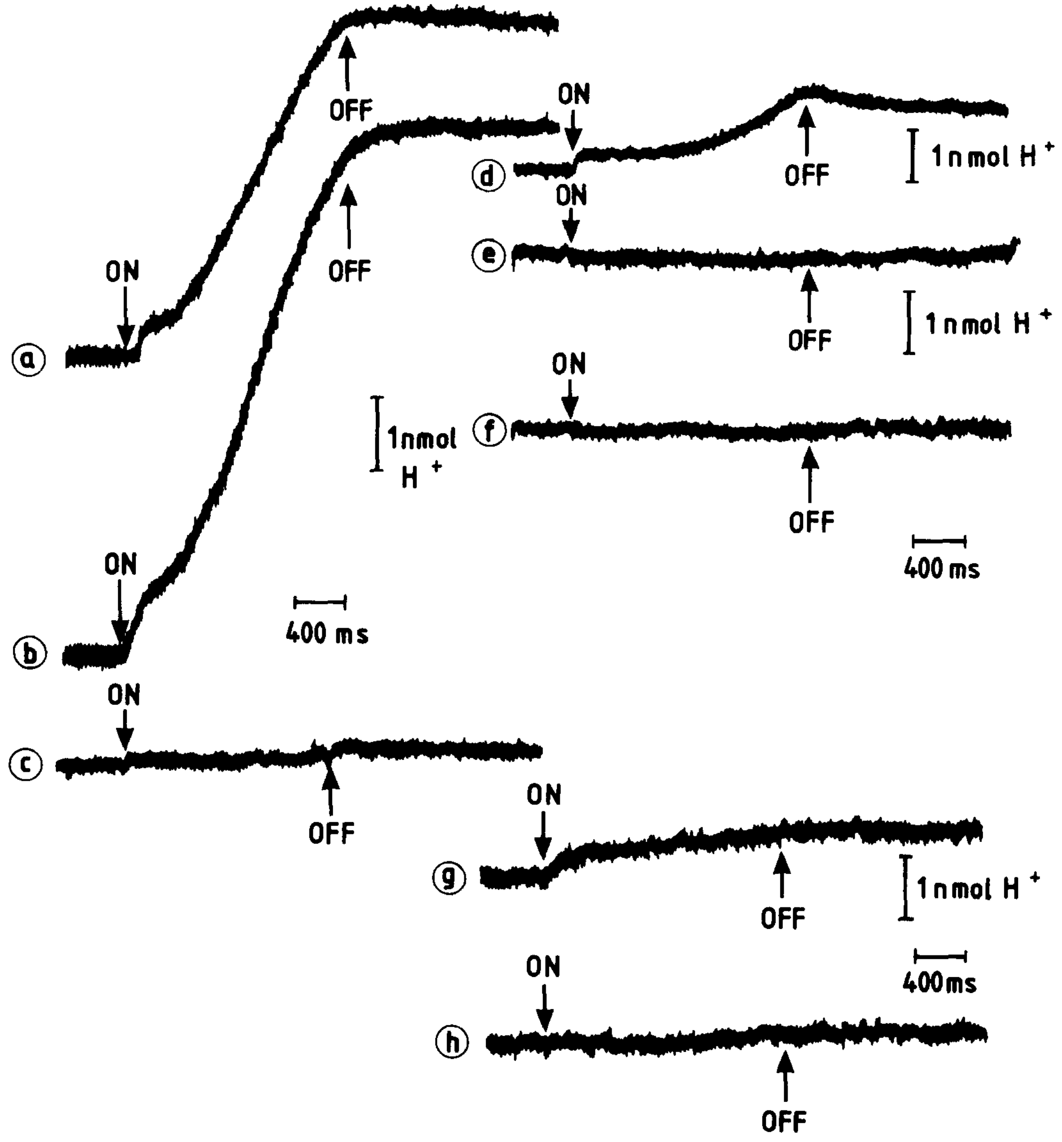

Fig. 4. The effect of venturicidin, FCCP and antimycin A on the light-induced H^+ release from intact cells of *Rps. capsulata* measured with cresol red. Conditions as in Fig. 1a, using the protocol for adding the inhibitors as described in Methods. (a) Control, no other addition, (b) plus 5 μg/ml venturicidin; (c) buffer control (10 mM phosphate) plus venturicidin; (d) plus 0.5 μM FCCP; (e) plus 5 μM FCCP; (f) buffer control plus 5 μM FCCP; (g) plus 2.5 μM antimycin A; (h) buffer control plus antimycin A.

pler may remove a barrier to proton diffusion from the cytoplasmic membrane to the external medium. The observation that even in dark, anaerobic suspensions of the photosynthetic bacteria, there is a substantial membrane potential [17,21] and the recognition that the ionic conductance of the membrane is non-ohmic [22], lead to another explanation for the effect of low concentrations of uncoupling agents, which is consistent simply with their behaviour as protonophores within the cytoplasmic membrane. Fig. 6A shows the changes in $\Delta\psi$ measured by electrochromism in an anaerobic intact cell suspension of *Rps. capsulata* (experiment in collaboration with N.P.J. Cotton). After the generation of $\Delta\psi$ during illumination and its decay in the subsequent dark

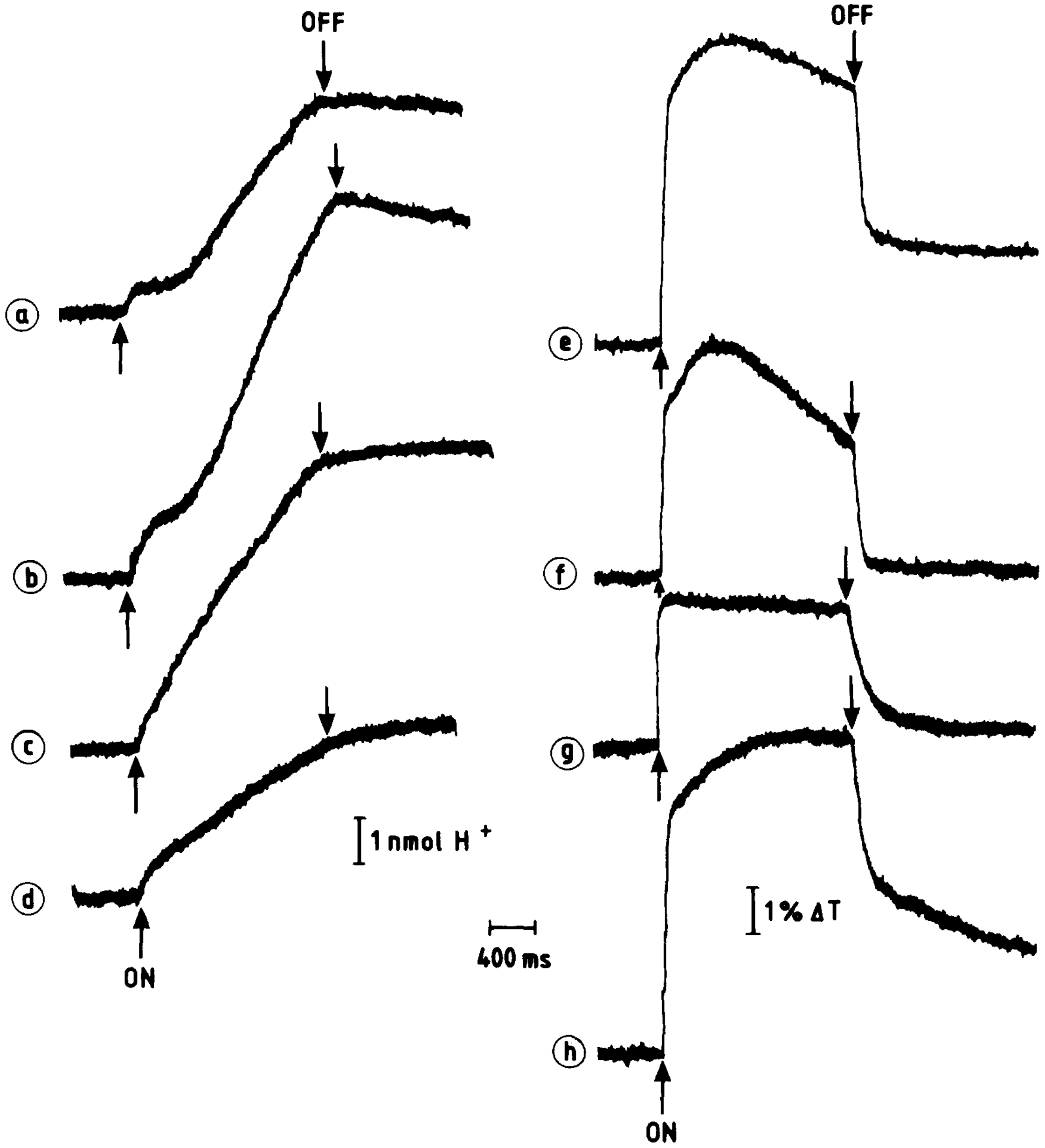

Fig. 5. The effect of permeant ions on the light-induced carotenoid absorbance change and H^+ release from intact cells of *Rps. capsulata* measured with cresol red. Conditions as in Fig. 1a, using the protocol for introducing the permeant ions as described in Methods for the addition of inhibitors. The permeant ions were added as a concentrated aqueous solution. (a)–(d) Cresol red absorbance changes; (e)–(h) carotenoid absorbance changes; (a) and (e) control, no further additions; (b) and (f) plus 20 mM NaSCN; (c) and (g) 200 μM BTPP; (d) and (h) 30 μM BTPP.

period, the addition of 0.01 μM FCCP led to a considerable decrease in the value of the residual dark, anaerobic $\Delta\psi$. During a second illumination period the absolute value of $\Delta\psi$ was similar to that reached during the first period but the apparent light-induced change in $\Delta\psi$ was greater simply because the base-line was lower. The addition of 10 μM FCCP led to a further drop in the base-line $\Delta\psi$ and almost completely abolished the light-induced change. The stimulatory effect of very low

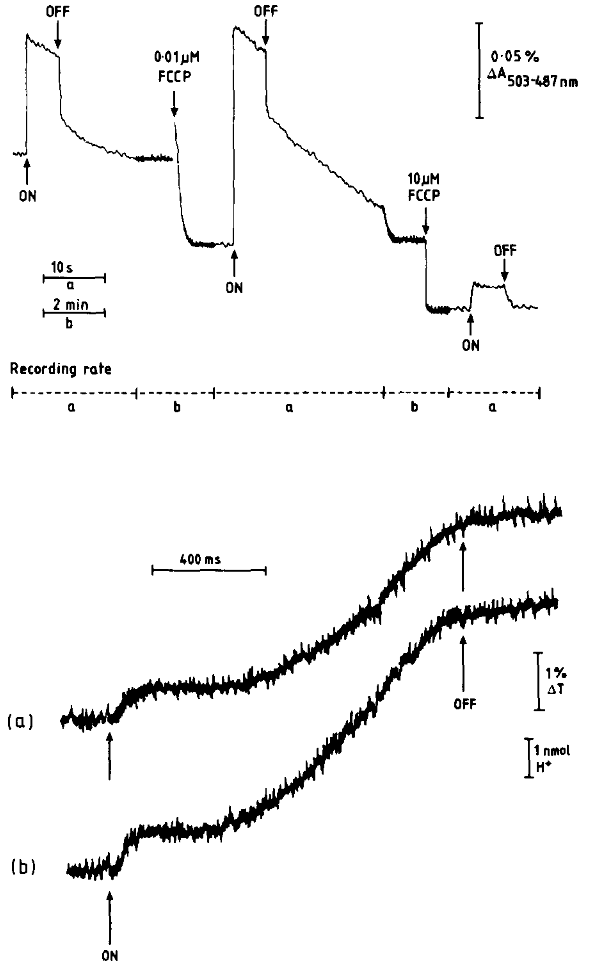

Fig. 6. (A) Apparent stimulation of light-induced generation of membrane potential by decreasing the base line potential in dark, anaerobic conditions with FCCP. Carotenoid absorbance changes recorded at 503–487 nm. Bacteriochlorophyll concentration 20 μM. The bacteria were incubated anaerobically in fresh growth medium as described in Ref. 19. Two recording rates were used as shown on the bottom axis and the two time bars. The light was switched on and off as shown. The final concentration of FCCP is shown above the arrows. (B) Stimulation of H^+ release by FCCP. Conditions as Figs. 1 and 4. (a) Control, no further additions; (b) plus 0.01 μM FCCP.

concentrations of FCCP on the light-induced phase A and phase C of H^+ efflux from intact cells is shown in Fig. 6B (contrast the inhibitory effects of higher FCCP concentrations demonstrated in Fig. 4).

Discussion

Four observations provide convincing evidence that cresol red and phenol red are reliable indicators of the external pH of intact cell suspensions of *Rps. capsulata.* (i) They do not bind significantly to the cells. (ii) Colour changes associated with the dyes can be completely suppressed with added hydrophilic buffer. (iii) The two dyes show similar light-induced absorbance changes in unbuffered intact cell suspensions. (iv) On a slow time scale the dyes show the same light-induced response as the glass electrode.

It is impossible on present evidence to assign each of the five phases that we have called A–E in the light-induced external pH changes of intact cells of *Rps. capsulata,* to specific chemical reactions. In view of the complexity of the pH changes it seems likely that they originate from a number of different, probably unrelated processes. Therefore only tentative suggestions about the nature of each phase can be offered, but we shall be particularly concerned whether the data can be used in a discriminating way to examine the mechanism of energy coupling by the bacteria.

Evidence for a chemiosmotic proton circuit in intact cells of photosynthetic bacteria

At the onset of illumination there is a period of acidification of the external medium measured with pH indicator dyes (phase A) which may correspond to electron-transport-driven, electrogenic proton translocation as predicted by the chemiosmotic hypothesis (see also Ref. 15). The reaction is rapid ($t_{1/2} = 30$ ms) and is completed at about the same time that the $\Delta\psi$-indicating carotenoid absorbance change reaches steady-state (Fig. 1). This suggests that proton ejection and $\Delta\psi$ generation on this time-scale are formally equivalent processes, and that there is no significant kinetic barrier to the diffusion of protons from the cytoplasmic membrane of the bacteria. When the photosynthetic light was switched off at the end of phase A of the H^+ release (i.e., after 50 ms) then a reuptake of protons was observed with similar kinetics to the relaxation of the electrochromic absorbance change (Fig. 3e). This suggests a close coupling between inward proton translocation and the dissipation of $\Delta\psi$ and it provides evidence of a complete chemiosmotic protein circuit. It should be emphasised that rapid proton re-uptake could be demonstrated only for short illumination periods (e.g., 50 ms in Fig. 3) and after 4 μs flashes as in Ref. 15). After prolonged periods of illumination the fast re-uptake of H^+ was not detected (Figs. 1 and 2): we conclude either that the chemiosmotic hypothesis fails to explain this observation or that a fast re-uptake of H^+ is masked by other pH changes (see below).

Although thiocyanate and butyltriphenylphosphonium may have other interfering effects, the stimulatory effect on phase A of both of these permeant ions also suggests that this period of proton ejection is primary and electrogenic (Fig. 5). Inhibition of the reaction by antimycin A demonstrates the necessity of uninterrupted electron transport through the cytochrome b/c_1 complex and inhibition by uncoupling agent can be simply explained as a result of an increased rate of return of protons back across the cytoplasmic membrane (Fig. 4). The ATP synthase inhibitor venturicidin has been shown to decrease the membrane proton conductance in chromatophores [25] and on this basis its stimulatory effect on phase A of light-induced proton ejection would also be readily explained (Fig. 4).

Complexities in the H^+-release reactions during prolonged illumination

On a longer time-scale (greater than 100 ms) the proton-releasing reactions become more complicated. The first consideration is that some of the features in the kinetics of the light-induced pH change (Fig. 1 and 2) may be explained by delays caused by limited diffusion of protons from their site of release. For example the possibility that the outer membrane of the bacteria constitutes a barrier to protons was considered but then rejected by Gould and Cramer [5]. Although these arguments seem to be sound, it is interesting that Hitchens and Kell [9] found that the H^+-electrode response to oxygen pulses was faster in protoplasts than in intact bacteria. These authors concluded that poorly characterised interactions between the cell wall and the cytoplasmic membrane can affect the ability of the cytoplasmic membrane to retain energised protons in a state inaccessible to the periplasmic space. Our results with cresol red show that protons which have been associated with $\Delta\psi$ formation can diffuse from the cytoplasmic membrane into the external phase on the (millisecond) time-scale of photosynthetic electron transport (phase A). The question arises as to whether the delayed release of H^+-during phase C could arise from a limited rate of diffusion. It should be emphasised that the outer membrane (cell wall) is not expected to behave as a permeability barrier to protons [9] and that the work of Gutman and collaborators [26,27] clearly shows that the release of H^+ from the surface of model membranes into the bulk aqueous phase is extremely rapid.

In photosynthetic bacteria additional limitations to proton diffusion could arise from tight constrictions of the extensively invaginated intracytoplasmic membrane [28]. It is possible that phase C might represent the slow appearance in the external phase of H^+ which had been released on the outer face of cytoplasmic membrane deep within the cell's interior. In this case, diffusionally isolated patches of membrane comprising for example one or more 'chromatophores' and not the entire, contiguous cytoplasmic membrane might represent the energy coupling unit. This is similar to the view that limited proton diffusion in opposed cristae membranes of mammalian mitochondria might confine the coupling unit [29] as described by the mosaic chemiosmotic coupling model of Westerhoff et al. [30]. A persuasive argument against adopting the view that the appearance of H^+ during phase C is limited by the existence of diffusion barriers is the observation that venturicidin and the lipid-soluble permeant anions (SCN^-) and cations ($BTPP^+$) accelerate this component of H^+ appearance (Figs. 4 and 5, respectively).

It is possible that some of the proton releasing and consuming reactions observed in Fig. 1 and 2 are not directly involved in energy coupling. Phase B was described as a 'lag period' in the light-induced pH decrease. In this sense it could represent a quiescent period at the end of phase A and before the (delayed) onset of phase C. Alternatively phase B might arise from a period of alkalinisation which cancels acidification at the end of phase A and at the beginning of phase C. The putative proton uptake would be electrically neutral (either a scalar process or a neutral transmembrane exchange), since the value of $\Delta\psi$, given by the electrochromic absorbance change, does not change significantly during the period of phase B. Countenance should be given to this alternative because it might explain why fast re-uptake of H^+ is observed when the actinic light is switched off at the end of phase A (see above and Fig. 3), but not after longer illumination period (Figs. 1 and 2): the fast relaxation of proton efflux upon extinguishing the light at the end of phase A is evident because during that period interference from other protolytic reactions has been minimal. In contrast, when the light is extinguished at the beginning of phase C (or later), the fast re-uptake of protons is masked by relaxation of the alkalinisation occurring during phase B.

Phase C in the light-induced pH change also has no equivalent in the kinetics of the electrochromic absorbance change and is therefore adjudged to be electrically neutral. The inhibitory effect of FCCP and the stimulatory effect of venturicidin and of high concentrations of the permeant ions SCN^- and $BTPP^+$ suggest that phase C is a component of vectorial proton translocation. It is possible therefore that phase C arises as a result of electron-transport-driven, charge-compensated H^+ efflux due to the action of a uniport system for either the relatively slow efflux of anions or influx of cations. The generation of $\Delta\psi$ by photosynthetic electron transport during the period of phase A and subsequently, would lead to the electrophoretic movement of anion/cation through the uniporter. This would lead to further acidification of the medium by electron-transport-driven proton translocation similar to stimulated H^+ uptake in chromatophores in the presence of K^+/valinomycin [16]. According to this view the extensive uptake of H^+ during phase C would represent the electroneutral anion efflux or cation influx driven by $\Delta\psi$, and would be entirely consistent with the inhibition by FCCP and antimycin A and with stimulation by venturicidin and by SCN^- and $BTPP^+$ (Figs. 4 and 5). It would not explain inhibition of phase C by low concentrations of $BTPP^+$ (Fig. 5d). Possibly this could be due to direct inhibition of the uniporter by the phosphonium salt. At higher concentrations of $BTPP^+$ (200 μM – Fig. 5c) only its effect as a lipid-soluble, permeant cation would be significant.

The electroneutral character of phase C explains why complete reversal of the light-induced pH change in the following dark period is so slow. When the light is extinguished, $\Delta\psi$ is dissipated rapidly by the return of only a few protons (cf. phase A) or other ions (cf. the carotenoid shift data Fig. 2b). Consequently, those protons remaining in the external medium (i.e., equivalent to the protons translocated electroneutrally during phase C) will be driven back across the membrane only very slowly by energetically insignificant concentration gradients. A similar explanation may account for the slow reversal of pH changes measured with glass electrodes after respiratory pulses in chemosynthetic bacteria [5,9].

The nature of the alkali-going period that we described as phase E was not studied in detail. It is too slow to be associated directly with the mechanism of energy coupling, but it is extensive and may contribute significantly to H^+ efflux from intact bacterial cells particularly after long (> 2 s) periods of electron transport. In the interesting experiments of Kobayashi and Nishimura [12–14] the reversal in some ionic media of the light-induced pH changes in intact cells of *Rhodospirillum rubrum* measured with a glass electrode may be associated with this phenomenon.

Do glass electrode recordings of pH changes during respiratory pulses in bacteria falsify the chemiosmotic hypothesis?

In the absence of added permeant ions the H^+/O ratio measured with a glass electrode in suspensions of intact chemosynthetic bacteria is low [1]. Within the framework of the chemiosmotic hypothesis this is explained by the supposition that the outward translocation of only a few protons across the cytoplasmic membrane will lead to the development of a large Δp and this will drive protons back across the membrane before they can be detected by the electrode. Gould and Cramer [5] and Hitchens and Kell [9] reasoned that it should be possible to reduce the size of the oxygen pulse to the point below which the full Δp is generated. In this condition Δp might be insufficient to inhibit proton-motive activity or to drive protons back across the membrane. Their experiments, however, showed that the pH change measured with a glass electrode depended linearly on the size of the oxygen pulse even for respiratory bursts calculated to lead to less than maximum Δp values. We obtained similar results with oxygen pulses given to intact cells to *Rps. capsulata* (data not shown).

Our experiments with light induced pH changes measured with cresol red, however, highlight the difficulties of using glass electrode data for this kind of experiment. Fig. 2 shows that during the first 2 s of illumination the glass electrode probably responds to a combination of phases A, B and C. The rapid electrogenic component, phase A is overwhelmed by the extensive phase C and the kinetics of phase A are not revealed. Furthermore, the electrically neutral character of the dominating phase C in the glass electrode response may provide a simple explanation for the anomalous results [5,9]. We assume that the features revealed during photosynthetic electron flow are similar to those during a burst of respiratory activity. When the period of electron transport ceases, $\Delta\psi$ will be fully dissipated by the re-entry of only a few protons (a reversal of phase A). The residual change in external pH is equivalent to the quantity of anion efflux (or cation uptake) which had occurred during the period of energisation (i.e., to phase C). The linear dependence of the residual change in external pH on the quantity of added oxygen [5,8,9] is, on this basis, a result of the relation between the quantity of anion extruded (or cation accumulated) and the period of respiration.

While this explanation can possibly account for the observations of earlier workers [5,9] there is still reason to expect an increase in the $H^+/2e$ ratio when the size of the electron transport burst is reduced, i.e. when the quantity of charge translocated is insufficient to generate a large $\Delta\psi$ [5,9]. As pointed out by Ferguson [32] a calculation of how small such a burst should be depends crucially upon the estimation of the electric capacitance of the bacterial membrane. Using a standard value for biological membrane capacitance and an estimate of the surface area of *E. coli* membrane, Gould and Cramer [5] calculated that H^+ efflux resulting from such small respiratory bursts should be detectable with a glass electrode. Applying the procedure described for chromatophores [33] to intact cells of *Rps. capsulata* and assuming one photosynthetic reaction centre per 100 bacteriochlorophyll, we calculate a membrane capacitance of $2.8 \cdot 10^{-18}$ F per reaction centre. To generate a light-induced $\Delta\psi$ of 290 mV [19] would therefore require translocation of five protons per reaction centre. The extent of phase A is 3.45 H^+ per reaction centre. In view of the assumptions this is good agreement. This calculation shows (a) that the extent of phase A is sufficient to generate a large $\Delta\psi$ (b) that each photosynthetic electron-transport chain need turn over only 2.5-times to generate a $\Delta\psi$ of 300 mV (assuming $H^+/e^- = 2$). Using the same capacitance value and assuming a similar number of respiratory chains ($H^+/O = 6$) to photosynthetic reaction centres, then a full respiratory $\Delta\psi$ of 230 mV [19] would require reduction of only 1.56 ngatom oxygen (0.13 ngatom O/ml) in the conditions of Fig. 2d. This is 30-fold less than the quantity of oxygen used in Fig. 2d. Proton release accompanying such a small respiratory burst would not be measurable with our glass electrode.

As a result of their experiments, Gould and Cramer [5] and Hitchens and Kell [8,9] concluded that in intact bacteria, observations on H^+-release were not consistent with the view that a delocalised protonmotive force can restrict the proton-translocating activity of the respiratory chain. Our interpretation of their results, however, is that such an effect cannot be ruled out. Moreover, using a variant of Gould's and Cramer's double pulse technique [5], the facility for driving photosynthetic reactions in defined short bursts and the time resolution of the cresol red response, we can provide positiv eevidence (Fig. 3) for this aspect of the chemiosmotic hypotheses in intact cells of *Rps. capsulata*. The prediction of the experiment was that if proton translocation can be limited by Δp then the proton efflux produced by the second of two closely consecutive periods of electron transport should be less than that produced by the first. The observation with the glass electrode [5] was that the pH change was similar after each of two oxygen pulses delivered a few seconds apart. However, this may simply reflect the predominantly electrically neutral nature of the measured pH changes (see above). In Fig. 3 we employed two periods of photosynthetic illumination which were sufficiently short (50 ms) to induce mainly only electrogenic proton efflux (phase

A). In accordance with the prediction, when the two periods were fired so close together (100 ms dark time) that $\Delta\psi$ had time to decay only slightly, then the H^+ appearance during the second illumination period was considerably reduced. When the two illumination periods were spaced further apart (200 ms in Fig. 3), such that $\Delta\psi$ decayed substantially in the intervening darkness, then H^+ release during the second illumination was almost equivalent to that during the first. These experiments show clearly that a persisting $\Delta\psi$ from the first period of electron transport can inhibit protonmotive activity during a subsequent period.

Apparent stimulation of proton translocation and $\Delta\psi$ formation by low concentration of uncoupling agents [7,11–14]

The stimulation of energy-dependent proton translocation by agents which are supposed to act by dissipating Δp is, at first sight, not consistent with the chemiosmotic hypothesis. However, the recognition that the ionic conductance of the bacterial membrane is non-ohmic [24] and the observation that even apparently de-energised bacteria maintain a significant Δp [19,23,31] lead to a simple explanation. Because permeant ion redistribution techniques become insensitive at low values of Δp, the existence of a residual Δp in deenergised bacterial suspensions may not have been generally appreciated (but see Ref. 31). In contrast the linearity of the electrochromic response in photosynthetic organisms clearly reveals a $\Delta\psi$ under such conditions. Conceivably this $\Delta\psi$ may arise from very low rates of electron transport even in apparently deenergised suspensions [19], from outward proton translocation by the F_0F_1-ATPase hydrolysis [23], or from diffusion potential gradients. Whichever mechanisms are operative, only very low generative currents are required to maintain this Δp (or $\Delta\psi$), because the conductance responsible for its dissipation is extremely small at low values of Δp [24]. Proton conduction through even very low concentrations of FCCP is sufficient to collapse the residual $\Delta\psi$ (Fig. 6A). Proton translocation by photosynthetic electron transport in fully illuminated bacteria is relatively powerful, but extensive increase in $\Delta\psi$ in the light is offset by a $\Delta\psi$-dependent increase in membrane conductance [24]. Consequently, the low concentrations of uncoupler which are sufficient to dissipate $\Delta\psi$ in the dark do not significantly lower the steady-state $\Delta\psi$ in the light. The change in $\Delta\psi$ and, by implication the quantity of protons needed to establish the ultimate value of the $\Delta\psi$, are increased (Fig. 6A). The observation shows that low concentrations of uncoupling agents by lowering the effective 'base line' can lead to an apparent stimulation of energy-linked events. Both phase A and phase C of the light-induced proton release were stimulated by such low concentrations of uncoupler (Fig. 6B). Higher concentrations of uncoupler (Figs. 4 and 6) are able to raise significantly the dissipative membrane ionic current achieved during illumination, and therefore lower or eliminate the light-induced $\Delta\psi$.

In conclusion, the complex pH changes observed upon energising intact bacterial cells are improperly understood, but in contrast to results with pH electrodes, measurements with pH indicators do reveal rapid components whose properties are largely consistent with the predictions of the chemiosmotic hypothesis.

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