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FAST CHANGES OF ENTHALPY AND VOLUME ON FLASH EXCITATION OF *CHROMATIUM* CHROMATOPHORES

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SUMMARY

We have used a capacitor microphone transducer to measure volume changes in suspensions of *Chromatium* chromatophores 100 μs to 20 ms after flash excitation. Volume changes in photochemical reactions can arise both from volume differences between reactants and products, and from enthalpy changes which heat or cool the solution. By measuring the volume changes at two temperatures, one can resolve the total changes into their two components.

In *Chromatium*, light-driven electron transfer from cytochrome C555 to the primary or secondary electron acceptors causes a contraction of approx. 33 Å^3 per electron transferred. The relaxation of the initial volume change is altered by the presence of gramicidin, valinomycin, *o*-phenanthroline, or phosphate. From the effects of these agents, we conclude that the volume change is localized at the photochemical reaction center, and that part of the relaxation involves transfer of a proton from the solvent to the chromatophores.

Electron transfer from cytochrome C555 to the primary or secondary electron acceptors does not cause a significant enthalpy change. We conclude that the free energy increase that accompanies this reaction is due to a negative entropy change.

INTRODUCTION

In the search for clues to the mechanism of oxidative phosphorylation, the thermodynamics of electron transport has received considerable attention. It is therefore surprising that most of the approaches to this topic have focused only on redox titrations of the electron carriers, and that the information which has emerged deals mainly with free energy changes. The underlying enthalpy and entropy changes generally have remained obscure.

Studying the photosynthetic bacterium *Chromatium*, Case and Parson[1] have resolved free energy changes into entropy and enthalpy, by measuring the midpoint redox potentials of the primary and secondary electron acceptors (X and Y) and donors (P870 and cytochrome C555) as a function of temperature. They have concluded, unexpectedly, that an entropy decrease accounts for all of the free energy

Abbreviation: PMS, *N*-methylphenazonium methosulfate.

which is stored in the light-driven transfer of an electron from cytochrome C555 to Y.

In evaluating the results of redox titrations, one must keep in mind that titrations such as

$$C555 \rightarrow C555^{+} + e^{-},$$

and

$$Y^{-} \rightarrow Y + e^{-}$$

are carried out separately at equilibrium. (Here $C555^{+}$ represents the ferricytochrome, and Y^{-} the reduced form of the secondary electron acceptor.) The ΔG, ΔH, and ΔS values measured in these titrations do not necessarily correspond to those of the process

$$C555 \cdot Y \rightarrow C555^{+} \cdot Y^{-}$$

As the symbolism $C555^{+} \cdot Y^{-}$ here suggests, $C555^{+}$ and Y^{-} within a photosynthetic unit could interact strongly with each other. This interaction might involve transient high-enthalpy states that the separate redox titrations would not reveal.

For this reason, one would like to have an independent measure of the thermodynamic changes that occur rapidly upon illumination of photosynthetic systems. An independent approach could come from calorimetric studies of light-induced enthalpy changes, and Callis *et al.*[2] recently have developed a flash calorimeter which is ideally suited for such measurements. The calorimeter employs a capacitor microphone to measure volume changes in a solution following flash excitation. With the flash calorimeter one can measure volume changes as small as $3 \cdot 10^{-10}$ cm^3 and enthalpy changes as small as 1 μcal, in the interval of 100 μs to 1 s after a flash.

In this paper we report on flash calorimeter studies of *Chromatium* chromatophores.

EXPERIMENTAL

Chromatium vinosum was grown as described elsewhere[1]. Chromatophores were prepared and stored with conventional techniques after sonication of the cells in 0.45 M sucrose, containing 0.1 M Tris–HCl (pH 7.7).

For most of the calorimetric experiments, the bacteriochlorophyll concentration was approx. 10^{-4} M. The chromatophores were generally suspended in 0.1 M KCl, 0.05 M Tris–HCl buffer (pH 7.4), containing 10^{-5} M *N*-methylphenazonium methosulfate. Anaerobic conditions were obtained by bubbling argon gas through the sample prior to the experiment.

A modified version of the flash calorimeter of Callis *et al.*[2] was used. The calorimeter cell, which is shown in Fig. 1, is constructed of 25 mm × 25 mm square Pyrex tubing. The top edge is formed round to accept a circular diaphragm of 25-mm diameter. Joined to the side is a 4-mm Pyrex–Teflon vacuum stopcock which serves for filling the cell and for relaxing slow pressure changes with a time constant long compared to the measuring time. The valve stem includes a spring, so that the time constant for pressure relief does not change as the temperature of the cell is varied between 23 and 4 °C.

Temperature control was obtained by mounting the cell in an aluminum block whose temperature was maintained by a thermoelectric module. A precision ther-

mistor (Yellow Springs Instrument Corp. No. 44003), mounted in a thin glass wall in the side of the cell, served as the sensing element for an indicating temperature controller.

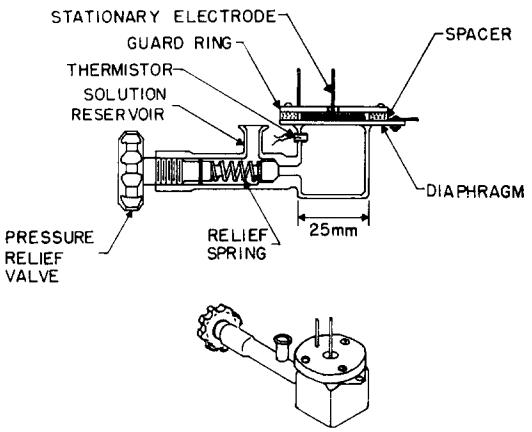

Fig. 1. Flash calorimeter cell. Components of the microphone are not drawn to scale.

The exciting source was a General Radio Strobotac, modified to discharge 3 J of energy with a pulse half width of 30 μs. The sample could be illuminated simultaneously with a 1 kW, water-cooled tungsten–halogen lamp. The two light beams entered the same face of the cell after merging at a beam splitter. The wavelength output of both lamps was limited to 750–950 nm by a Schott RG-9 filter and 2 cm of water. The incident irradiance from the tungsten lamp was 10^{-3} W/cm^2.

The electronics and signal-averaging equipment have been described previously[2]. Because of the low signal-to-noise ratio of the output, the calorimetric data which are presented generally represent the average of 230 flashes at 6.5-s intervals.

The apparatus employed in the spectroscopic studies has been described previously[3]. Digital data acquisition was accomplished by means of a Biomation Transient Recorder model 610. For signal averaging, the digitized signals could be summed and deposited in an auxiliary ferrite core memory.

DATA ANALYSIS AND CALIBRATION

Volume changes due to a chemical reaction can arise in two ways. First, there may be a volume difference ΔV_r between reactants and products. Second, there may be an expansion or contraction of the solution through heating or cooling. If the reaction involves no work other than pressure–volume work, the heat absorbed is equal to the enthalpy change of the reaction, ΔH_r. For reactions in solution, ΔH_r is essentially indistinguishable from the energy change in the reaction, ΔE_r. Thus the total volume change is

$$\Delta V = \Delta V_r - \Delta E_r(\alpha/\rho C_p), \quad (1)$$

where α is the thermal expansion coefficient, ρ the density and C_p is the heat capacity per g of solution.

In the case of a flash-initiated reaction, ΔV depends also on the energy of the photons absorbed and the quantum yield of the reaction. We shall assume that, upon absorption of photons of average energy E_i, the chromatophore either returns radiationlessly* to the ground state or converts to a product which has energy E_p. (We define the energy of the ground state before the flash as zero. E_p is that part of E_i which is not converted to heat.) If n is the number of systems excited by the flash and N is the number that reaches the product state, then the observed volume change, D, is:

$$D = (nE_i - NE_p)(\alpha/\rho C_p) + N\Delta V_r \tag{2}$$

If the sample is illuminated simultaneously with continuous light of sufficient intensity to saturate all of the photochemical reaction centers, then all of the flash energy will be degraded radiationlessly to heat. The volume response, L, is then:

$$L = nE_i(\alpha/\rho C_p) \tag{3}$$

Thus if the volume change on flash excitation is measured both in saturating background light and in the dark we obtain

$$L - D = N[E_p(\alpha/\rho C_p) - \Delta V_r] \tag{4}$$

If E_p and ΔV_r are independent of temperature** while $(\alpha/\rho C_p)$ has a strong temperature dependence, then measurement of L—D at two different temperatures, T_1 and T_2, allows one to obtain $(N/n)\cdot(E_p/E_i)$ from Eqn 5.

$$\frac{(L-D)_{T_1} - (L-D)_{T_2}}{L_{T_1} - L_{T_2}} = \frac{N}{n}\frac{E_p}{E_i} \tag{5}$$

For aqueous solutions it is advantageous to perform one experiment near 4 °C where α approaches zero.

N, the number of systems that reach the product state, depends on the total number of reactive systems N_t, on the number of absorbed photons n, on the limiting quantum yield for product formation at low light intensity Φ, and on the absorbance. Because our experimental conditions included a high absorbance and an uncollimated exciting beam, the dependence of N upon n is complicated. It is intuitively apparent that at very high flash intensities $N \to N_t$, and at very low intensities $(N/n) \to \Phi$. A detailed equation for intermediate intensities has been obtained (J. B. Callis and M. P. Gouterman, unpublished), but will not be discussed here.

At low light intensities, the limiting forms of Eqns 4 and 5 are as follows:

$$\lim_{n\to 0} \frac{L-D}{n} = \Phi[E_p(\alpha/\rho C_p) - \Delta V_r] \tag{6}$$

$$\lim_{n\to 0} \frac{(L-D)_{T_1} - (L-D)_{T_2}}{L_{T_1} - L_{T_2}} = \Phi\frac{E_p}{E_i} \tag{7}$$

* The fluorescence yield from *Chromatium* is less than 5% (ref. 4). In addition, our samples are concentrated enough so that most fluorescence is reabsorbed.

** The linear temperature dependence of the midpoint redox potentials of C555, P870, X and Y[1] supports the assumption that E_p is constant between 0 °C and 40 °C. Volume changes due to the formation of ions in water (see Discussion) also are essentially independent of temperature over this range[5].

We could determine absolute volume changes from

$$S = G\Delta V(B/Ax) \tag{8}$$

where S is the output signal (L or D), G the total gain, B the polarizing voltage across the microphone, A the microphone diaphragm area, and x the distance between the stationary electrode and diaphragm[2]. All quantities except ΔV are known.

The number of photons absorbed can be obtained from S measured with the light on:

$$L = G(B/Ax)(\alpha/\rho C_p)nE_i \tag{9}$$

For the apparatus used in these experiments, G was $1.1 \cdot 10^4$, B was 90 V, A was 2.55 cm^2, x was $7 \cdot 10^{-3}$ cm, $\alpha/\rho C_p$ was $5.6 \cdot 10^{-5}$ cm^3/J at 23 °C, and E_i was 32 kcal/einstein. Thus a volume change of 10^{-8} cm^3 gave an output of 0.55 V before signal averaging and absorption of 10^{-9} einstein at 890 nm gave an output of 0.41 V in the presence of actinic light.

CALORIMETRIC RESULTS

(*A*) *Basic observations*

Fig. 2 gives the volume responses of *Chromatium* chromatophores to weak flashes under four conditions: light (23 °C), dark (23 °C), light (4 °C) and dark (4 °C). At 23 °C in the presence of the background actinic light a volume increase occurs within the risetime of the apparatus. As discussed in the previous section, such a response is expected if the continuous light causes complete oxidation of the P870, and the photons absorbed during the flash are converted rapidly to heat. The magnitude of the volume increase, L, varies linearly with the flash intensity, as predicted by Eqn 3. Reduction of the primary electron acceptor by addition of $Na_2S_2O_4$, or replacement of the chromatophores by an inert, absorbing material ($CuSO_4$ or black ink), has the same effect as saturating illumination, within experimental error.

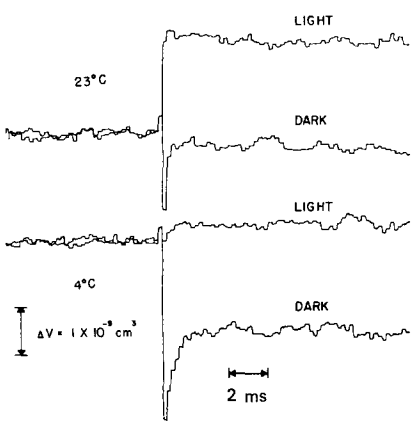

Fig. 2. Volume changes of *Chromatium* chromatophores in response to flash excitation. Flash intensity 0.32 nanoeinsteins. Bacteriochlorophyll concentration approx. 100 μM; buffer: 0.1 M KCl, 0.05 M Tris–HCl (pH 7.4), 10^{-5} M PMS. Traces are the waveform eductor average of 230 flashes. Traces labeled "LIGHT" were results of experiments performed in the presence of continuous actinic light.

At 4 °C in the presence of the continuous light there is a very small positive volume response. The response at 4 °C is so small because the coefficient $\alpha/\rho C_p$ for the solution is nearly zero at the lower temperature.

In the dark at 23 °C the flash causes an instantaneous volume decrease which is then followed by a fast recovery ($\tau_{1/2}$ approx. 100 μs) almost to the base line. Stronger flashes under these conditions gave instantaneous volume increases, followed by further expansion. (See Fig. 10 for example.)

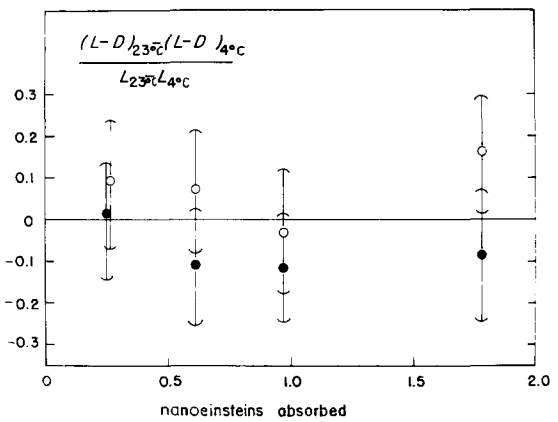

Fig. 3. $[(L-D)_{23\,°C} - (L-D)_{4\,°C}]/[L_{23\,°C} - L_{4\,°C}]$ as a function of the flash intensity. Results taken from experiments similar to those shown in Fig. 2. ○ and ● represent experiments with two different preparations of chromatophores.

At 4 °C in the dark we observe an instantaneous volume decrease, about half of which recovers by a first-order process with $\tau_{1/2}$ approx. 250 μs.

In order to separate the observed volume changes into the contributions due to ΔV_r and E_r, Fig. 3 plots the quantity

$$\frac{(L-D)_{23\,°C} - (L-D)_{4\,°C}}{L_{23\,°C} - L_{4\,°C}}$$

versus n for the volume change remaining after the partial recovery. It is apparent from Figs 2 and 3 that this quantity is zero within experimental error (± 10%). Referring to Eqn 7, assuming that $\Phi = 1$ (ref. 6), and noting that E_i is approx. 32 kcal/mole, we conclude that $E_p = 0$ within ± 3 kcal/mole. The possibility that E_p is not zero, and that a temperature dependence of ΔV_r fortuitously balances that of $\alpha/\rho C_p$ seems unlikely. The conclusion that E_p is approx. 0 appears to apply to the instantaneous volume change as well, although the recovery at 23 °C is too rapid to allow an accurate measurement of the initial contraction.

Fig. 4 shows the quantity $(L—D)$ as a function of the absorbed intensity both at 4 °C and 23 °C. With increasing flash intensity, $(L—D)$ approaches a maximum value as predicted by Eqn 4 when $N \to N_T$. To determine ΔV_r we plotted $(L—D)/n$ *versus* n and extrapolated to $n = 0$ according to Eqn 6. The straight lines in Fig. 4 give the limiting slopes obtained from these plots. Assuming again that $\Phi = 1.0$, and that $E_p = 0$, the limiting slope yields ΔV approx. —33 Å^3 per photon for the

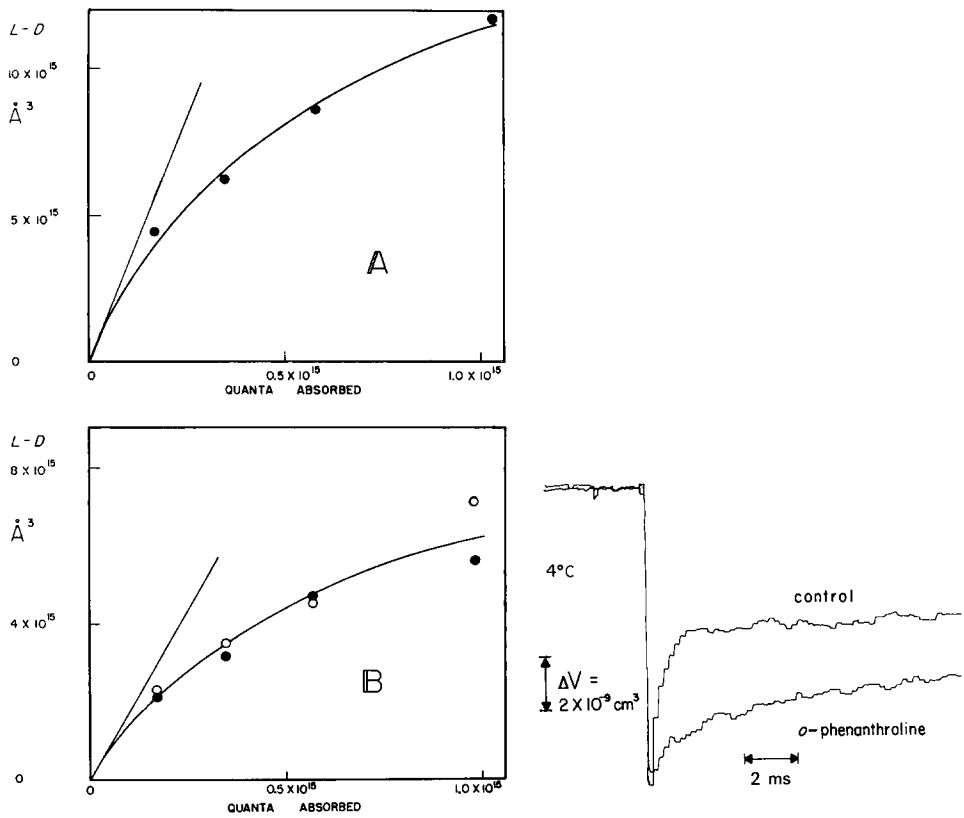

Fig. 4. (*L–D*) as a function of the flash intensity. ○, 23 °C; ●, 4 °C. A, initial volume change; B, volume change remaining after the partial recovery. Results taken from experiments similar to those shown in Fig. 2.

Fig. 5. Volume responses in the presence and absence of 10^{-3} M *o*-phenanthroline at 4 °C. Both measurements were performed without continuous actinic light. Flash intensity 1.6 nanoeinsteins. Buffer was the same as in Fig. 2.

initial volume change and ΔV_r approx. —19 Å^3 per photon for the volume change remaining after the partial recovery.

Preliminary measurements with slower sweep rates indicate that a further contraction occurs with a half-time of about 50 ms at 23 °C. We could not study this transition critically, because the AC coupling of the detection electronics imposed a time constant of 300 ms on the signal.

(B) Effect of o-phenanthroline

o-Phenanthroline blocks electron transfer between the primary and secondary electron acceptors in *Chromatium* chromatophores[7]. In addition, it inhibits the fast binding of protons which occurs after a single flash[8,9]. Fig. 5 compares the volume responses at 4 °C in the presence and absence of 10^{-3} M *o*-phenanthroline. *o*-Phenanthroline does not affect the initial volume decrease, but it dramatically inhibits the expansion which normally follows.

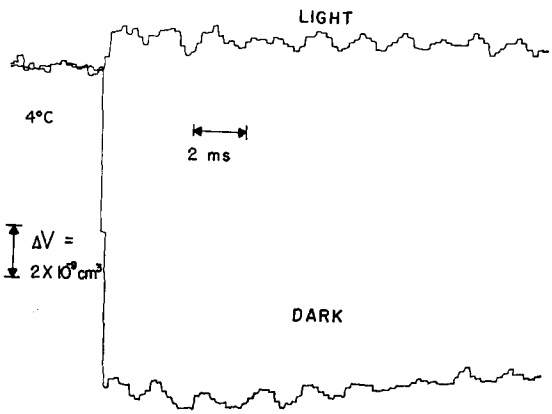

Fig. 6. Effect of phosphate buffer on the volume response of *Chromatium* chromatophores at 4 °C. Flash intensity 1.6 nanoeinsteins. Buffer: 0.05 M KCl, 0.04 M potassium phosphate (pH 7.4), 10^{-5} M PMS.

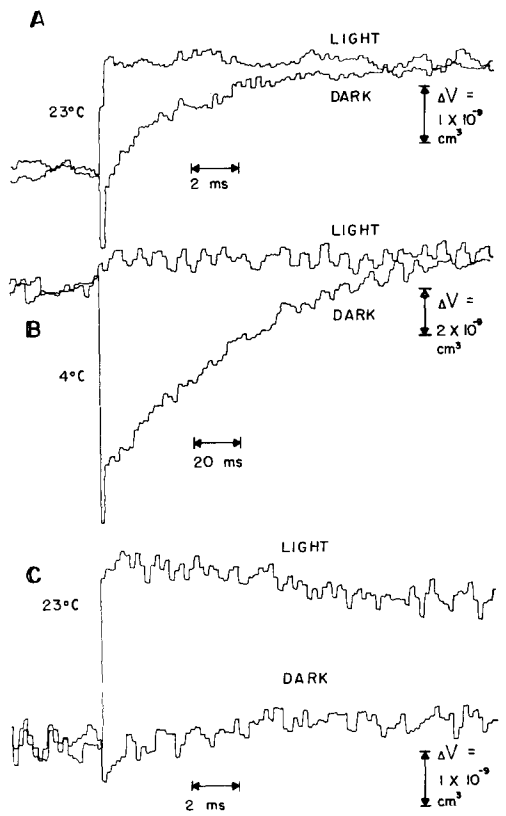

Fig. 7. Effect of valinomycin. A and B, volume responses at 23 °C and 4 °C, under experimental conditions identical to those of Fig. 2 except for the addition of 2.1 μM valinomycin. Flash intensity for A, 0.32 nanoeinsteins; for B, 0.96 nanoeinsteins. C, volume response at 23 °C in an experiment identical to A except buffer contained 0.1 M NaCl instead of 0.1 M KCl.

(C) Effect of various buffers

In all of the experiments that have been discussed so far, the buffer was 0.05 M Tris–HCl. Substitution of glycylglycine or phosphate for Tris does not affect the magnitude of the initial contraction. However, there is no partial recovery in phosphate buffer (Fig. 6). Fig. 9C shows this secondary volume change in an experiment with very dilute glycylglycine buffer (see below).

Replacement of 20 % of the water of the standard buffer by ethylene glycol increases the coefficient $(\alpha/\rho C_p)$ substantially, so that the initial volume changes are positive even in the dark at 4 °C. However, the quantity $(L—D)$ is just the same as that in aqueous solution, as is the rate of the expansion which follows the initial volume change.

Addition of $MgCl_2$ and ADP to the phosphate buffer does not affect the volume response of the chromatophores.

(D) Effect of valinomycin

Valinomycin specifically increases the permeability of natural and artificial membranes to potassium ions[10–12]. Figs 7A and 7B show the volume response of the chromatophores in an experiment identical to that of Fig. 2 except for the presence of 2.1 μM valinomycin. Valinomycin causes the volume change remaining after the

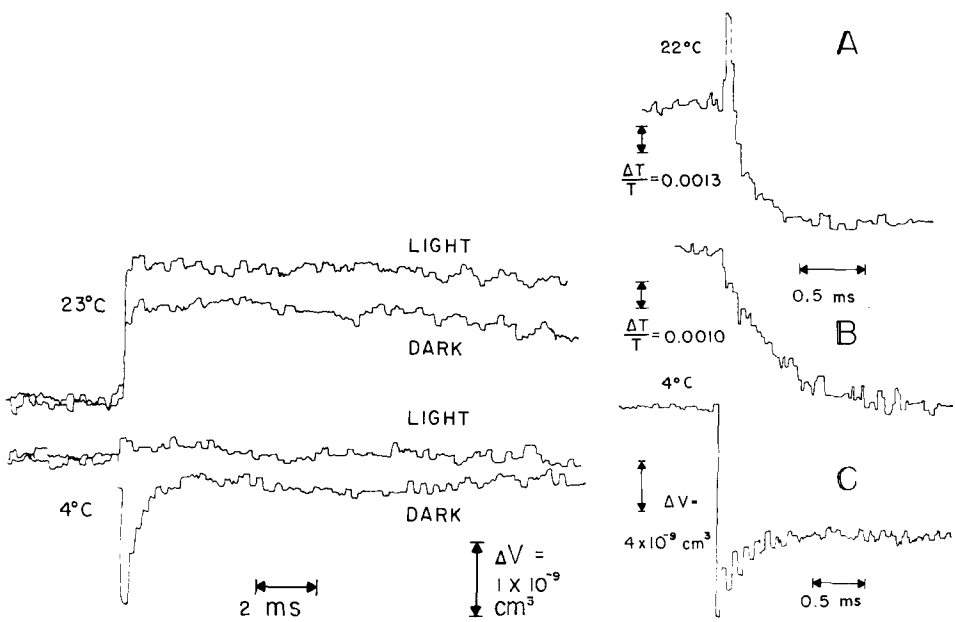

Fig. 8. Effect of gramicidin. Volume response under experimental conditions identical to those of Fig. 2, except for the addition of 1 μg/ml gramicidin D.

Fig. 9. A. pH change following a saturating, 20 ns, 830 nm flash from a dye laser. A downward deflection of the trace represents a pH increase, as indicated by an increase in the optical absorbance at 580 nm. The indicator is 3 μM bromocresol purple. The *Chromatium* chromatophores are suspended in 0.1 M KCl, 0.1 mM glycylglycine, pH approx. 6.2. The traces are digital averages of 15 measurements. B. Same as A, but at 4 °C, an average of 20 measurements. C. Volume measurements at 4 °C under conditions similar to those of B. Flash intensity 1.6 nanoeinsteins; no background illumination.

fast transient to decay to zero with first-order kinetics: at 4 °C $\tau_{1/2}$ = 30 ms and at 23 °C $\tau_{1/2}$ = 4 ms. However, the initial volume change and the fast partial relaxation are unchanged. Comparison of light and dark responses shows that again $E_p = 0$.

Replacing the KCl of the buffer by NaCl greatly decreases the effect of valinomycin, as Fig. 7C shows.

(E) Effect of gramicidin D

Gramicidin D greatly increases the permeability of natural and artificial membranes to protons and alkali metal cations[10–12]. Fig. 8 shows the volume response of the chromatophores in an experiment identical to that of Fig. 2 except for the addition of 1 μg/ml gramicidin. Gramicidin has no effect on the volume response in the presence of continuous light, but it has a large effect on the dark response. A large positive volume change occurs in the dark at 23 °C. In the dark at 4 °C the immediate contraction is smaller than that which occurs without gramicidin, and a fast recovery returns the volume almost to the base line. The kinetics and magnitude of the recovery are identical with those in the absence of gramicidin. Following this transition, (*L*—*D*) is the same at 4 °C as it is at 23 °C, indicating that, as in the absence of gramicidin, $E_p = 0$.

Gramicidin therefore must either prevent the occurrence of part of the initial contraction, or cause a partial recovery in a time too short for us to measure.

SPECTROSCOPIC STUDIES

We undertook the studies detailed below in an effort to see whether spectroscopic transients correlated with the volume transients.

(A) Proton binding

Chance *et al.*[8,9] have found that *Chromatium* chromatophores bind approx. 1 equiv of protons per mole of photosynthetic units, with a half-time of about 200 μs, after a flash at room temperature. Following their procedure, we measured proton binding with chromatophores which were suspended in a medium containing 0.1 M KCl and 10^{-4} M glycylglycine. The pH was adjusted to 6.0–6.3 by addition of HCl. Absorbance changes of the bromocresol purple indicator are shown as a function of time at 23 °C and 4 °C in Figs 9A and 9B. After a flash the pH in the medium increases by a first-order process with $\tau_{1/2}$ approx. 130 μs at 23 °C and $\tau_{1/2}$ approx. 200 μs at 4 °C. The half-time at 23 °C is shorter than that observed by Chance *et al.*[8,9] under similar conditions. The reason for the discrepancy is unclear.

For comparison, Fig. 9C gives the volume response of the chromatophores suspended in the same buffer at 4 °C. The time constant for the expansion which follows the initial contraction is the same as that of the proton uptake, within experimental error.

(B) Carotenoid shift

Because valinomycin and gramicidin are known to increase the rate of reversal of the light-induced carotenoid spectral shifts in chloroplasts[13,14] and *Rhodopseudomonas spheroides* chromatophores[12,15], we studied the effects of these antibiotics on carotenoid shifts in *Chromatium*.

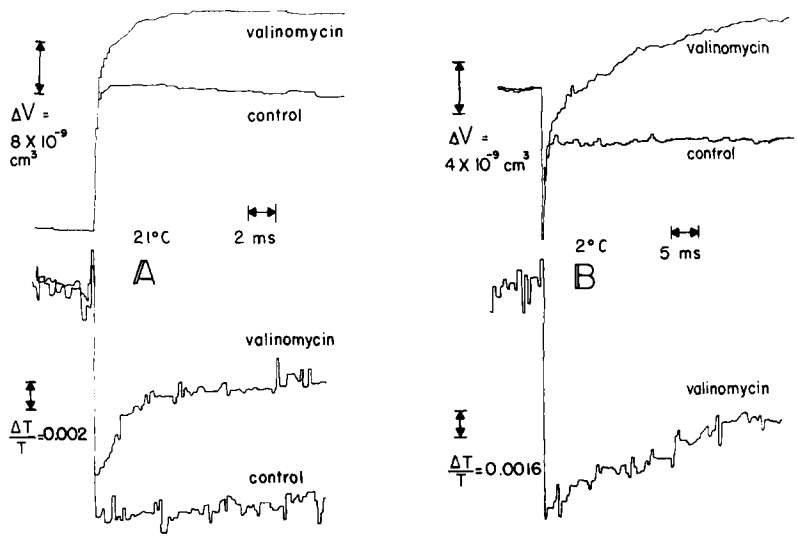

Fig. 10. Carotenoid shift kinetics. Top traces are volume responses of *Chromatium* chromatophores in the presence and absence of 2.2 μM valinomycin. Flash intensity approx. 2 nanoeinsteins; no background illumination. Bottom traces give absorbance changes at 482 nm, measured with the same chromatophore suspensions as were used for the volume measurements. A downward deflection indicates an absorbance increase. About half of the initial absorbance change represents the carotenoid shift. Conditions as in Fig. 2. The actinic flash for the absorbance measurements was a saturating, 20-ns, 694-nm flash from a ruby laser. The traces are digital averages of 40 measurements.

Fig. 10 shows the kinetics of the absorbance change at 482 nm following an actinic flash. In untreated chromatophores, the recovery of the initial absorbance increase is very slow, and is multiphasic. In chromatophores treated with 2.2 μM valinomycin, about half of the initial absorbance change at this wavelength recovers much more rapidly. Measurements at other wavelengths reveal that the spectrum of the portion of the absorbance change which is sensitive to valinomycin is similar to that of the carotenoid shift in *R. spheroides*, though the magnitude is much smaller in *Chromatium*. The portion of the initial absorbance change that is insensitive to valinomycin probably arises mainly from cytochrome oxidation.

Within experimental error, the rate of the carotenoid shift recovery that valinomycin mediates is the same as the rate of the reversal of the volume change that remains, after the initial, rapid phase of reversal (Fig. 10).

In the presence of 1 μg/ml gramicidin D, no carotenoid shift could be detected. The initial absorbance change at 482 nm was smaller than that which occurred in the absence of gramicidin, and appeared to be due solely to cytochrome oxidation. If a carotenoid shift occurred in the presence of gramicidin, it must have recovered within 10 μs. The effect of gramicidin on the carotenoid shift thus was closely parallel to its effect on the initial volume decrease (Fig. 8). Gramicidin D had no effect on the the amount of cytochrome oxidation that resulted from either of two saturating actinic flashes 1 ms apart, nor on the rate of recovery of the reduced cytochrome following the flashes.

DISCUSSION

Mechanism of the initial contraction

The flash calorimeter experiments reveal a new feature of the initial events in bacterial photosynthesis: a substantial volume decrease accompanies the transfer of an electron from C555 to X. The finding that *o*-phenanthroline has no effect on the initial volume change shows that the secondary reaction between X^- and Y involves little or no further volume change.

In an attempt to account for the initial volume decrease, let us first consider a simple model of the photosynthetic apparatus. Junge and his colleagues[13,14] and Jackson and Crofts[12,16] have suggested that the primary electron donor and acceptor lie on opposite sides of a membrane, so that electron transfer between them generates a transmembrane electrical field. They interpret the light-induced shift in the carotenoid absorption spectrum as an indicator of this field. In this view, valinomycin and gramicidin reverse the carotenoid shift because they discharge the transmembrane potential by facilitating the movement of cations across the membrane. Because the dramatic effects of valinomycin and gramicidin on the volume changes parallel their effects on the carotenoid shift (Fig. 10), the two phenomena must be intimately related.

In the model of Junge and colleagues[13,14] and Jackson and Crofts[12,16]. the chromatophore membrane acts as a capacitor with capacitance, C, given by

$$C = \frac{\kappa \varepsilon A'}{d} \tag{10}$$

where κ is the effective dielectric constant of the membrane, d the thickness of the membrane, A' is the area of one surface of the membrane and $\varepsilon = 8.85 \cdot 10^{-12}$ F·m^{-1}. This model requires that, as the electron carriers gain or lose electrons, they also bind ions or release ions to the solution. The charge on the electron carriers themselves remains constant, and mobile ions distribute the electric field uniformly over the entire surface of the membrane.

Volume changes would accompany the transfer of ions between the electron carriers and the solvent. However, this probably cannot be the origin of the initial volume change, because ion-transporting antibiotics presumably would not reverse such volume changes. Further, since the addition of 20% ethylene glycol to the buffer does not affect the magnitude of the initial volume decrease, it seems likely that the volume change originates mainly within the membrane and not in the solution. We shall therefore neglect this possible source of volume changes for the moment, and consider whether a uniform transmembrane electrical field could cause a contraction of the membrane sufficient to account for the initial volume change.

The free energy stored in the membrane capacitance is

$$\Delta G = Q^2/2C \tag{11}$$

where Q is the net charge generated on either side of the membrane. Combining relations 10 and 11 with the thermodynamic relationship

$$\Delta V = (\partial \Delta G/\partial P)_T \tag{12}$$

yields the following expression for the volume change:

$$\Delta V = -\Delta G \left\{ \left(\frac{\partial \ln \kappa}{\partial P} \right)_T + \beta \right\} \tag{13}$$

We have assumed that the dielectric material has an isotropic volume compressibility β, and that A' is constant.

If $A' = 8.5 \cdot 10^{-11}$ cm^2 (ref. 17), $\varepsilon = 2$, and $d = 30 \cdot 10^{-8}$ cm (ref. 18), then the membrane capacitance is approx. $5 \cdot 10^{-17}$ F. In the limit of very weak, short flashes, only one electron moves across the membrane of each chromatophore. Then $Q = 1.6 \cdot 10^{-19}$ C, and from Eqn 11, $\Delta G = 2.6 \cdot 10^{-22}$ J per chromatophore.

Although we have no information on the values of $(\partial \ln \kappa / \partial P)_T$ and β for natural membranes, the values for non-polar organic liquids may offer an upper limit. For *n*-heptane at 1 atm, the former quantity is $8.2 \cdot 10^{-11}$ cm^2·dyne^{-1} (ref. 19) and the latter is $15 \cdot 10^{-11}$ cm^2·dyne^{-1} (ref. 20). For more polar liquids, the values of both quantities are smaller. Using the values for *n*-heptane, Eqn 13 gives $\Delta V = -0.6$ Å^3 per photon. This value is much smaller than the volume decrease of —33 Å^3 per photon that we actually found (Fig. 4). Even if 10 electrons were to move across the membrane during a weak flash, the theory would predict a volume change of only —6 Å^3 per photon.

Two other predictions of the membrane capacitor model seem at odds with our experiments. The first of these concerns the rates of the carotenoid shift recovery and the volume increase in the presence of valinomycin. If the carotenoid shift is an electrochromic effect[13,14,21], its magnitude should be proportional to the voltage across the membrane, which is in turn proportional to the charge Q. The volume change, on the other hand, should be proportional to Q^2 (Eqn 13). Thus, the flow of charge across the membrane should cause the volume change and the carotenoid shift to decay at different rates. Fig. 10 shows, however, that the secondary volume increase and the reversal of the carotenoid shift have identical kinetics. The second, and related problem is that the quantity L—D should exhibit a quadratic dependence on flash energy, for weak flashes. Fig. 4 shows no indication of this behavior.

The simple membrane capacitor model thus fails to predict the correct magnitude of the initial volume change, the correct dependence on flash energy, or the correct kinetics of the valinomycin effect. All of these difficulties could follow from the assumption that an exchange of ions between the electron carriers and the solution immediately distributes the electric field over the entire membrane. Suppose, instead, that the initial volume change and the carotenoid shift reflect an electric field which is confined to the vicinity of the photochemical reaction center. If neighboring reaction centers do not interact, the volume changes occurring in different centers would be additive. The total volume change would then depend linearly on the total number of electrons transferred in a short flash, rather than quadratically. Decreasing the area over which the electric field is distributed would decrease the effective capacitance of the membrane (Eqn 10). This in turn would increase the free energy that is stored in the field (Eqn 11), and the volume change that results (Eqn 13).

Volume changes due to local electrostatic interactions are well known[5,22,23]. The electric field of an ionic or polar molecule causes a local ordering of the dipoles of the solvent and a compression of the solvent cavity in which the molecule resides.

As an example, a contraction of approx. 13 cm^3/mole accompanies the formation of the zwitterions of α-amino acids in aqueous solution[24]. The contraction is larger (18 cm^3/mole) with ε-amino acids, in which the interaction of the two charged groups with each other is less. It is still larger in nonaqueous solvents with greater compressibility and greater values of $(\partial \ln \kappa/\partial P)_T$ (refs 5, 22 and 23).

The analogy between zwitterion formation and the transfer of an electron from C555 to X is obvious. In the electron transfer reaction, of course, the "solvent" is a complex and ordered structure, including components of the membrane as well as the aqueous solution. In addition, the electrical charge on C555 or X may differ from zero before the photochemical electron transfer.

The main distinction between the membrane capacitor model and the zwitterion model is that, in the latter, strong, local electrical fields persist in the region of the electron carriers. This feature may appear to conflict with the conclusion of Case and Parson[1] that electrostatic interactions between X^- and C555, or between different C555 hemes, do not perturb the midpoint redox potential of C555. To avoid this conflict one may assume either that the charged species are far apart, or that the binding or release of ions does, in time, neutralize the local electrical fields. In the latter case, the flash calorimeter experiments would examine the chromatophore at a time preceding this relaxation, the redox titrations would examine a later state of the system. By carrying cations into the membrane, gramicidin and valinomycin could hasten the transition between the two states. A useful model for the volume increase that would accompany this relaxation might be the volume increase that occurs[25] upon the binding of cations to polyelectrolytes.

Several other types of phenomena might account for a local volume decrease[5,22]. First, the intrinsic volume of the products might be less than that of the reactants. This would be the case if the transfer of an electron from C555 to X involved the formation of new covalent bonds, or hydrogen bonds, or conformation changes that open new pockets for the penetration of solvent molecules. The suggestion that the carotenoid shift arises from a conformation change has been made previously[26].

As a dramatic example of such a conformation change, the oxidation of mitochondrial ferrocytocrome *c* occurs with a restructuring of major portions of the polypeptide chain, creating a new hydrogen bond and a new pocket for solvent or ion inclusion[27]. Similar conformation changes occur on the binding of O_2 to hemoglobin[28].

Mechanism of the secondary expansion

Several lines of evidence show that the secondary volume increase which follows a flash in the dark accompanies the transfer of a proton from the aqueous solution to the chromatophores. First, the increase in the pH of the solution following a flash has the same kinetics as the volume increase, both at 23 °C and at 4 °C (Fig. 9). Second, *o*-phenanthroline blocks both phenomena (Fig. 5 and ref. 9). Valinomycin and gramicidin have no pronounced effect on either. And, finally, phosphate blocks both the volume change (Fig. 6) and the proton uptake (M. Baltchefsky, personal communication). Chance *et al.*[8] suggest that the proton uptake reflects a change in the conformation of a component of the chromatophore membrane. The volume increase could be a direct indicator of the same event.

Negentropy storage

The basic conclusion from the studies reported here is that the metastable state which prevails at 1 ms after flash excitation shows no significant increase in enthalpy over the ground state. This result is a seemingly unambiguous conclusion from the observation that $L—D$ is independent of temperature in aqueous solution. Since it is known that the chromatophores undergo an increase in free energy after flash excitation, then (from the definition $\Delta G = \Delta H—T\Delta S$) we must conclude that $\Delta S < 0$. Thus the free energy available from the photon is stored in the form of negentropy rather than enthalpy. This conclusion corroborates the results of the potentiometric studies[1] and extends them by showing that no transient high-enthalpy state lasts as long as 1 ms after the flash.

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