Light-Induced Interactions of *Rhodospirillum rubrum* Chromatophores with Bromothymol Blue*

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ABSTRACT: When chromatophores of *Rhodospirillum rubrum* are incubated with bromothymol blue and the system is illuminated with red light, reversible changes in absorbancy of bromothymol blue are observed at 6100 A. These changes are equivalent to about 0.04 pH unit/3 $\times$ 10^{-5} M bacteriochlorophyll, and are not reflected in pH changes of the suspending medium monitored with a glass electrode. In the presence of adenosine diphosphate, orthophosphate, and magnesium chloride, the system will carry out light-induced phosphorylation resulting in an increased alkalinity of the suspending medium; at the same time color changes of the bromothymol blue indicate an apparent pH change toward acidity. Metabolic inhibitors, such as carbonyl cyanide *m*-chlorophenylhydrazone or antimycin A, completely or partially inhibit these color changes; methylamine, a substance which "uncouples" light-induced phosphorylation from electron transport in chloroplasts, produces an increased color change of the bromothymol blue–chromatophore system upon illumination. Bromothymol blue is weakly adsorbed by the chromatophores, and the color changes appear to reflect either internal pH changes or reversible changes in the structure of the chromatophore exposing or occluding active groups in the chromatophore proteins. The onset of the light-induced color changes of bromothymol blue and their decay in the dark follows first-order kinetics with a pattern similar to that observed by Packer for changes in light scattering of whole cells and chromatophores.

The observations that shifts in hydrogen ion concentration accompany light-induced reactions in chloroplasts (Jagendorf and Hind, 1963) have created considerable interest in the role such pH changes play in the operation of certain partial photosynthetic reactions. Furthermore, when chloroplasts are subjected to certain externally applied pH changes, both dark phosphorylating activity (Jagendorf and Uribe, 1966) and luminescence activity (Mayne and Clayton, 1966) can be demonstrated.

Changes in pH have been used indirectly as a measure of light-induced phosphorylation in chromatophores (Frenkel, 1956). Since that time, Nishimura, using weakly buffered chromatophore suspensions, was able to follow the small and rapid pH changes associated with light-induced phosphorylation (Nishimura *et al.*, 1962). He did not report on any changes in the absence of ADP[1] and orthophosphate.

Though we were able to repeat Nishimura's measurements, we decided that we wanted to find a method which would permit us to follow, simultaneously, pH changes and chromatophore absorption changes upon illumination. For this reason we selected BTB which is a useful pH indicator near neutrality. This dye has also been used to demonstrate pH changes in mitochondrial preparations (Chance, 1965; Mela, 1966).

When we began to use this indicator we were surprised to find that the apparent pH changes were of opposite sign to those expected from Nishimura's measurements, and it was then observed that these "apparent" pH changes were observed even under nonphosphorylating conditions when no pH change could be detected with a glass electrode. Baltscheffsky has recently reported that he has detected pH changes in illuminated chromatophores, when these changes were monitored with a glass electrode (Baltscheffsky, 1966).

This paper deals with a kinetic analysis of the BTB color changes upon illumination and cessation of illumination of chromatophores; it presents evidence that the color changes described below are not due to the release of protons into the surrounding medium in the absence of net phosphorylation. Since the observed color changes of BTB reported here also are sensitive to metabolic inhibitors, the possible relation of the observed reactions to light-induced electron transport and phosphorylation will be discussed.

Methods

Chromatophores were prepared as previously reported (Frenkel and Hickman, 1959), and in some instances without added buffer. Reactions were run

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[1] Abbreviations used: ADP, adenosine diphosphate; *m*-Cl-ccp, carbonyl cyanide *m*-chlorophenylhydrazone; BTB, bromothymol blue or 3,3′-dibromothymolsulfonphthalein; PMS, phenazine methosulfate; τ, inverse of the first-order rate constant.

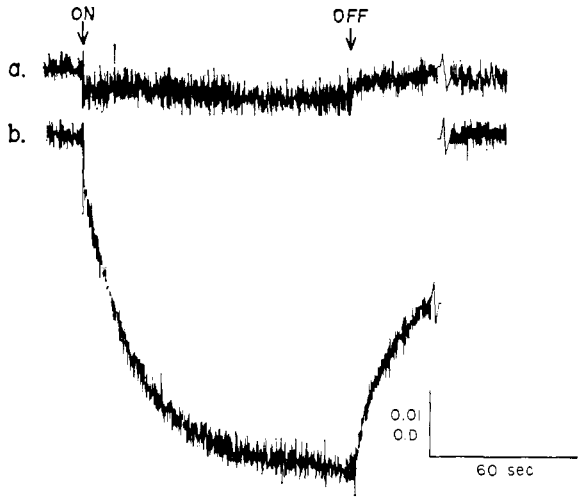

FIGURE 1: Photograph of the ΔOD of the light-induced reversible color changes of chromatophores. (a) Minus BTB. (b) BTB (2×10^{-5} M), 3×10^{-6} M bacteriochlorophyll, 2×10^{-2} M glycylglycine, pH 7.75. For Figures 1–6 all concentrations given as final concentrations. Final volume of all preparations 4.0 ml.

under anaerobic conditions in Thunberg-type cuvets with an optical path length of 1 cm. Measurements were made with a Cary Model 14 spectrophotometer equipped with a 0.0–0.1- and 0.1–0.2-optical density slide-wire assembly. Illumination was through a CS7-69 deep red Corning filter and was at right angles to the measuring beam. When necessary the pH of the additions was adjusted to the same value as the chromatophore BTB mixture in advance of their addition to the reaction mixture. Spectra of chromatophores plus BTB showed a maximum change in light minus dark absorption at 6100 A, which is near a maximum in the absorption spectrum of BTB and close to a minimum in the absorption spectrum of the chromatophores. Light intensities were measured with a Yellow Springs Radiometer, and all experiments unless otherwise stated were carried out at saturating light intensities. Bacteriochlorophyll was measured by a standard method (Cohen-Bazire *et al.*, 1957).

A Texas Instruments, Servo-Riter recorder attached to a Radiometer pH meter type TTT1c was used for recording pH measurements. Stirring was accomplished by continuously bubbling argon through the reaction mixture.

A potentiometer attached to the recorder allowed an expansion of 0.05 pH unit over an 8-in. chart width.

Experimental Results

Figure 1a is a photograph of the recorded absorbancy changes of chromatophores showing a rapid light-induced change and the decay of this absorbancy change upon termination of illumination. Figure 1b represents the absorbancy changes seen in the presence of BTB. The light-induced change occurs in two distinct steps, the first being rapid, the second being slow and following first-order kinetics (Figure 6). The decay of the light-induced signal in the dark is relatively slow (Figure 6).

As indicated earlier, most absorbancy changes were measured at 6100 A near the maximum of the absorption spectrum of BTB. An objection might be raised that illuminated chromatophores show appreciable light-induced absorbancy changes in this region (with a maximum at 6050 A). The rapid absorbancy changes in the presence of BTB, however, are three to four times larger than those of the control chromatophores without BTB; also they are clearly observable between 6200 and 6400 A, in which region light-induced optical density changes in the chromatophores are minimal. The magnitude of the total absorbancy change in the presence of BTB at the completion of the slow change

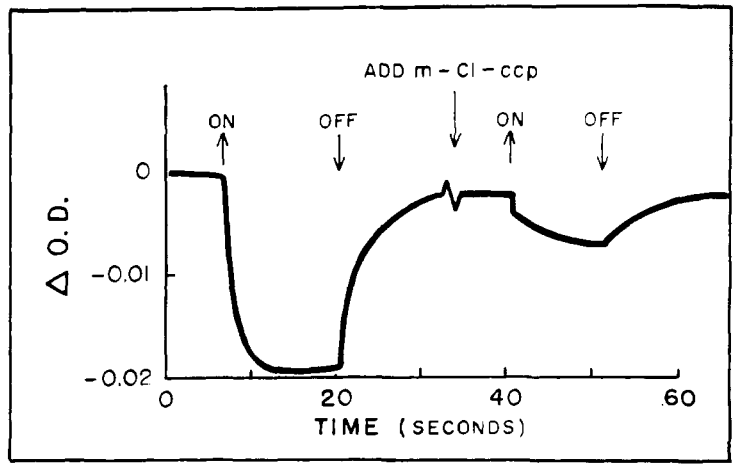

FIGURE 2: Effects of the addition of *m*-Cl-ccp on the light-induced color changes of BTB (tracing). *m*-Cl-ccp (1.0×10^{-6} M) was added to reaction mixture given in Figure 1b. *m*-Cl-ccp (1.0×10^{-5} M) caused no further effect.

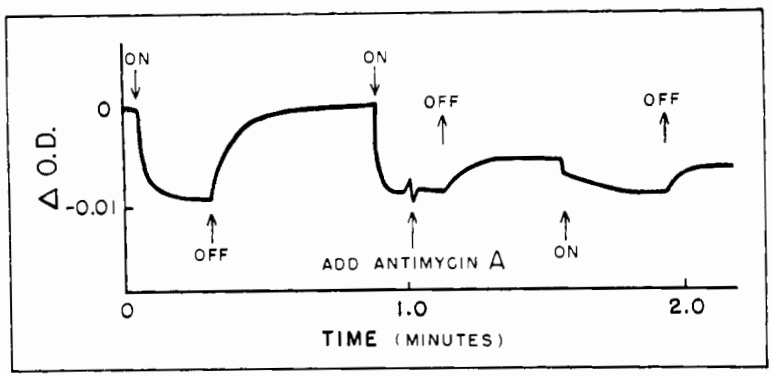

FIGURE 3: Effect of the addition of antimycin A on the light-induced color changes of BTB (tracing); 4×10^{-6} M antimycin A added to reaction mixture given in Figure 1b. Addition of 4×10^{-5} M antimycin caused no further effect.

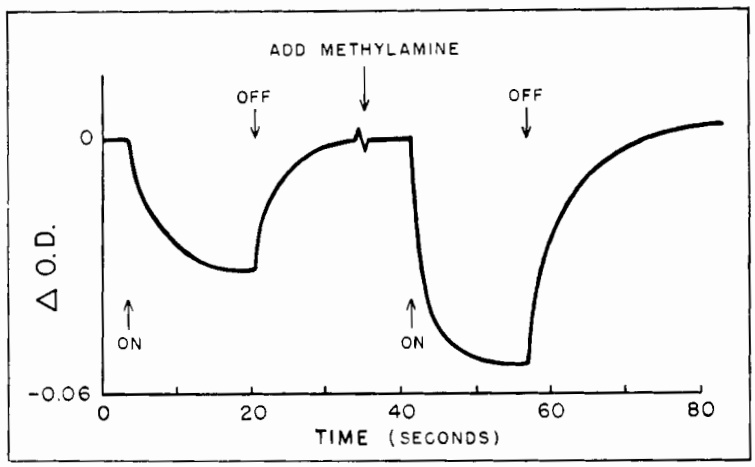

FIGURE 4: The effect of the addition of methylamine on the light-induced color change of BTB (tracing). CH_3NH (2.5×10^{-3} M), pH 7.7, added to reaction mixture given in Figure 1b.

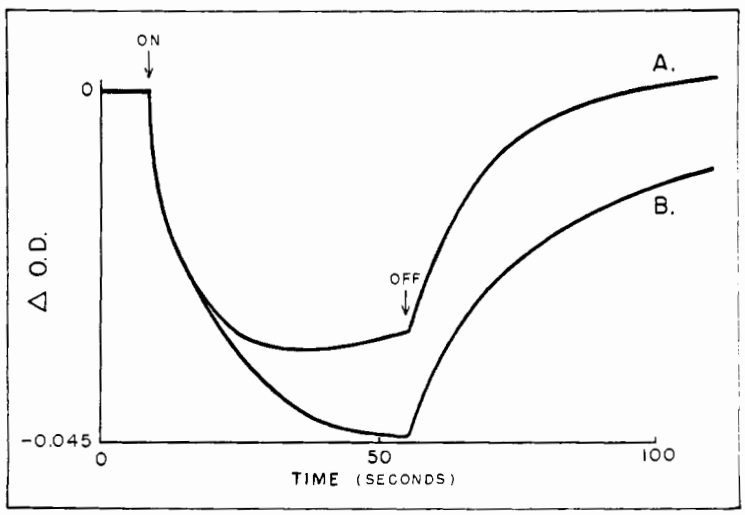

FIGURE 5: Time course of the light-induced BTB color change in the presence of high buffer concentration (tracing). (A) Bacteriochlorophyll (3×10^{-5} M), BTB (2×10^{-5} M), and glycyclglycine (4×10^{-1} M) buffer, pH 7.7. (B) Same as A but with 5×10^{-1} M Tris buffer, pH 7.7.

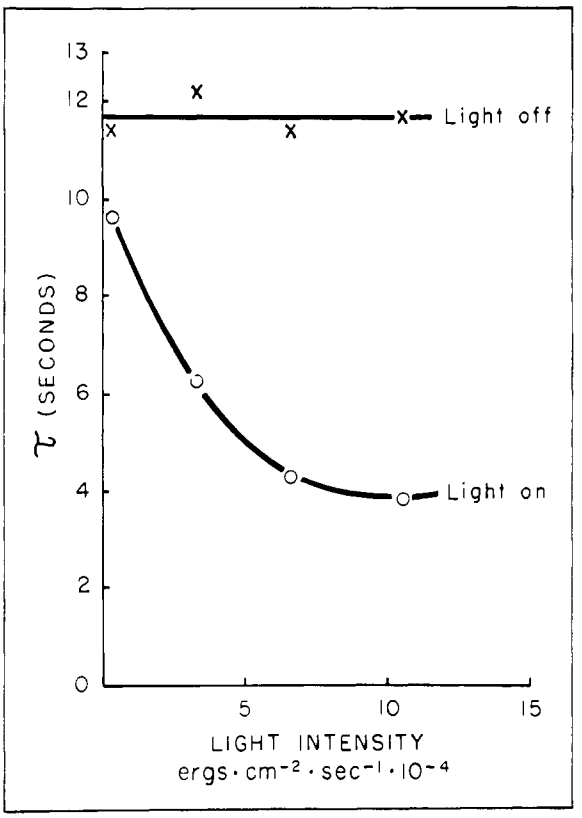

FIGURE 6: The effect of light intensity on the kinetics of the rise of the light-induced color changes (O—O) and on the kinetics of the decay of the reverse reaction in the dark (×—×). τ applies only to the second "slow" absorbancy change.

differs by an order of magnitude from the control change without BTB (Figure 1).

BTB is blue at alkaline pH and orange at acid pH; it has a pK of 7.0. The dye is not strongly bound to the chromatophores, since after several washings by centrifugation the 6100-A peak of the dye disappears from the chromatophore sediment.

Measurements of pH changes were carried out using an apparatus with an expanded pH scale similar to that cited above (Nishimura *et al.*, 1962). In a non-phosphorylating reaction mixture, without added buffer, no pH changes could be detected with this system (limit of detection 6×10^{-4} of a pH unit). When orthophosphate, ADP, and $MgCl_2$ were added in order to convert the system to a phosphorylating one, the pH meter did record an increase in pH upon illumination, but the optical density changes due to BTB were not affected. In a control experiment, it was observed that BTB, at the low concentrations used in these experiments, had no effect on the rate of light-induced phosphorylation as judged from pH changes monitored with a glass electrode. In order to determine the relationship of these changes in BTB absorption to light-induced electron flow and phosphorylation in the chromatophores, several inhibitors of electron transport and substances considered to be uncouplers of phosphorylation from electron transport were tested in the reaction mixture containing chromatophores plus BTB. From Figure 2, it is evident that after addition of the "uncoupler" of photophosphorylation, *m*-Cl-ccp (Heytler, 1963), the color change due to BTB was diminished, leaving only about 24% of the light-induced changes observed in the control system not containing *m*-Cl-ccp, and the rapid absorbancy change diminished to the control value without the dye.

Figure 3 illustrates the effect of antimycin A, an inhibitor of electron flow (Nishimura, 1963; Baltscheffsky *et al.*, 1960). The concentration of antimycin A was adjusted to assure almost complete inhibition of photophosphorylation (Baltscheffsky and Baltscheffsky, 1960). It is apparent that the magnitude of the reaction in the presence of antimycin A is diminished, while the rapid jump seen in the presence of BTB returned to the control value. Addition of oxidized PMS to the antimycin A inhibited mixture caused a complete reversal of the inhibition, while PMS alone caused only a slight increase in the magnitude of the absorbancy change. Methylamine, which Good has shown to be a potent "uncoupler" of photophosphorylation in chloroplasts (Good, 1962), causes the absorbancy change to proceed at a greatly enhanced rate and also causes the magnitude of the change to increase as seen in Figure 4.

In order to assure that there was no pH change in the suspending medium, the reactions were carried out in buffer solutions of increasing concentrations. Figure 5A illustrates the reaction as it occurred in 4×10^{-1} M glycylglycine buffer at pH 7.75 and in 5×10^{-1} M Tris buffer at pH 7.6. It is evident that the reactions were not inhibited with increased buffer capacity; there was even a stimulation of the magnitude of the reaction. Extreme caution had to be taken in the use of these buffers, since the dissociation constant of the amino group of glycylglycine is temperature dependent; thus, changes in temperature can alter the absorbancy of BTB dissolved in such a buffer. Once this was recognized, it became possible to utilize the dye also as an internal temperature indicator. Under no circumstances could heating and cooling of the reaction mixture produce the kinetics of the color changes observed in the light-induced reaction. The light reaction was inactivated 75% when incubated at 60° for 2 min.

Figure 6 illustrates the relationships of the rates and magnitude of the color changes to light intensity. Upon illumination, the rates increase with increasing light intensity, while the rates of the reverse reaction upon cessation of illumination are independent of the intensity of previous illumination and have τ values comparable to those seen at low light intensity. Color changes were not observable below an incident intensity of red light of 2×10^3 ergs cm^{-1} sec^{-1}.

Discussion

It is evident from the results presented above that

upon illumination of chromatophores in the presence of BTB, reactions occur in the system which lead to absorbancy changes primarily due to the dye. These absorbancy changes occur in two stages, a rapid one followed by a relatively slow one. Both these changes are susceptible to "uncouplers" of light-induced phosphorylation as well as to inhibitors of electron transport; this points to the requirement of an operational electron transport system and possibly certain components of the phosphorylation system for these reactions.

The fact that this reaction is observable in the presence of strong buffers indicates that the color change of BTB does not occur in the suspending medium. It does not, however, preclude the movement of H^+ to and from the surrounding medium as would presumably be seen in weakly buffered chromatophore suspensions. When more information becomes available, it is possible that the differences between our observations regarding pH differences measured with glass electrodes as reported above and those of other laboratories (Baltscheffsky, 1966) may be readily resolved. Should the observed absorbancy change be the result of a pH change, it is in the opposite direction of the pH changes observed in a photophosphorylating system (Nishimura *et al.*, 1962). Upon illumination the color change is in the direction of an apparent acid production, while the reverse is true upon cessation of illumination. Since under conditions of strong buffering pH changes in the suspending medium would be minimized, the color changes in the absence of net phosphorylation could not be due to a pH change in the medium. Thus, the color change may be due to a localized change in proton concentration which, in the case of the changes in the presence of the BTB, would not involve excretion of free protons into the surrounding medium. The absorbancy could be limited to a region of the chromatophores which is accessible to the BTB. These observations may be a reflection of structural changes comparable to those reported to occur in intact *Rhodospirillum rubrum* and also in chromatophores prepared from these cells (Packer *et al.*, 1963). Such light-induced structural changes could conceivably expose a variety of reactive groups of the chromatophore proteins which would cause the observed color changes. The difficulty of measuring pH changes colorimetrically in the presence of proteins has long been recognized, and has been the subject of some discussion (Haurowitz, 1963). Consequently, discretion must be used in interpreting the observed color changes in the BTB–chromatophore system.

In connection with the observations reported here, it is of interest that Antonini used this dye in an attempt to study the kinetics of proton production by hemoglobin during oxygenation, only to find that the indicator reacted with hemoglobin and had an effect on the oxygen equilibrium (Antonini *et al.*, 1963). These investigators suggested that the "kinetics of the dye reaction reflect in a most sensitive way, differences or changes of conformation of the protein molecules involved," and that such kinetic studies appear to be a useful experimental method for the study of such conformational differences or changes.

Acknowledgment

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Added in Proof

The following publication dealing with pH changes in *Rhodospirillum rubrum* chromatophores has appeared since the submission of this paper: Chance, B., Nishimura, M., Avron, M., and Baltscheffsky, M. (1966), *Arch. Biochem. Biophys. 117*, 158.

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