The Effects of Phenazine Dyes and $N,N',N'-$Tetramethyl-$p$-phenylenediamine upon Light-induced Absorbance Changes and Photophosphorylation in *Rhodospirillum rubrum* Extracts

(David M. Geller)

*(Received for publication, September 30, 1968)*

From the Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

**SUMMARY**

The effects of *N*-methylphenazonium methosulfate (PMS) have been compared with three other phenazine dyes and $N,N',N'-$tetramethyl-$p$-phenylenediamine (TMPD). All accelerate the recovery of the initial absorbance changes in *Rhodospirillum rubrum* extracts induced by a 10-μsec flash, although only *N*-ethylphenazonium ethosulfate and TMPD stimulate photophosphorylation to the same extent as PMS. *N*-Ethyl-1,2-benzophenazonium perchlorate and *N*-ethyl-1,2,6,7-dibenzophenazonium chloride are less active.

Absorbance changes with the dyes give evidence for the presence of at least three steps. The first is the light reaction, occurring at the instant of a flash ($<25$ μsec), generating an oxidant and a reductant. This is represented by the initial flash-induced absorbance change, which is unaffected by the presence of dyes.

The second step, a slower reaction (complete in approximately 100 μsec with 0.5 mM reduced PMS), is the reduction of the photo-oxidant at the expense of reduced dye. This is evidenced by the marked stimulation of recovery of the initial flash-induced absorbance change by reduced dye, coupled with the appearance of oxidized dye (TMPD). The kinetics is monophasic first order, in contrast to the slow polyphasic kinetics of recovery in absence of dye. The initial rate of recovery of absorbance change is directly proportional to reduced dye concentration. The oxidation of TMPD induced by a single saturating 10-μsec flash is directly proportional to extract concentration and represents one-thirtieth of the bacteriochlorophyll content. Double flash experiments show that the initial flash-induced absorbance changes signal saturation of photochemical reaction centers. Maximal utilization of a second flash for dye photo-oxidation requires complete recovery of the initial absorbance changes induced by the first flash.

The third step is the reduction of oxidized dye at the expense of reductant formed in the light reaction, completing the cyclic process of electron transport. Reduction of photo-oxidized TMPD is stimulated markedly by repetitive flashes. Reduction of TMPD following a single flash is very slow ($t_1 \sim 1$ sec), whereas reduction in repetitive flashes is complete in less than 50 msec (with a flashing rate of 20 per sec). The slowness of dye reduction compared to dye photo-oxidation is evidenced by the accumulation of oxidized dye during repetitive excitation. In continuous illumination the level of oxidized dye ($\sim 20$-fold that formed in by single 10-μsec flash) approximates the bacteriochlorophyll content.

A study of photophosphorylation in flashing illumination indicates that addition of PMS or TMPD lowers the yield of ATP formed in a 10-μsec flash. With TMPD, the 10-μsec flash yield represents 0.05 ATP per electron (TMPD photo-oxidized). Double flash experiments show that maximal utilization of a second flash for phosphorylation (as with dye photo-oxidation) requires complete recovery of the initial absorbance change induced by the first flash. These experiments show that the rapid recovery of photochemical reaction centers induced by both TMPD and PMS allows accumulation of intermediates involved in photophosphorylation during repetitive excitation. The dyes thus increase the flash yield of ATP obtained with 2-msec flashes. The rate of phosphorylation in darkness following a 2-msec flash also is increased by the dyes.

*This work was supported by Grant GM-07023-09 from the National Institutes of Health, United States Public Health Service.*
stimulates photophosphorylation resistant to antimycin A (3) and accelerates the conversion of flash-induced intermediates to ATP (6). The dye-mediated pathway also may bypass sites involved in photophosphorylation since the PMS system has a lowered quantum efficiency (7).

This paper is a report of work initiated by the interesting report of Parson (8), who found (in accordance with Cost, Bolton, and Frenkel (9)) that reduced PMS markedly stimulated the recovery of flash-induced absorbance changes in R. rubrum extracts. Moreover, he did not observe oxidation of PMS following a flash, although the dye was oxidized by continuous illumination.

In the present work the involvement of PMS in photophosphorylation in R. rubrum has been studied by observing the effects of other phenazine dyes and N,N',N',N'-tetramethyl-p-phenylenediamine upon light-induced absorbance changes. TMPD was especially interesting because it stimulated photophosphorylation to the same degree as PMS and had more desirable spectral characteristics.

Light-induced absorbance changes attributed to TMPD have been compared to (a) light-induced absorbance changes of pigment(s) in R. rubrum extracts and to (b) effects of TMPD upon the flash-induced formation of ATP. Parallel experiments with PMS also are reported. The results support the concept of the cyclic nature of the photo-induced electron transport with the dye supplemented system. Three steps may be distinguished:

Bacteriochlorophyll + A \rightarrow \text{bacteriochlorophyll}^+ + A^- (1)

Bacteriochlorophyll^+ + dye \rightarrow \text{bacteriochlorophyll} + dye^+ (2)

Dye^+ + A^- \rightarrow dye + A (3)

The first step, the light reaction, which may be very rapid (<1 \mu sec (8)), is represented by the initial flash-induced absorbance change. This is followed by two slower dark reactions. The more rapid of these is Step 2, with an initial rate directly proportional to reduced dye concentration. The slower second phase (Step 3) completes the cycle of electron transport, with reduction of the dye at the expense of photoreductant.

METHODS

Synthesis and Properties of Phenazines

N-Ethylphenazonium Ethosulfate—EPES (Fig. 1) was prepared by heating phenazine in diethyl sulfate and was crystallized from ethanol (10). Its molar absorptivity in water was assumed to be identical with PMS (11); the absorption spectrum (Fig. 2) was identical with PMS.

N-Ethyl-1,2-Benzophenazonium Perchlorate 1,2 Benzophenazine was synthesized by a procedure based upon the method of Witt (12). 1,2-Naphthoquinone (7.9 g, 50 mmole) and o-phenylene diamine (5.4 g, 50 mmole) were dissolved in 100 ml of warm glacial acetic acid. The solution was taken to dryness (water pump) and the residue was crystallized twice from ethanol, yielding 6.3 g of yellow needles, m.p. (uncorrected) 142-143°. The phenazine (1.5 mmole, 350 mg), dissolved in a mixture of dimethylformamide (6 ml) and diethyl sulfate (6 ml), was heated for 20 min at 160-170°. The cooled reaction mixture was applied to a silica gel column (50 mm diameter \times 30 mm, silica gel "for chromatography," 0.06 to 0.2 mm, E. Merck). After a wash with benzene, the ethylated phenazine was eluted with absolute ethanol. The ethanolic solution was concentrated to \sim 15 ml,
made 0.7 m in lithium perchlorate, chilled (0°C) and filtered. The residue was recrystallized from water (120 mg of orange crystals). With an assumed molecular weight of 359, the apparent molar absorptivity of the perchlorate salt (Fig. 1) is 1.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} at 436 nm in water (Fig. 2).

**N-Ethyl-1,2,6,7-Dibenzophenazonium Chloride**—The dibenzophenazine was prepared by fusion of 1,2-naphthalenediamine with 2,3-naphthalenediiodide under nitrogen, followed by oxidation in chronic acid (13). The phenazine was crystallized from chloroform and treated with diethyl sulfate with the procedure described with 1,2-benzophenazine. The ethanolic eluate from the silica gel column was concentrated and treated with excess saturated aqueous picric acid. The picrate, recrystallized from ethanol, was converted to the chloride by dissolving it in 6 M HCl and extracting the liberated picric acid with chloroform. The aqueous phase, taken to dryness under reduced pressure, yielded the chloride (brown crystals) (Fig. 1). In water, the apparent molar absorptivity at 475 nm was 1.3 to 1.4 \text{ M}^{-1} \text{ cm}^{-1} at 25°C for 5 to 30 min. Phosphorylation was measured by disappearance of inorganic phosphate (17) in the supernatant fluid obtained after the system was made 0.3 M in HClO₄ or 5% in trichloroacetic acid.

**Properties of Phenazine Dyes**

All three compounds were reduced by sodium hydrosulfite (Fig. 2) and (nonenzymatically) by DPNH. The dyes were autoxidizable: the reduced forms were unstable in air. The absorption spectra of the dibenzophenazonium salt were difficult to measure because of the apparent tendency to adhere to glass at the air-water interface.

**Synthesis of Oxidized TMPD** (Wurster's Blue)

Wurster's Blue was synthesized by oxidation of TMPD with bromine (14). TMPD (3.4 millimoles), 800 mg, was dissolved in a mixture of 10 g of sodium perchlorate in 14 ml of water and 23 ml of methanol and cooled to –10°C. To this solution was added (dropwise, with stirring) 25 ml of cold aqueous 0.12 M HCl and extracting the liberated picric acid with chloroform. The aqueous phase, taken to dryness under reduced pressure, yielded the chloride (brown crystals) (Fig. 1). In water, the apparent molar absorptivity at 475 nm was 1.3 to 1.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} (assumed molecular weight, 345) (Fig. 2).

**Preparation and Purification of Extracts**

Batch cultures of *R. rubrum* (strain S-1 of van Niel) were grown in the light and harvested, and crude extracts were made in 0.2 M glycylglycine (pH 8, 0°C) with a French pressure cell as previously described (15). The crude extract (37,000 \times g supernatant) was centrifuged for 3 hours at 100,000 \times g. The supernatant fluid was discarded and the precipitate was resuspended in an equal volume of glycylglycine buffer and centrifuged again for 1 ½ hours at 100,000 \times g. The precipitate was resuspended in 0.2 M glycylglycine buffer and stored in the dark at 0°C. These preparations retained at least 80% of their initial activity (phosphorylation) for 1 month (after which time they were discarded).

**Measurement of Photophosphorylation in Continuous Illumination**

The basic system used consisted of *R. rubrum* particles (not in excess of 60 \mu M bacteriochlorophyll) in 50 mM glycglycine, 5 mM MgCl₂, 5 mM potassium phosphate, 20 mM glucose, 0.1 mM ADP, and hexokinase (1.7 \mu M per ml), final pH 7.0. The molar absorptivity for bacteriochlorophyll in aqueous suspensions of *R. rubrum* particles was 1.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} at 880 nm (16). For aerobic assay, 100-\mu l volumes were placed in test tubes, 10 \times 75 mm. For anaerobic assays, 500-\mu l volumes in 15-ml Warburg flasks (with particles initially in the side arm) were flushed for 4 min with previously purified argon (Linde) washed with 10% alkaline pyrogallol. In all experiments with TMPD, the flasks were gassed for 2 min before the addition of TMPD, followed by an additional 4 min afterward. At concentrations of 1 mM or above, TMPD solutions (freshly prepared) were neutralized with KHCO₃ (at 0°C) just before addition to the flasks.

Illumination was provided by red light (in excess of saturation) at 25°C for 5 to 30 min. Phosphorylation was measured by disappearance of inorganic phosphate (17) in the supernatant fluid obtained after the system was made 0.3 M in HClO₄ or 5% in trichloroacetic acid.

**Preparation of Samples for Light-induced Absorbance Changes**

All observations were made under anaerobic conditions with a cuvette of conventional design (19). The cuvette contained *R. rubrum* particles in the basic system used for photophosphorylation, with appropriate additions. The cuvette was flushed 10 min with argon (previously purified, Linde, washed with 10% alkaline pyrogallol) bubbled through the contents of the body of the cuvette; the gas was vented from the side arm which contained a shallow layer of *R. rubrum* particles and hexokinase. With TMPD, the gassing procedure was modified. The side arm was removed and the body alone was flushed with argon for several minutes; TMPD (freshly neutralized with KHCO₃ if the final concentration was to be 1 mM or greater) then was added to the body, the side arm assembly was attached, and the entire cuvette was flushed for 10 min. The cuvette was sealed under pressure, mixed, and placed in the photometer.

**Rapid Recording Photometer**—This instrument was a modification of that previously described (20). The intensity of the measurement beam (360 to 640 \mu M band width 1 \mu M) transmitted by the cuvette containing the extract (optical path, 2 mm) was monitored by an EM1 95248 photomultiplier positioned directly behind the cuvette. The photomultiplier was protected from infrared flash light by Corning filter 4784. The cuvette was irradiated at 90° to the measurement beam by infrared light (filtered through two Wratten 88A filters).
The infrared light intersected the measurement beam (an image of the monochromator slit, ~1 mm wide) after traversing 1 mm of the solution in the cuvette.

The infrared beam was provided either by an Osram xenon 450-watt lamp or by a xenon flash tube. The xenon lamp was provided with lenses, a heat filter (3 cm of flowing water), and a mechanical shutter (20). The flash tube (FX-12-25) was provided with a parabolic mirror and lenses. The power supply for the flash tube consisted of a 4-µF capacitor bank (charged to a maximum of 1.4 kv for 4-J flashes). The tube was ignited by a pulse from a TM-11 Trigger Module. The flash was 10 µsec (one-peak to one-third peak) with a tail of 15-µsec duration.

The power supply also was modified to provide two flashes of equal intensity. Two 4-µF capacitor banks were provided, each isolated from the flash tube by a GP-16B spark gap. Each spark gap was controlled by a separate Trigger Module. Each Trigger Module also was connected to the flash tube by an RCA 1X2B diode. This design permitted one Trigger Module to ignite one spark gap and the flash tube simultaneously without also firing the second spark gap. The Trigger Modules were controlled by a Tektronix 160 series pulse generator. With this arrangement the minimum separation between two flashes was 50 µsec. The flash tube, spark gaps, and Trigger Modules were obtained from Edgerton, Germeshausen, and Grier, Inc. (Boston, Massachusetts). The circuits were patterned after Edgerton, Germeshausen, and Grier data sheets No. 1002B and No. TSG-100.

The flash was monitored by an SGD-100 silicon photodiode (Edgerton, Germeshausen, and Grier, Inc.) which triggered the oscilloscope to record the change in photomultiplier output following the flash. With suitable shielding of the flash tube (grounded sheet metal enclosure) it was possible to observe absorbance changes within 25 µsec of the onset of the flash.

Repetitive Flash Apparatus—The mechanical flash generator (used for 2-msec flashes) consisted of a 450-watt Osram xenon lamp, fitted with lenses and 3 cm of flowing water (heat filter), controlled by the double sector disc combination previously (90). The fast disc was operated at 2700 rpm. The image of the arc (filtered by a single Wratten 88A filter) was focussed upon the sample (2-mm optical path) held in a 25° thermostat (described above). Light intensity was measured as previously described (16).

The electronic flash apparatus consisted of two flash tubes 4 cm apart with the sample (2-mm optical path) held in a 25° thermostat (described above) midway between them. Each flash tube was provided with one Wratten 88A filter. For double flash experiments two FX-11-25 tubes were used, each supplied with a 4-µF capacitor (charged to 1 kv) and a TM-10 Trigger Module controlled by a Tektronix 160 series pulse generator. One flash tube was fired once every 400 msec; the second tube was fired at a set interval (up to 200 msec) after the first. The sequence resulted in one flash of double intensity per 400 msec (simultaneous firing) at one extreme, to one flash per 200 msec (200-msec delay) at the other. A delay in firing the second tube of less than 200 msec gave one pair of flashes every 400 msec.

The electronic flash apparatus also was used for multiple flash experiments. For this purpose two FX-108B tubes (Edgerton, Germeshausen, and Grier, Inc.) were used. Each tube could deliver a maximum of seven 1-J flashes at one flash per 20 msec. When operated so that the second tube fired 10 msec after the first, the result was a train of 14 1-J flashes with one flash per 10 msec. This multiple firing overtaxed the power supply, with the result that the first two flashes were 10% more intense than each of those which followed. Separate experiments indicated that this was not significant since the weaker flashes were in excess of saturation. The apparatus delivered one train of flashes per sec.

Reagents

Hexokinase and glucose-6-P dehydrogenase were crystalline (Boehringer). Chemicals used for synthetic work were the purest grades available, from Eastman Kodak and Aldrich. Silica gel “for chromatography,” 0.05 to 0.2 mm, E. Merck, was obtained from Drickmann Instruments. TMPD as the dihydrochloride was a product of Eastman Kodak, and PMS was obtained from Sigma.

RESULTS

Effects of Dyes upon Light-induced Absorbance Changes—The failure of Parson (8) to observe oxidation of reduced PMS with R. rubrum extract following a flash was based upon the observation that dichlorophenol-indophenol also gave similar spectral changes in the region associated with PMS oxidation (388 mp). These results, confirmed in the course of this work, suggested that a component of R. rubrum extract may be responsible for these absorbance changes. This led to a study of the effects of other phenazine dyes.

The first phenazine tested was EPES. This had an absorption spectrum identical with PMS (I in Fig. 2). EPES, reduced in the presence of succinate (see "Methods"), markedly stimulated the decay of initial flash-induced absorbance changes (0.025 msec after a flash) which are attributed to pigment(s) in R. rubrum extract. The same absorbance changes are found in the absence of dye. These initial bands are replaced by slowly recovering (4 ~ 1 sec) bands at 385, 425, and 450 mp (measured at 2 msec after a flash). Reduced PMS gave the same results. The 387 mp band thus might represent oxidized dye, with the 450 mp band a contribution of the semiquinone (II).

The second phenazine examined was the monobenzophenazine, N-ethyl-1,2-benzophenazonium perchlorate (II in Fig. 2). This dye, reduced in the presence of succinate, also markedly stimulated the decay of the usual flash-induced bands (q ~ 100 µsec at 0.1 mm dye). The magnitude of the initial difference spectrum shown in Fig. 2 is somewhat depressed because of the late observation time (0.05 msec). Slowly decaying bands (q ~ 150 msec) then appear at 385 mp and 435 mp, with a shoulder at 450 mp (1 msec following the flash). The novel 435 mp band here may represent oxidized dye.

Analogous results were obtained with the third phenazine, N-ethyl-1,2,6,7-dibenzophenazonium chloride (III in Fig. 2), in the presence of succinate. The initial flash-induced bands (measured at 0.1 msec) decayed more slowly (q ~ 20 msec with 0.033 mm dye) and were replaced by bands at 385 mp and 477 mp (450 mp shoulder), decaying with q ~ 500 msec (measured at 200 msec). The 477 mp band represents oxidized dye.

The results of the experiments with the benzophenazines favor the view that oxidation of dye follows a 10-µsec flash. The absorbance changes due to oxidized PMS or EPES are partially masked by absorbance changes due to a component of
the extract at 385 μ. With all of these dyes, however, it still is necessary to distinguish between absorbance changes due to extract and the dye. This problem has been solved with TMPD. TMPD is most useful in the analysis of the effect of dyes upon light-induced absorbance changes, for two reasons: (a) at 585 μ, which is an isosbestic point for absorbance changes associated with R. rubrum extract, the molar absorptivity of oxidized TMPD is 8.6 × 10³ M⁻¹ cm⁻¹ (the reduced dye is colorless), and (2) TMPD stimulates photophosphorylation to the same degree as PMS.

The results of the effects of TMPD upon flash-induced absorbance changes are shown in the upper half of Fig. 3. In the presence of 3 mM dye the initial flash-induced bands (measured at 0.2 msec following the flash) decay rapidly (t₁ = 1.5 msec), with the simultaneous appearance of slowly decaying bands (t₂ = 1.5 sec) at 565 to 615 μ and 385 μ. The former broad band is due to oxidized TMPD (Wurster’s Blue) with maxima at 566 and 613 μ (14, 21). The spectrum of Wurster’s Blue is the dashed curve in the lower half of Fig. 3. The 385 μ band, observed with every dye (cf. Fig. 2), must represent a component of R. rubrum extract.

At this point it was of interest to compare the effects of continuous illumination with a 10-μsec flash. The concentration of oxidized TMPD formed in continuous illumination was about 20-fold the maximal level induced by a single 10-μsec flash (Fig. 3). This difference could not be explained on the basis of insufficient flash energy. Halving the intensity of the 10-μsec flash produced the same result. The other difference between the two modes of illumination concerned the duration of illumination. This led to a series of experiments concerned with the effects of multiple flashes, which are discussed below.

**Relationship between TMPD Photo-oxidation and Light-induced Absorbance Changes**—The first experiments with TMPD were concerned with its effects upon absorbance changes induced by single 10-μsec flashes. The results strongly support the concept that the initial photochemical event consists of the generation of an oxidant which then reacts with reduced dye in a second step.

Both the maximal amount of TMPD oxidized (followed at 585 μ) and the magnitude of the initial flash-induced absorbance change attributed to extract (followed at 435 μ) are directly proportional to extract concentration (Fig. 4). Furthermore, the recovery rate of the flash-induced change at 435 μ and the appearance of oxidized dye (at 585 μ) are (within experimental error) both first order and apparently simultaneous (Fig. 5). The first order rate constants are directly proportional to TMPD concentration (Fig. 6). Added oxidized TMPD (up to 1 mM) has no effect upon the rates of absorbance changes. It should be noted that parallel experiments with PMS show that the effects promoted by PMS are much more rapid (Fig. 6).

**Effects of Repetitive Flashes upon TMPD Oxidation and Reduction**—These experiments show that the increased oxidation of TMPD found in continuous illumination compared to a 10-μsec flash (Fig. 3) is due to the prolonged duration of illumination. With a short saturating flash, all of the primary photochemical centers are utilized. Re-excitation of these sites must await their recovery by reaction with “trapping systems” which utilize electrons provided by the dye. An extension of the period of illumination thus allows repeated excitation of these photochemical reaction centers.
Absorbance Changes and Photophosphorylation in R. rubrum

The effects of repeated excitation initially were studied in double flash experiments with TMPD (Fig. 7). Two saturating flashes of equivalent energy were generated. The interval between flashes was varied, as absorbance changes were measured at 435 nm (R. rubrum pigment) and 585 nm (TMPD). Since photo-oxidized dye is reduced slowly ($t \sim 1$ sec in Fig. 7), a doubled increment in absorbance at 585 nm would be expected with two flashes. This in fact occurs if the flashes are spaced 10 msec or more apart (with a TMPD concentration of 3 mM). However, as the interval between flashes is diminished there is a progressive decrease in yield of oxidized dye induced by the second flash. With the second flash, the additional yield of oxidized dye is directly proportional to the increment of absorbance at 435 nm. Maximal utilization of light for dye oxidation thus requires prior removal of the initial photo-oxidant generated by the first flash.

In order to extend the results of the double flash experiments, the effects of repetitive flashing upon extract supplemented with TMPD (3 mM) were followed. This work disclosed that repetitive flashing markedly stimulated the reduction of TMPD oxidized following a flash. An example of this is an experiment in which the absorbance at 585 nm was followed as the preparation was exposed to two flashes per sec (Fig. 8a). Initially the absorbance at 585 nm increases with each flash. After some seconds of flashing a steady state is reached: the absorbance recovery between each flash is equivalent to the flash-induced increment. It should be noted that the flash induced increment in the steady state is equivalent to that produced by the first flash (upper and lower curves, respectively, in Fig. 8a). In a separate experiment with a more rapid flashing rate (20 per sec, Fig. 8, b to e), the results at 585 nm were compared to absorbance changes at 435 nm. At the start of the experiment the effect of a single flash was recorded at 435 nm (Fig. 8d) and 585 nm (Fig. 8e). The absorbance changes at 585 nm induced by one flash (Fig. 8d) decays very slowly. After repetitive flashing for several seconds to allow a steady state to be achieved at 585 nm, the absorbance changes at 435 (Fig. 8c) and 585 nm (Fig. 8e) were recorded again. Reduction of photo-oxidized TMPD now is complete within the 50-msec interval between flashes. The kinetics at 435 nm is unaffected by repetitive flashing. For technical reasons it was not possible to produce saturating flashes at a frequency in excess of 20 per sec.

At the present time positive evidence regarding the source of the stimulatory effect of repetitive flashing upon dye reduction is lacking. The stimulation is not due to the increased concentration of oxidized dye which invariably accompanies repetitive excitation. Added oxidized TMPD (up to 1.1 mM) in the presence of TMPD (3 mM) fails to accelerate dye reduction following a single flash; repetitive flashing is still required. The most likely factor in stimulation of dye reduction therefore would seem to be the elevated concentration of a photoreductant during repetitive excitation. Accumulation of a photoreductant under these conditions would be expected as a consequence of the elevated concentration of oxidized dye. The system is closed, under anaerobic conditions, and the amount of dye reduced following cessation of illumination is equivalent to that oxidized during repetitive excitation.

Fig. 6. The effect of dye concentration upon the first order rate constant ($k$, sec$^{-1}$) for the recovery of flash-induced absorbance change. Q, the effect of TMPD concentration upon absorbance recovery at 435 nm (30 μM bacteriochlorophyll); □, the effect of PMS concentration upon absorbance recovery at 600 nm in the presence of 5 mM succinate (90 μM bacteriochlorophyll).

The effects of repeated excitation initially were studied in double flash experiments with TMPD (Fig. 7). Two saturating flashes of equivalent energy were generated. The interval between flashes was varied, as absorbance changes were measured at 435 nm (R. rubrum pigment) and 585 nm (TMPD). Since photo-oxidized dye is reduced slowly ($t \sim 1$ sec in Fig. 7), a doubled increment in absorbance at 585 nm would be expected with two flashes. This in fact occurs if the flashes are spaced 10 msec or more apart (with a TMPD concentration of 3 mM). However, as the interval between flashes is diminished there is a progressive decrease in yield of oxidized dye induced by the second flash. With the second flash, the additional yield of oxidized dye is directly proportional to the increment of absorbance at 435 nm. Maximal utilization of light for dye oxidation thus requires prior removal of the initial photo-oxidant generated by the first flash.

In order to extend the results of the double flash experiments, the effects of repetitive flashing upon extract supplemented with TMPD (3 mM) were followed. This work disclosed that repetitive flashing markedly stimulated the reduction of TMPD oxidized following a flash. An example of this is an experiment in which the absorbance at 585 nm was followed as the preparation was exposed to two flashes per sec (Fig. 8a). Initially the absorbance at 585 nm increases with each flash. After some seconds of flashing a steady state is reached: the absorbance recovery between each flash is equivalent to the flash-induced increment. It should be noted that the flash induced increment in the steady state is equivalent to that produced by the first flash (upper and lower curves, respectively, in Fig. 8a). In a separate experiment with a more rapid flashing rate (20 per sec, Fig. 8, b to e), the results at 585 nm were compared to absorbance changes at 435 nm. At the start of the experiment the effect of a single flash was recorded at 435 nm (Fig. 8d) and 585 nm (Fig. 8e). The absorbance change at 585 nm induced by one flash (Fig. 8d) decays very slowly. After repetitive flashing for several seconds to allow a steady state to be achieved at 585 nm, the absorbance changes at 435 (Fig. 8c) and 585 nm (Fig. 8e) were recorded again. Reduction of photo-oxidized TMPD now is complete within the 50-msec interval between flashes. The kinetics at 435 nm is unaffected by repetitive flashing. For technical reasons it was not possible to produce saturating flashes at a frequency in excess of 20 per sec.

At the present time positive evidence regarding the source of the stimulatory effect of repetitive flashing upon dye reduction is lacking. The stimulation is not due to the increased concentration of oxidized dye which invariably accompanies repetitive excitation. Added oxidized TMPD (up to 1.1 mM) in the presence of TMPD (3 mM) fails to accelerate dye reduction following a single flash; repetitive flashing is still required. The most likely factor in stimulation of dye reduction therefore would seem to be the elevated concentration of a photoreductant during repetitive excitation. Accumulation of a photoreductant under these conditions would be expected as a consequence of the elevated concentration of oxidized dye. The system is closed, under anaerobic conditions, and the amount of dye reduced following cessation of illumination is equivalent to that oxidized during repetitive excitation.

Effect of Dyes upon Photophosphorylation in Continuous Illumination—The dyes used in absorbance experiments were tested for their effects upon photophosphorylation (Fig. 9). In the presence of succinate (3), the ethylated phenazine (EPES) stimulated photophosphorylation in continuous illumination to the same degree as PMS. Much higher levels of TMPD (1 to 3 mM) were required for maximal stimulation of photophosphoryla-
Effect of TMPD and PMS upon Photophosphorylation in Flashing Light—It was of interest to compare the results of the absorbance experiments with the effect of the dyes upon the kinetics of photophosphorylation. These experiments initially used a rotating sector disc which generated 2-msec flashes for measurements of phosphorylation following a flash (see "Methods"). The report that PMS accelerates phosphorylation following a flash (6) has been confirmed in the present experiments (Fig. 10). TMPD also shows this effect. A maximal flash yield with either dye requires more than 40 msec of darkness between flashes. The results with succinate are in agreement with earlier experiments which indicate that a dark

![Fig. 8. Accumulation of photo-oxidized TMPD in a series of flashes: acceleration of reduction of photo-oxidized TMPD during repetitive flashes.](image)

![Fig. 9. The effect of PMS, EPES (PES), and TMPD upon photophosphorylation in continuous illumination.](image)

![Fig. 10. The influence of dyes upon the relationship of flash frequency to flash yield of ATP.](image)
period of at least 150 msec is required for maximal phosphorylation following a flash (20). All three curves follow first order kinetics, with values of \( k \) of 20 sec\(^{-1} \) (succinate), 60 sec\(^{-1} \) (PMS + succinate), and 87 sec\(^{-1} \) (TMPD).

It should also be noted that the dyes not only accelerate phosphorylation following a flash, but also increase the maximal flash yield of ATP (Fig. 10). At the light intensity used (1.5 \( \times 10^4 \) ergs per cm\(^2\) per sec) the yield of a 2-msec flash apparently represents the maximum with this order of magnitude of light intensity; increasing the intensity to 2.5 \( \times 10^4 \) ergs per cm\(^2\) per sec did not change the flash yield. The increase in maximal flash yield by dyes varied with different extracts: in a separate experiment the maximal yield with PMS + succinate was twice that obtained with succinate.

Attention now turned to measurements of phosphorylation following short (10-\( \mu \)sec) flashes. This work duplicated the illumination conditions used in the flash-induced absorbance experiments. The results were of special interest in view of the report of Nishimura (23) of the marked increase in flash yield of ATP obtained in short intense flashes (compared to the mechanical sector disc apparatus).

Phosphorylation following a 10-\( \mu \)sec flash is directly proportional to extract concentration (Fig. 11). In marked contrast to the results with 2-msec flashes (Fig. 10), however, both TMCP and PMS lower the flash yield of ATP. The yield per flash with succinate alone in 2-msec flashes (Fig. 10) is equivalent to that obtained with a 10-\( \mu \)sec flash (Fig. 11) at equal bacteriochlorophyll concentrations.

These results with 10-\( \mu \)sec flashes differ markedly from comparable experiments of Nishimura (23), who reported a flash yield of 0.047 ATP per bacteriochlorophyll. The comparable value calculated from the succinate data of Fig. 11 is 0.022. Attempts to increase the flash yield with succinate so far have failed: (a) doubling the flash intensity (at 20 \( \mu \)mols bacteriochlorophyll) by firing two flash tubes simultaneously instead of in alternation (see "Methods"); (b) increasing the flash energy 5-fold (to 5 J), with a 2.5-fold increase in flash duration (to 25 \( \mu \)sec), with a flash rate of one per sec; and (c) increasing the duration of the 5-J flash to 0.5 msec. Nishimura used a 45-J 0.5-msec flash, with an irradiant energy of 0.086 J per cm\(^2\), at a rate of one flash per sec. In the present work a 1-J 10-\( \mu \)sec flash with an irradiant energy of 0.02 J per cm\(^2\), has been used (increased to a maximum of 0.1 J per cm\(^2\) in the 5-J flash experiments). It is most likely that differences in results must be related to the extract or the analytical methods used. At low levels of phosphorylation the direct fluorometric analytical methods (used here) may give a more reliable result than the measurement of pH change used by Nishimura.

In subsequent work the kinetics of flash-induced phosphorylation was measured. This was accomplished by a double flash system patterned after the double flash absorbance experiments (Fig. 12). Two flash tubes were used (see "Methods"); a pair of flashes was generated repetitively by firing the first tube every 400 msec, followed by the second within 200 msec or less. With an interval of 200 msec, this procedure generated a series of single flashes at five per sec; with simultaneous firing, a series of single flashes of double intensity was generated at one per 400 msec. With this procedure, "100\%" in Fig. 12 represented the flash yield of ATP at five flashes per sec. Theoretically "0\%" (one flash every 400 msec) would give 50\% of the maximal yield. The 75\% point would thus represent half-utilization of the second flash.

The results obtained with succinate (Fig. 12) resemble the flash yield curves obtained with the sector disc (Fig. 10 and Reference 20), with a yield equal to 75\% of the maximum at 19 to 20 msec between flashes. This result is consistent with the kinetics of phosphorylation following a 2-msec flash (Fig. 10). With PMS and TMCP, however, the flash yield curves differ markedly from the results in Fig. 10. Furthermore, although both dyes induce a similar time course of phosphorylation following a 2-msec flash (Fig. 10), the flash yield curve of PMS in Fig. 12 rises more rapidly than that of TMCP. The 75\% maxima are reached at 0.2 and 2 msec, respectively. Preliminary experiments indicate that this time course in the presence of dye may be directly proportional to dye concentration: with a PMS concentration of 0.04 mM (one-tenth that in Fig. 12) the 75\% maximum is attained in 1.5 msec.

With the dyes the time course of the flash yield curve (Fig. 12) is very similar to the kinetics of the flash-induced changes (Fig. 6). The flash-induced change in absorbance with 3 mM TMCP recovered with a half-time of 1.5 msec (Fig. 5); a separate experiment with 0.4 mM PMS (measured at 600 mp) gave a corresponding half-time of 0.1 msec. These are to be compared...
FIG. 13. The influence of PMS and TMPD upon the flash yield of ATP in multiple flashes. The procedure followed was the same as Fig. 11 except for the difference in mode of flashing. Flashes (1-10 msec) were applied as a train, recurring one per sec. The number of flashes in a train varied from one to a maximum of 14. The interval between flashes in each train was 10 msec (see "Methods"). For example, at 14 flashes per see, the samples received a train of 14 flashes (with a dark interval of 10 msec between each flash) which was repeated once every second. Each sample (in duplicate) was exposed to a total number of flashes sufficient for analytical purposes: this varied from 900 (15 min of flashing at one flash per sec) to 2940 (3 hr min of flashing at 14 flashes per sec). With a bacteriochlorophyll concentration of 20 \( \mu \text{M} \), circles represent 0.4 mM succinate, squares 3 mM TMPD, and triangles 0.4 mM succinate-0.4 mM PMS.

with the noted values for (the 75% maxima of) the 10-msec flash yield of ATP of 2 and 0.3 msec, respectively. At this time it may be tentatively concluded that with the dyes the absorbance recovery and flash yield curves coincide, within experimental error.

One unexpected result in these experiments was the low flash yield repeatedly found with PMS at low flash frequencies. With simultaneous flashes (0 msec in Fig. 12) the flash yield with PMS is 30% of that obtained with flash pairs in which flashes were a minimum of 1 msec apart. This is almost half of the theoretical yield (50%). These results raise the question of the effect of higher flash frequency upon flash yields with the dyes, the calculated flash yield of ATP rises with flash frequency. This is especially marked with PMS. The maximal value for the flash yield with both dyes is apparently equivalent to that obtained with succinate (at one flash per sec). The results with succinate show the marked decline in flash yield with increasing flash frequency consistent with the kinetics of phosphorylation following a flash (Fig. 10), and the results of the double flash experiment (Fig. 12).

**DISCUSSION**

The study of the effects of dyes upon photophosphorylation in *R. rubrum* extract has been greatly facilitated by the choice of TMPD: (a) the absorption spectrum of this dye makes possible the observation of changes in electronic state of the dye without interference from absorbance changes associated with pigments in the extract; (b) TMPD stimulates photophosphorylation to the same extent as PMS; and (c) only two species of TMPD molecules (reduced and free radical form (29)) exist, whereas three (reduced, semiquinone, and fully oxidized) forms must be considered with PMS (11).

The dye-supplemented extract displays evidence for at least two delayed events which follow the initial excitation by a saturating flash of light. In this work only absorbance changes in the visible region have been followed; it is tentatively presumed that this change show parallel effects (this has been observed with PMS (8)). As indicated in the introductory section, the initial stage, the light reaction, which may be very rapid (<1 msec (8)), is represented by the initial flash-induced absorbance change. The second step, a dark reaction, is a rapid reaction associated with oxidation of dye in which the rate is determined by the concentration of reduced dye. This may be complete in less than 100 msec with high levels (~0.5 mM) of reduced PMS or EPES. The third step is a slower sequence of reactions by which photoreductants reduce oxidized dye. The result is a cyclic process of electron transfer.

The evidence for the second step following the light reaction consists primarily of the accelerated recovery of flash-induced absorbance changes, coupled with oxidation of dye (TMPD, Figs. 5 and 6). The fact that the photo oxidation of TMPD is proportional to extract concentration (Fig. 4) favors the concept that the dye is interacting stoichiometrically with some component of the system. The data in Fig. 4 show 1.75 \( \mu \text{M} \) TMPD photo-oxidized following a flash in the presence of extract containing 50 \( \mu \text{M} \) bacteriochlorophyll. This represents 1 TMPD molecule photo-oxidized for every 30 bacteriochlorophyll molecules. The photosynthetic unit in intact *Rhodospirillum* cells has been estimated to contain 50 bacteriochlorophyll molecules (24). It is of interest also to note that the value of 30:1 approximates the estimated ratio of bacteriochlorophyll to cytochrome (19:1, according to Nishimura (23)). The behavior of this system in double flash absorbance and phosphorylation experiments (Figs. 7 and 12) indicates that this might represent one photochemical site (bacteriochlorophyll in a specialized environment (25)) in 30 bacteriochlorophyll molecules. With a saturating flash, the initial absorbance changes signal saturation of photochemical systems involved in phosphorylation. Useful absorption of light does not occur until recovery of these initial absorbance changes.

The nature of the third step, reduction of oxidized dye, has been inferred only from negative evidence. Reduction of TMPD is markedly stimulated by repetitive flashes (Fig. 8). Although this process of repetitive excitation is invariably associated with an elevated concentration of oxidized dye, added oxidized dye does not stimulate dye reduction following a single flash. This is a closed system in which the amount of dye photo-oxidized during repetitive flashes is reduced following cessation of illumination. The elevated concentration of oxidized dye thus must reflect the accumulation of a photoreductant which may thereby reduce oxidized dye at a more rapid rate.

The process of dye reduction would appear to be slower than the oxidation. This is indicated by the elevated steady state concentration of oxidized dye found in continuous illumination compared to that oxidized by a single 10-msec flash (Fig. 3). The data of Fig. 3 show that continuous illumination induces the oxidation of 32 \( \mu \text{M} \) TMPD in the presence of extract con-
yields the semiquinone upon photo-oxidation; the semiquinone a special case of this side reaction may occur. Reduced PMS electron donors other than the photoreductant. With PMS may undergo dismutation to oxidized and reduced PMS (11, 29). With sufficiently intense illumination for a prolonged period, intermediates accumulate, presumably as a result of the rapidity of photo-oxidation of dye compared to the reaction sequence which follows. The results of the effects of PMS and TMPD upon flash yields of ATP in double (10-μsec) flash experiments (Fig. 12) favor this and are consistent with the increased flash yields with the dyes in long (2-msec) flashes (Fig. 10).

With TMPD, an estimate of electron flux induced by a flash may be obtained and related to the flash yield of ATP. For example, as indicated above, from Fig. 4 it is calculated that 1 TMPD molecule is phot-oxidized for every 30 bacteriochlorophyll molecules. The flash yield of ATP with TMPD with the same extract preparation (Fig. 11) is 0.05 ATP/30 bacteriochlorophyll molecules. Thus the estimate is 0.05 ATP formed per electron transferred (TMPD photo-oxidized). This value is consistent with measurements of the quantum efficiency of photophosphorylation. With a value of 0.85 mole per Einstein for TMPD photo-oxidation (this value was reported for photo-oxidation of horse heart cytochrome c by R. rubrum extracts (28)), the calculated quantum efficiency is 24 Einsteins per mole of ATP, assuming 0.05 ATP per electron. The values observed (reported for PMS) varied from 8 ± 1 to > 11 (7).

The results of the effects of dyes upon photophosphorylation in 10-μsec flashes also agree with the decreased quantum efficiency reported for the PMS system (7) and support the view that more than one site may be involved in phosphorylation in the absence of dye. In all cases (except in Fig. 13, in which the flash yield of ATP with succinate was unusually low) the dyes result in lowered flash yields (e.g. Fig. 11). However, the concept of multiple sites in phosphorylation fails to explain the curious decline in flash yield of ATP with the dyes at low flash frequency (Fig. 13). It is tempting to assume that this may be due to slow side reactions reducing photo-oxidized dye with electron donor other than the photoreductant. With PMS a special case of this side reaction may occur. Reduced PMS yields the semiquinone upon photo-oxidation; the semiquinone (presumably the species reduced by the photoreductant) also may undergo dissipation to oxidized and reduced PMS (11, 29).

Acknowledgments—I am indebted to Mr. James Burgess of the Medical Electronics Shop, Washington University School of Medicine, for assistance in the design of some of the electronic equipment. I also thank Dr. O. H. Lowry for his critical analysis of this manuscript.

REFERENCES

The Effects of Phenazine Dyes and \(N,N,N',N'-\text{tetramethyl-p-phenylenediamine}\) upon Light-induced Absorbance Changes and Photophosphorylation in \textit{Rhodospirillum rubrum} Extracts

David M. Geller

\textit{J. Biol. Chem.} 1969, 244:971-980.

Access the most updated version of this article at \url{http://www.jbc.org/content/244/4/971}

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at \url{http://www.jbc.org/content/244/4/971.full.html#ref-list-1}