BBA 46385

THE EFFECT OF DIAMINODURENE ON THE DELAYED LIGHT AND THE CAROTENOID BAND SHIFT IN *RHODOPSEUDOMONAS SPHEROIDES*

LOUIS A. SHERMAN*

Section of Genetics, Development and Physiology, Cornell University, Ithaca, N.Y. 14850 (U.S.A.)

(Received July 5th, 1972)

SUMMARY

1. When 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene), which is an activator of cyclic electron flow, was added to chromatophores isolated from the photosynthetic bacterium, *Rhodopseudomonas spheroides*, it caused a large increase in the emission of delayed light measured at 5–10 ms after excitation. This increase was pH dependent, and ranged from 5–100 times the control intensity. Substances that counteract light-induced proton uptake, such as ammonium salts, amines and nigericin, caused a further increase in the delayed light emission. These compounds also markedly slowed a characteristic decline of the delayed light that occurs during sustained illumination. This decline in the delayed light may be related to the quenching of prompt fluorescence that is seen in the presence of diaminodurene. Substances, like valinomycin, that dissipate the membrane potential, almost completely abolish the diaminodurene-catalyzed increase in the delayed light.

2. Increases in the carotenoid band shift (measured between 450–550 nm) of between 1.8- and 3.0-fold were also found upon the addition of diaminodurene. As was the case with the delayed light, this effect was pH dependent, and was maximal at diaminodurene concentrations between 50–100 μM. The carotenoid band shift has been found to be an indicator of membrane potential in photosynthetic bacteria, and the above experiments also imply a strong connection between the membrane potential and the delayed light.

INTRODUCTION

Recent studies in photosynthesis have shown that a number of optical phenomena that monitor the primary photochemical reactions can be related to the high energy state necessary for the synthesis of ATP. According to Mitchell's chemiosmotic hypothesis of energy coupling[1,2], the high energy state is dependent upon a gradient in the electrochemical activity of H^+ across a membrane which has two energetic components: an electrical activity (membrane potential), and a chemical activity (ΔpH). The importance of these components for ATP synthesis varies

Abbreviations: diaminodurene, 2,3,5,6-tetramethyl-*p*-phenylenediamine; CCCP, carbonylcyanide 3-chlorophenylhydrazone; MES, (*N*-morpholino)ethanesulfonic acid.

* Present address: Division of Biological Sciences, University of Missouri, Columbia, Mo. 65201, U.S.A.

between green plants and photosynthetic bacteria, with the chemical term providing most of the energy in chloroplasts, and the membrane potential the most in bacteria[3–5]. In the preceding paper, we have discussed the relationship between prompt fluorescence and the proton gradient[6]. We shall now deal with the influence of the high energy state on the spectral shifts of carotenoid absorption (carotenoid band shifts) and delayed light emission.

Witt and his collaborators[7,8] have studied in detail the 515-nm shift found in chloroplasts, and have shown that these spectral changes are energy linked. The rise of this shift is extremely rapid and, though the decay is much slower, it can be greatly accelerated under conditions that dissipate the high energy state. From these considerations, Junge and Witt[9] concluded that this 515-nm change is an indicator of electrical potential difference that developed across the chloroplast membrane as a result of charge separation occurring in the primary photochemical reactions. Similar arguments have been used in discussing the carotenoid band shift in chromatophores of photosynthetic bacteria. In particular, Jackson and Crofts[10] have studied the kinetics of the shift and the sensitivity to antibiotics, and were able to show that the carotenoid shift could be related to a change of the membrane potential. More importantly, they were able to induce spectral shifts similar to those induced by light, by means of ionic pulses calculated to induce diffusion potentials[11]. The close relationship between the primary photochemistry and the carotenoid shift is seen in a mutant of *Rhodopseudomonas spheroides* which lacks P870, reaction centers, and all light-induced absorption changes. None of the carotenoid changes can be induced in this mutant, even with pulses of KCl in the presence of valinomycin[12]. It is now generally accepted that the carotenoid band shift can be used as an indicator of membrane potential in chromatophores, but the presence of reaction centers is required even when the change is induced by non-photochemical means.

After having been exposed to light, photosynthetic tissues emit light for some minutes after the cessation of illumination[13]. This delayed light emission has a spectrum identical to that of prompt fluorescence, and, presumably, requires the reformation of the excited singlet state of chlorophyll or of bacteriochlorophyll from metastable precursors[14]. The properties of the delayed light at various times of illumination are quite different, but the emission 1–10 ms after illumination has been shown to be related to the high energy state[14–16]. Wraight and Crofts[16] have recently shown how the delayed light depends on both components of the electrogenic H^+ gradient in chloroplasts, while Barber and Varley[17] have related the delayed light to the membrane potential. This relationship has also been shown to exist in chromatophores by Clayton and Fleischman[14,15,18].

The correspondence between the carotenoid band shift and the delayed light emission was first discussed in the work of Fleischman and Clayton[15]. This relationship will be verified and extended in the present study. We will show that diaminodurene causes a large increase in the delayed light emission, and a corresponding increase in the carotenoid band shift. From these results we will conclude that the stimulated delayed light emission is dependent on the membrane potential. We will then discuss this finding in the context of a theory for delayed light emission proposed by Crofts *et al.*[16,19].

MATERIALS AND METHODS

Cells of *R. spheroides* 2.4.1 (van Niel) were grown and chromatophores were prepared as described in the accompanying paper[6]. In some instances, chromatophores were prepared in 100 mM choline chloride, 10 mM Tricine, pH 7.5, or in 10 mM Tris, pH 7.8. Chromatophores were stored under argon at 4 °C for no more than 48 h.

The spectrometer used to measure the changes in the carotenoid spectra was a home-made device that has been described by Bolton *et al.*[20]. Actinic light was provided by a 650-W tungsten–iodide lamp (Sylvania Sun Gun) filtered through an 800-nm Baird-Atomic Interference Filter. The reaction mixture (in 3 ml) contained: chromatophores at a concentration of 5 μg bacteriochlorophyll/ml, 100 mM KCl (or 100 mM choline chloride) + 20 mM (*N*-morpholino)ethanesulfonic acid (MES) (pH 6.0–7.1) or 20 mM Tricine (pH 7.1–8.5).

Delayed light was measured in a modified Becquerel phosphoroscope that has been described by Clayton[21]. The delayed light was generally measured 5 ms after excitation, though measurements between 4 and 10 ms yielded almost identical results. Actinic light was provided as above and filtered through a Corning 2-64 filter. The reaction mixture was the same as above, except that the chromatophores were at a concentration of 75 μg bacteriochlorophyll/ml, based on the extinction coefficient of Clayton[22].

The other compounds used were obtained as described in the accompanying paper[6].

RESULTS

Effect of diaminodurene on the 5-ms delayed light

The addition of 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene) to a suspension of chromatophores led to a large increase in the intensity of delayed light, as seen in Fig. 1A. At the peak, this increase could vary between 5 and 100 times the control level. This rapid rise was followed by a decline to a new steady state value that was generally 2–3 times greater than the control. At times, chromatophore preparations showed quite different kinetics which resembled those seen in chloroplasts without electron transport cofactors[16]. This is manifest as a rapid spike followed by a slow rise (of 1–2 min) back to the level of the spike. This is then followed by a decay, which though similar in many ways to that seen in Fig. 1, had a longer half time. These kinetics were seen only in freshly prepared chromatophores from cells grown photosynthetically for 3 days or longer. Due to the complexity of the kinetics, these preparations were not studied quantitatively.

The increase in delayed light emission was highly pH dependent, as can be seen in Tables I, III and V. The stimulated emission was highest at pH 6.0 and declined to more or less a plateau level between pH 7 and 8. The diaminodurene-catalyzed increase in delayed light was 2–4-fold higher at pH 6.0 than at pH 7.1. Under the conditions utilized in these experiments, the delayed light of chromatophores without diaminodurene showed only a slight pH dependence. In this case, the delayed light was about 50 % higher at pH 8.0 than at pH 6.0.

The dependence of the delayed light increase on the diaminodurene concentration is shown in Fig. 2A. At pH 7.1, there was no increase until the diaminodurene

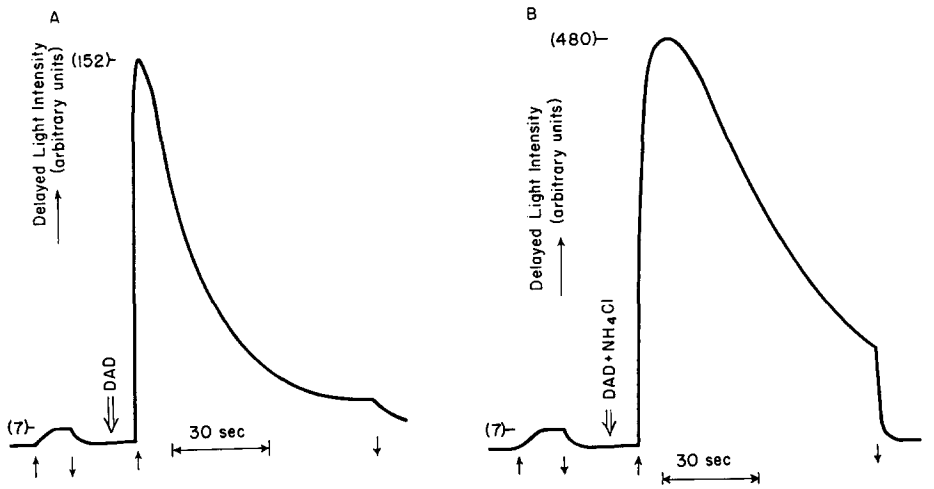

Fig. 1. The time course of delayed light emission measured at 5 ms after excitation. The clear sided cuvette of 1-cm pathlength contained (in 3 ml): chromatophores at 75 μg bacteriochlorophyll/ml, 20 mM MES, pH 7.1 and 100 mM KCl. The actinic light of intensity = 6 mW/cm^2 was filtered through a Corning 2-64 filter, as was the measured light. A control without any cofactor was measured in both cases. (A) 100 μM diaminodurene (DAD) was added; and (B) 100 μM diaminodurene + 6.0 mM NH_4Cl was added. The vertical scale for the diaminodurene + NH_4Cl experiment is expanded 3-fold.

TABLE I

INCREASE IN DELAYED LIGHT INTENSITY IN THE PRESENCE OF DIAMINODURENE AND NH_4Cl

Chromatophores, at 75 μg bacteriochlorophyll/ml, were suspended in 20 mM MES (pH 6.0–7.1) or 20 mM Tricine (pH 7.4–8.0) + 100 mM KCl. Diaminodurene (DAD) was added to a final concentration at 100 μM and NH_4Cl to 6.0 mM. Measurements were done as in Fig. 1, with a light intensity = 6 mW/cm^2.

pH	*Delayed light intensity*	
	$\frac{(+DAD)}{(-DAD)}$	$\frac{(+DAD+NH_4Cl)}{(-DAD-NH_4Cl)}$
6.0	87	110
6.5	48	105
7.1	22	73
7.4	21	47
8.0	21	23

concentration had reached approximately 50 μM, while at pH 6.0 there was over a 5-fold increase at 33 μM. However, in all cases, increasing the diaminodurene concentration above 100 μM had no further effect on the delayed light. This should be compared to the hyperbolic relationship between the diaminodurene concentration and prompt fluorescence quenching[6,23]. The delayed light both with and without diaminodurene varied as I^2 over a range from 1–9 mW/cm^2.

Effect of uncouplers and ionophorous antibiotics

Uncouplers and ionophorous antibiotics had pronounced effects on the diaminodurene-catalyzed delayed light which could be correlated with their effect on the

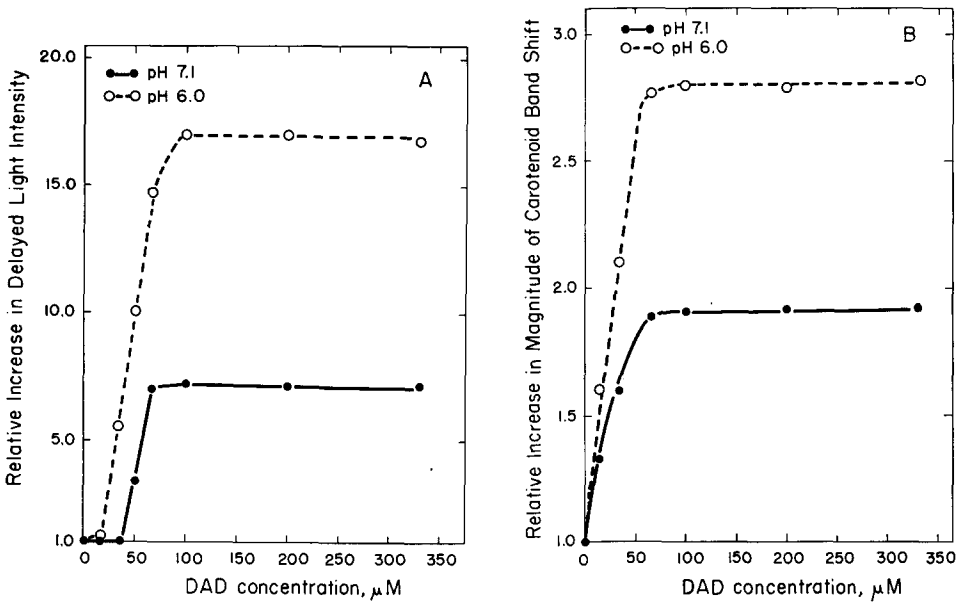

Fig. 2. Concentration dependence of relative increases of delayed light and the carotenoid band shift. In both cases, the chromatophores were suspended in 20 mM MES and 100 mM KCl. (A) The delayed light was measured as in Fig. 1 and the ratio between the control and +diaminodurene (DAD) peaks were used as the relative increase. The chromatophores were at a concentration of 75 μg bacteriochlorophyll/ml and the actinic light intensity was 5.9 mW/cm^2. (B) The carotenoid band shift was measured as in Fig. 3, and the relative increases were obtained as described in the text. Chromatophore concentration = 5 μg bacteriochlorophyll/ml and actinic light intensity = 5.75 mW/cm^2.

high energy state[1-3,6]. Those substances, such as NH_4Cl, amines or nigericin, which are known to inhibit proton uptake in chromatophores and chloroplasts, caused the delayed light to be increased over the level seen with diaminodurene alone. However, compounds known to effect the membrane potential, such as valinomycin, gramicidin, and carbonylcyanide 3-chlorophenylhydrazone (CCCP), drastically reduced the emission of delayed light. These effects will be discussed separately in the succeeding paragraphs.

The time course of the delayed light emission after the addition of diaminodurene and NH_4Cl is seen in Fig. 1B. There is once again a relatively fast rise, followed by a slower decay to a steady-state level. However, the magnitude of the rise is over 3 times higher than seen in the presence of diaminodurene alone. The kinetics of the decline during continuous illumination were also changed in the presence of NH_4Cl, with the $t_{1/2}$ increasing from 15 to 35 s at pH 7.1. The time courses of the delayed light measured in the presence of nigericin and ethylene amine were identical to that seen with NH_4Cl.

The effect of these compounds was pH dependent, as shown in Tables I and V. In Table I, we see that the increase in delayed light in the presence of diaminodurene and NH_4Cl was over 100 times the control level at pH 6.0, declining to a factor of about 20 times at pH 8.0. In Table V, we see that similar results were obtained for nigericin.

Nigericin and NH_4Cl also altered the kinetics of the decline in the level of the

delayed light. In Table II, we see that NH_4Cl slows down this decay in a pH-dependent manner. In both cases, however, this decay is fastest at pH 6.5 and slowest at pH 8.0. The dark decay (the decay after the exciting illumination is turned off) was substantially faster when nigericin or NH_4Cl is present (compare Figs 1A and 1B).

The dark reversibility of the diaminodurene-catalyzed delayed light was also markedly changed by NH_4Cl and nigericin. In their absence, the delayed light declined to a steady state value 2–3 times the control level; a higher level was regained only very slowly in the dark. NH_4Cl allowed about 50% restoration, with a $t_{1/2}$ of approx. 1–2 min. Furthermore, with NH_4Cl there was no further decline after 2–3 min of illumination, the delayed light emission remaining at 20–50 times the control level. The dark restoration with NH_4Cl was pH dependent and, at pH 8.0, the delayed light returned to near its peak level after a 2-min dark period. Nigericin produced similar effects, but with less pH dependence.

Those compounds that inhibit formation of the membrane potential, such as valinomycin and gramicidin, greatly inhibit the diaminodurene-catalyzed delayed light. The inhibition caused by valinomycin and the pH dependence of this effect is given in Table III. In all cases, valinomycin inhibits at least 75% of the delayed light, but the inhibition is greater at higher pH. At least 90% inhibition was obtained

TABLE II

KINETICS OF THE DECLINE OF THE DELAYED LIGHT INTENSITY DURING PROLONGED ILLUMINATION

Conditions were those given in Table I. The decay was determined from the peak of the light-induced curve. DAD, diaminodurene.

pH	$t_{1/2}$ (*s*)	
	+DAD	*+DAD+NH₄Cl*
6.0	19	38
6.5	14	33
7.1	15	35
7.4	16	42
8.0	22	63

TABLE III

INHIBITION OF THE INCREASE IN DELAYED LIGHT BY VALINOMYCIN

Conditions were as given in Table I. Valinomycin (Val) (0.33 μM final concentration) and nigericin (Nig) (0.2 μM) were added at the same time as diaminodurene (DAD).

pH	*Delayed light intensity*			
	$\frac{(+DAD)}{(-DAD)}$	$\frac{(+DAD+Nig)}{(-DAD-Nig)}$	$\frac{(+DAD+Val)}{(-DAD-Val)}$	$\frac{(+DAD+Val+Nig)}{(-DAD-Val-Nig)}$
6.0	36.5	47.5	9.5	2.9
6.5	27.6	35	5.8	2.7
7.1	16.5	31.2	2.0	2.8
7.4	23.5	32.0	1.6	3.3
8.0	22.5	31.5	1.8	6.5

at pH 8.0, but values as high as 98% were found. Gramicidin had similar effects, while CCCP at high concentrations (5–10 μM) totally abolished the delayed light emission.

Relationship between delayed and prompt fluorescence

The relationship between the emission of delayed and prompt fluorescence has been studied by a number of people, notably Lavorel[24,25] and Clayton[21]. In particular, Clayton was able to demonstrate that the delayed fluorescence could be related to the "live"* component of prompt fluorescence in chloroplasts under specific conditions. Since most of the prompt fluorescence in chromatophores is usually considered to be "live"[21], one would imagine that this relationship could also be shown in bacteria. Though the correlation appears dubious for the fast rise components, the decline during prolonged illumination of both delayed and prompt fluorescence appears to be related, as can be seen by comparing Fig. 1 of this paper with Fig. 1 of the accompanying article[6].

This decline in the delayed light emission, like the prompt fluorescence quenching, appears to be related to the uptake of protons. This can be seen by its sensitivity to NH_4Cl and nigericin, which tend to slow the decline in the same manner that they prohibit the prompt fluorescence quenching. The half time of the decline of the delayed light is appropriate to this hypothesis (Table II), since it is fastest at pH 6.5, a pH where the uptake of protons is near maximal in rate and extent[26]. Furthermore, the diaminodurene-catalyzed increase in the delayed light is usually minimal at the same pH (7.1) where the prompt fluorescence quenching is optimal. This would imply that proton uptake is acting in some way to compete with the delayed light increase, thereby causing the initial magnitude to be lower. The dark reversibility of this slow decline in the emissions, both in the presence and absence of uncouplers, is consistent with this hypothesis. These conclusions are similar to those reached in chloroplasts by Wraight and Crofts[16], though they were not able to achieve a correlation in the presence of uncouplers as shown here.

Under certain conditions, a decline after an initial spike is also seen in the carotenoid band shift[15] (see later), and it is likely that this decline is also due to proton uptake. This induction peak was not seen under the conditions of the present experiment to a great degree, either for the delayed light without diaminodurene or for the carotenoid band shift with and without cofactor. This phenomenon, however, does complicate any quantitative relationship between the delayed light decline and fluorescence yield lowering.

Effect of diaminodurene on the carotenoid band shift

The addition of diaminodurene to a suspension of chromatophores also had a distinct effect on absorption changes in the carotenoid region, as shown in Fig. 3. The usual very rapid rise in ΔA was increased about 2–3-fold over the control level, and was followed by a smaller and slower rise lasting a few seconds. From this level, the absorption change generally declined slowly ($t_{1/2}$ approx. 2 min). For comparison purposes, only the very fast component of the absorption changes was measured, which was obtained from oscilloscope traces during the first 200 ms of illumination.

* "Live" means emitted by chlorophyll or bacteriochlorophyll that can transfer energy to the photochemical traps.

This corresponds reasonably well to the kinetic components P_1 and P_2 of Jackson and Crofts[10], determined by flash illumination. This effect of diaminodurene was pH dependent as shown in Table V and Fig. 2B. The increase in the absorption changes was greatest at pH 6.0 and declined at more alkaline pH, in a manner similar to that found in the delayed light experiments. The concentration dependence of the increase in the carotenoid band shift is also shown in Fig. 2B, where it can be seen that the effect is near optimal at a diaminodurene concentration of 66 μM. There is also a large effect at much lower concentrations, in contrast to the delayed light studies where no change was found at these low concentrations. This is probably due to the different concentrations of chromatophores used in the two types of experiments.

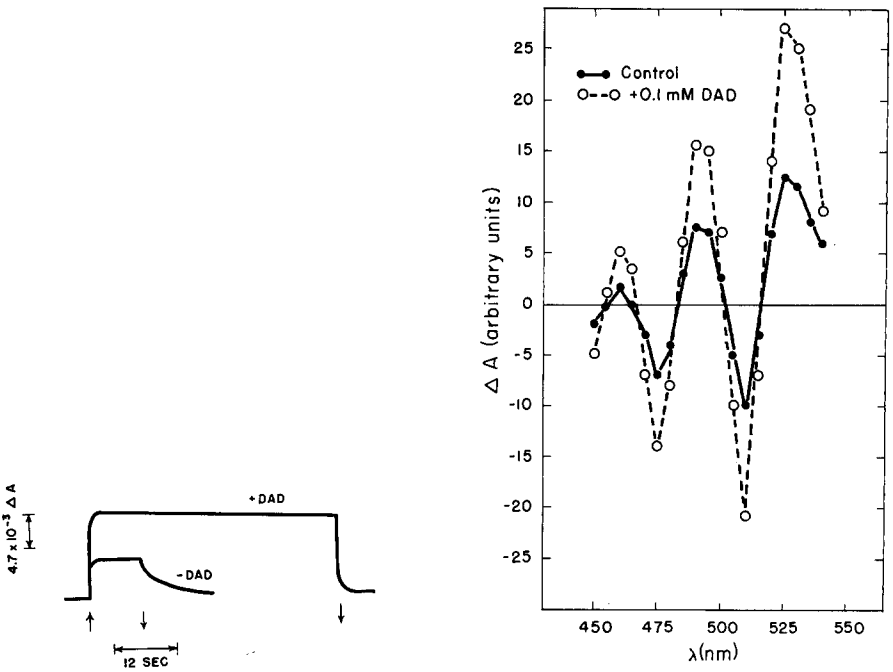

Fig. 3. The time course of the absorption change in the carotenoid spectra. Chromatophores at 5 μg bacteriochlorophyll/ml were suspended in 20 mM MES, pH 7.1 and 100 mM KCl. The actinic light, filtered through an 800-nm interference filter, had an incident intensity of 5.4 mW/cm^2. The absorption change was measured at 525 nm. The lower curve was made in the absence of cofactor, while the upper curve was made after the addition of 100 μM diaminodurene. The dark decay kinetics were always faster in the presence of diaminodurene (DAD).

Fig. 4. Spectra of the carotenoid band shift in the presence and absence of diaminodurene (DAD). Conditions were the same as in Fig. 3 and measurements were made every 5 nm. The ratio of the diaminodurene-catalyzed increase was taken at each point and an average was obtained. For this experiment, the average increase was 2.2 times.

When the absorption changes were determined every 5 nm throughout the carotenoid spectrum, it was apparent that diaminodurene did not change the spectrum of the carotenoid band shift, but only made it larger (Fig. 4). For the experiment in Fig. 4, diaminodurene caused an average increase in the carotenoid band shift of 2.2 times.

Relationship between increases in the delayed light and the carotenoid band shift

Since the delayed light emission and the carotenoid band shift have both been shown to be related to the high energy state, it would be expected that they would respond similarly to a variety of conditions. This correlation was originally demonstrated qualitatively by Fleischman and Clayton[15]. More recently, Jackson and Crofts[10,11] have suggested that the carotenoid band shift may be in response to the membrane potential. That the delayed light emission is also dependent on the membrane potential is now quite clear from the experiments presented in this paper and by others. It would, therefore, be expected that a direct relationship between the two processes could be found. Crofts *et al.*[16,19] have proposed a model in which the carotenoid shift is a linear function, and the delayed light intensity an exponential function of the membrane potential (see Discussion). Since we were not able to measure the delayed light under conditions identical to those used to observe the carotenoid band shift, it is premature to attempt a quantitative correlation. However, the above relationship does predict that for a "linear" increase in the carotenoid band shift, the delayed light should increase exponentially. This prediction appears to be valid when the relative diaminodurene-catalyzed increases of the respective phenomena are measured under a variety of conditions.

The different values for the diaminodurene-catalyzed increases seen in Table IV were obtained by preparing chromatophores in different ways or by using a slightly aged chromatophores. In Table IV, Expts 5 and 6 were done using the same chromatophored suspension, prepared in 0.25 M sucrose, except that in Expt 6 the chromatophores were fresh (about 2 h old), while in Expt 5 they were 24 h old. Similarly, for chromatophores isolated in 100 mM choline chloride, 10 mM Tricine, pH 7.5,

TABLE IV

RELATIONSHIP OF THE INCREASES IN THE DELAYED LIGHT INTENSITY AND THE CAROTENOID BAND SHIFT AT pH 7.1

Measurements were made as described in Figs 1–3, in 20 mM MES, pH 7.1 + 100 mM KCl. For the carotenoid band shift, the chromatophore concentration was 5 μg bacteriochlorophyll/ml and the actinic light intensity was 6.0 mW/cm^2. For the delayed light emission these values were 75 μg bacteriochlorophyll/ml and 6.8 mW/cm^2, respectively. The chromatophores used in this experiment were isolated in the following buffers: (1) 10 mM Tris, pH 7.8, fresh; (2) 100 mM choline chloride + 10 mM Tricine, pH 7.5, 1 day old; (3) same as (2), except fresh; (4) 0.25 M sucrose, 10 mM Tricine, pH 7.5, fresh; (5) 0.25 M sucrose + 10 mM Tricine, pH 7.5, different isolation than (4), 1 day old; (6) same preparation as (5), fresh. Fresh implies that measurements were made within a few hours of the preparation of the chromatophores. All of the chromatophore preparations are twice-washed crude extracts. Light chromatophores were always prepared in sucrose at each step and, when fresh, yielded results like those in Expts 5 and 6. Abbreviation: DAD, diaminodurene.

Expt	*Delayed light intensity*	
	$\frac{A_{525}\,(+DAD)}{A_{525}\,(-DAD)}$	$\frac{(+DAD)}{(-DAD)}$
1	1.82	6.4
2	1.92	6.9
3	2.2	9.3
4	2.33	11.0
5	2.78	16.5
6	3.0	20.5

TABLE V

EFFECT OF pH ON INCREASES IN DELAYED LIGHT AND THE CAROTENOID BAND SHIFT

The experimental conditions are the same as Table IV; 20 mM MES was used for pH 6.0–7.1, and 20 mM Tricine for pH 7.4–8.0. Chromatophores were prepared in 0.25 M sucrose, 10 mM Tricine (pH 7.5) and were fresh. When present, nigericin (Nig) was added at a final concentration of 0.2 μM. Abbreviation: DAD, diaminodurene.

pH	$\frac{A_{525}(+DAD)}{A_{525}(-DAD)}$	*Delayed light intensity*	
		$\frac{(+DAD)}{(-DAD)}$	$\frac{(+DAD+Nig)}{(-DAD-Nig)}$
6.0	3.15	25.0	32.0
6.5	2.78	15.5	20.0
7.1	2.33	11.0	21.0
7.4	2.50	12.5	21.5
8.0	2.3	10.5	22.5

Expt 3 was performed with fresh chromatophores and Expt 2 a day later. This sensitivity on the state of the chromatophores was such that comparable measurements on the delayed light and the carotenoid band shift had to be performed within 2 h of each other if the correlation was to be satisfied within reasonable accuracy. It can be seen that both the delayed light and the carotenoid band shift varied in a systematic fashion in a particular chromatophore preparation. Variations of the delayed light intensity were much more striking than those of the carotenoid band shift, as would befit an exponential relationship. When the pH dependence of a single chromatophore preparation was determined (Table V), a similar relationship between the delayed light and the carotenoid band shift was observed. In both cases, the relative increases are greatest at pH 6.0. The effect of pH on the increase of the delayed light in the presence of diaminodurene and nigericin is also given in Table V. However, nigericin did not change the carotenoid band shift in a systematic way. It had no affect on the rapid absorption change, except at pH 8.0, where an increase of about 15 % was obtained. NH_4Cl behaved in a similar fashion. This anomalous increase in delayed light without a concomitant increase in the carotenoid band shift remains to be explained.

DISCUSSION

The results described above are consistent with the delayed light being intimately dependent on the membrane potential developed during the primary light reaction. The addition of diaminodurene to a suspension of chromatophores induces a large increase in the delayed light, which is paralleled by a corresponding increase in the carotenoid band shift, used here as a measure of membrane potential. That both of these processes are dependent on the integrity of the membrane, as originally determined by Fleishmann and Clayton[15] and Clayton[27], was shown by their sensitivity to age and preparation procedure. Fresh chromatophores prepared in 0.25 M sucrose consistently produced the largest changes. When these chromatophores were aged, even a day or two, the increases became much smaller, and resembled increases

from chromatophores isolated in less ideal media. The kinetics of the light-induced changes were particularly sensitive to membrane integrity.

These results for *R. spheroides* suggesting that a linear increase in the carotenoid band shift, used as a monitor of membrane potential, corresponds to an exponential increase in the delayed light are consistent with the model suggested by Fleischman[18] and Crofts *et al.*[19]. The main postulate is that the primary donor (P) and donor (A) of the reaction center be situated on opposite sides of the membrane. Light would produce the reaction $P,A \rightarrow P^+A^-$, and this charge separation would generate a potential across the membrane. The necessity for the involvement of the reaction center in the carotenoid band shift induced by light or by ionic pulses has recently been shown by study of the reaction center-less mutant of *R. spheroides*[12]. Since it is thought that delayed light depends on the return of an electron from A^- to P^+, so as to give the first excited singlet P^*[14], the intensity of delayed light will involve a Boltzmann factor $\exp(-\Delta E/kT)$. Crofts and Fleischman hypothesize that the energy differential, ΔE, between P^* and A^- can be decreased by the membrane potential. This would lower the energy barrier making the transition more probable and cause an increase in the emission of delayed light.

The effect of ionophorous antibiotics and uncouplers lend further confirmation to this hypothesis. Nigericin, in the presence of K^+, mediates an electrically neutral, stoichiometric exchange of H^+ for K^+ across the membrane, which results in the dissipation of the transmembrane ΔpH in favor of a potassium gradient[3]. This results in loss of the chemical component of the high energy state without any change in the electrical component. Entirely analogous arguments can be made for the action of NH_4Cl[28]. With both compounds, it is entirely possible that the loss of ΔpH causes a corresponding increase in the membrane potential, leading to an even greater emission of delayed light. Valinomycin, which dissipates the membrane potential, substantially inhibits the diaminodurene-catalyzed increase.

The mechanism by which diaminodurene produces these effects is unknown, though it is presumed that as a cyclic cofactor diaminodurene increases cyclic electron flow[4,28]. However, diaminodurene may have other properties that cause the pronounced effects detailed here[6]. The concentration of diaminodurene necessary to produce large increases in the delayed light and the carotenoid band shift was much lower than that necessary for optimal quenching of prompt fluorescence[6,26,29] or for cyclic phosphorylation[30]. This may imply that the membrane potential component of the high energy state is more easily affected by diaminodurene than proton uptake. Indeed, from the concentration dependence, one can conclude that only a small number of diaminodurene molecules per reaction center are necessary. It is possible that diaminodurene has an effect as an amine as well as a mediator of electron flow.

These experiments tell us only that, under the conditions used here, the delayed light is primarily affected by the membrane potential (in general, the high energy state). They do not imply that the delayed light is dependent on this factor alone. In fact, delayed light emission is a highly complex process with a large number of variables, none of them totally indeyendent[14,16,19,31]. The data do provide further evidence that delayed light emission can be stimulated by a change in the membrane potential, and show that dramatic increases in the intensity of delayed light are possible with the right combination of reagents.

ACKNOWLEDGEMENTS

We wish to thank Dr R. K. Clayton for stimulating discussions and help in the preparation of this manuscript. This investigation was supported by A.E.G. contract No. AT (30-1) 3759 awarded to Dr R. K. Clayton. L.A.S. is a postdoctoral Fellow of the National Institutes of Health.

REFERENCES

1 P. Mitchell, *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Bodmin, Cornwall, 1966.
2 P. Mitchell, *Chemiosmotic Coupling and Energy Transduction*, Glynn Research, Bodmin, Cornwall, 1968.
3 J. B. Jackson, A. R. Crofts and L.-V. von Stedingk, *Eur. J. Biochem.*, 6 (1968) 41.
4 G. A. Hauska, R. E. McCarty and E. Racker, *Biochim. Biophys. Acta*, 197 (1970) 206.
5 D. A. Walker and A. R. Crofts, *Annu. Rev. Biochem.*, 39 (1970) 389.
6 L. A. Sherman and W. S. Cohen, *Biochim. Biophys. Acta*, 283 (1972) 54.
7 H. T. Witt, in S. Claesson, *Fast Reactions and Primary Process in Chemical Kinetics*, Nobel Symp., Vol. 5, Almquist and Wiksell, Stockholm, 1967, p. 261.
8 H. T. Witt, B. Rumberg and W. Junge, in *Colloquium der Gesselschaft für Biologische Chemie*, Mosbach, Springer-Verlag, Berlin, 1968, p. 262.
9 W. Junge and H. T. Witt, *Z. Naturforsch.*, 23b (1968) 244.
10 J. B. Jackson and A. R. Crofts, *Eur. J. Biochem.*, 18 (1971) 120.
11 J. B. Jackson and A. R. Crofts, *FEBS Lett.*, 4 (1969) 185.
12 L. A. Sherman and R. K. Clayton, *FEBS Lett.*, 22 (1972) 127.
13 B. Strehler and W. Arnold, *J. Gen. Physiol.*, 34 (1951) 809.
14 R. K. Clayton, *Adv. Chem. Phys.*, 19 (1971) 353.
15 D. E. Fleischman and R. K. Clayton, *Photochem. Photobiol.*, 8 (1968) 287.
16 C. A. Wraight and A. R. Crofts, *Eur. J. Biochem.*, 19 (1971) 386.
17 J. Barber and W. J. Varley, *Nature New Biol.*, 234 (1971) 188.
18 D. E. Fleischman, *Photochem. Photobiol.*, 14 (1971) 277.
19 A. R. Crofts, C. A. Wraight and D. E. Fleischman, *FEBS Lett.*, 15 (1971) 89.
20 J. R. Bolton, R. K. Clayton and D. W. Reed, *Photochem. Photobiol.*, 9 (1969) 209.
21 R. K. Clayton, *Biophys. J.*, 9 (1969) 60.
22 R. K. Clayton, in H. Gest, A. San Pietro and L. P. Vernon, *Bacterial Photosynthesis*, Antioch Press, Yellow Springs, Ohio, 1963, p. 498.
23 C. A. Wraight and A. R. Crofts, *Eur. J. Biochem.*, 17 (1970) 319.
24 J. Lavorel, in H. Metzner, *Progress in Photosynthesis Research*, Vol. II, H. Laupp Jr, Tübingen, 1969, p. 883.
25 J. Lavorel, *Biochim. Biophys. Acta*, 153 (1968) 727.
26 L.-V. von Stedingk, *Arch. Biochem. Biophys.*, 120 (1967) 537.
27 R. K. Clayton, *Photochem. Photobiol.*, 1 (1962) 313.
28 A. R. Crofts, *J. Biol. Chem.*, 242 (1967) 3352.
29 W. S. Cohen and L. A. Sherman, *FEBS Lett.*, 16 (1971) 319.
30 A. Trebst, E. Pistorius and H. Baltscheffsky, *Biochim. Biophys. Acta*, 143 (1967) 257.
31 W. Arnold and J. Azzi, *Photochem. Photobiol.*, 14 (1971) 233.