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DELAYED LIGHT STUDIES ON PHOTOSYNTHETIC ENERGY CONVERSION.

II. EFFECT OF ELECTRON ACCEPTORS AND PHOSPHORYLATION COFACTORS ON THE MILLISECOND EMISSION FROM CHLOROPLASTS

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SUMMARY

1. The delayed light emission from chloroplasts in the presence and absence of electron acceptors has been measured from 1 to 20 msec after the center of repeating flashes of exciting light.

2. The emission from chloroplasts to which neither hydrogen acceptors nor phosphorylation cofactors had been added showed a very slow dark decay compared to the dark decay of the emission from whole leaves.

3. Activation of noncyclic electron transport by either $K_3Fe(CN)_6$, $NADP^+$ + ferredoxin, 2,6-dichlorophenolindophenol, or methylviologen (pseudo-cyclic) resulted in increased intensity of emission at 1 msec and in more rapid dark decay of the emission, so that the shape of decay curves from activated chloroplasts approached that from whole leaves.

4. Activation of cyclic electron transport by phenazine methosulfate had no effect on the delayed emission of chloroplasts, whereas activation by pyocyanine had an effect similar to that of the noncyclic cofactors, but reduced in magnitude.

5. Inclusion of ADP or of ADP + P_i reduced the emission without markedly changing the kinetics of dark decay.

6. Inclusion of ascorbate had an effect similar to that of the noncyclic cofactors.

7. These results lend support to the previous conclusion that delayed light emission is dependent on that part of photosynthesis which DUYSENS has termed Photoreaction II. The intensity of emission is interpreted as reflecting energy storage by Photoreaction II. According to this interpretation, activation of noncyclic electron transport results both in more rapid utilization of energy stored by the photoreaction, and in an increase in energy stored by the photoreaction at dark times somewhat shorter than the turnover time of the multienzyme system. The increase in energy storage does not persist beyond times about equal to the enzymatic turnover time, presumably because the additional stored energy is rapidly utilized by the activated electron transport system. Nevertheless, the increase in energy storage at Photo-

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Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate.

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reaction II is directly opposed to the assumption that utilization of energy by the electron transport chain would result in less energy storage by the photoreaction at all times after excitation.

INTRODUCTION

The delayed light of photosynthetic organisms has been shown to be emitted from the first excited singlet of chlorophyll[1–5]. This low-intensity emission may be measured as a function of time after cessation of illumination. In the present studies illumination consisted of repeated flashes, and the intensity of emitted light was measured during the dark time between flashes. The emission of delayed light depends on the integrity of that part of the photosynthetic apparatus which DUYSENS *et al.*[6] have defined as Photoreaction II. This conclusion was drawn from the study of a mutant alga which was deficient in Photoreaction II and which showed a lack of delayed light emission[7]. The same conclusion follows from the present investigations with isolated chloroplasts.

Delayed light emission in the millisecond time range reflects energy storage in some metastable form before stabilization in the form of chemical products. The time scales relating to fluorescence of chlorophyll *in vivo*, 1 nsec, and to steady-state turnover time of the enzymatic systems, 0.01 sec, are seven orders of magnitude apart[8–14]. This means that although the excitation energy was no longer freely available to excite chlorophyll within 1 nsec after the initial absorption act, this energy did not appear as stabilized chemical products until 0.01 sec after absorption. It would follow that in the intermediate time range there is a storage of energy in metastable forms. The delayed emission in the millisecond region represents a low yield of reexcitation of chlorophyll by the stored metastable energy.

With the isolated chloroplast system used in the present investigation, the addition of an electron acceptor not only increased the rate of decay of the emission, but also increased the light emitted at 1 msec. The effects induced by electron acceptors can be explained in terms of charge separation and migration at reaction centers, in agreement with earlier results with whole cells which indicate that the delayed light reflected coupling between solid state processes and enzymatic processes in the early steps of photosynthesis[15]. On the other hand, our present results can also be explained in terms of luminescence resulting from reduction of photo-oxidized chlorophyll, if we assume the presence of reducing power which is not used in photosynthesis. These two possibilities are discussed elsewhere[16].

EXPERIMENTAL

"Broken" chloroplasts were prepared from garden-grown Good King Henry (*Chenopodium bonus-henricus*) by a variation of the method of WALKER[17]. In some cases chloroplasts were prepared from other plants; spinach (*Spinacia oleracea*), spinach beet (*Beta vulgaris*) or pea (*Pisum sativum* var. Laxton Superb). All data presented were taken with Good King Henry chloroplasts unless otherwise stated. Leaves were kept in running water for about 1 h, after which 50 g of leaf tissue were ground in a cold Waring Blendor by means of 3 high-speed blasts of 5 sec per blast.

The blender contained 200 ml of the following ice-cold grinding buffer: 20 mM Tris-HCl (pH 7.4), 0.35 M sucrose, 1 mM $MgCl_2$, 1 mM NaCl. The resulting leaf homogenate was squeezed through 8 layers of muslin (equivalent to about 16 layers of cheese cloth) into a cold beaker. A volume of 200 ml of the strained homogenate was centrifuged in four tubes for 10 min at 1200 × *g* at about 1°. The supernatant was discarded, and the pellets in the tubes were rinsed with resuspending medium. The pellets were resuspended and combined, using 2 ml of the following resuspending medium: 1 mM $MgCl_2$, 1 mM NaCl. These "broken" chloroplasts were stored in ice until use. A volume of 0.1 ml of these chloroplasts was included in a 3-ml reaction mixture, of which only 2 ml were actually used in the delayed light measurement. The final reaction mixture usually contained about 50 μg of chlorophyll per ml. The exact components of each reaction mixture are given in the figure legends.

To insure that active chloroplasts were used in the delayed light experiments, tests for the relevant reactions were made. The rate of $K_3Fe(CN)_6$ reduction varied between 90 and 140 μmoles/mg chlorophyll per h, that of $NADP^+$ reduction between 36 and 51 μmoles/mg chlorophyll per h. In both these cases addition of $ADP+P_i$ increased the rate of reduction by 1.7–3 times, while addition of $(NH_4)_2SO_4$ increased the rate by 1.9–4.2 times. The rate of 2,6-dichlorophenolindophenol (DCIP) reduction varied between 40 and 73 μmoles/mg chlorophyll per h, that of *p*-benzoquinone reduction between 38 and 64 μmoles/mg chlorophyll per h. Photophosphorylation in the presence of methylviologen, ADP and P_i varied between 110 and 180 μmoles/mg chlorophyll per h, that in the presence of pyocyanine, ADP, and P_i between 200 and 380 μmoles/mg chlorophyll per h. In both these cases addition of ascorbate increased the rate of phosphorylation by 1.5–2.3 times.

Rates of O_2 evolution were measured on a Rank-type electrode, which has been described by CHAPPELL[18]. Saturating red light was obtained using a 35-mm slide projector with filters which cut off wavelengths shorter than 6000 Å. Photophosphorylation was measured on 3-ml reaction mixtures with a molybdate color test, as described by HILL AND WALKER[19]. $Fe(CN)_6^{3-}$ reduction, $NADP^+$ reduction, and DCIP reduction were done by standard spectroscopic methods as described by WHATLEY AND ARNON[20].

The delayed light from 1 to 20 msec after the center of a flash of exciting light was measured by a modified Becquerel phosphoroscope, which has been described previously[7]. The instrument consisted of a pair of spinning discs mounted on a common shaft, with the discs acting as shutters. The reaction mixture was held stationary between the two discs in a 2-ml cellulose nitrate test tube. In order to provide adequate light baffling, the discs were mounted so that they spun in narrow slots within an aluminum housing. The reaction mixture was illuminated through two oppositely spaced holes in the front disc, so that the chloroplasts received two evenly spaced flashes of exciting light per revolution of the discs. The reaction mixture was viewed by a photomultiplier placed behind the back disc. This back disc was shaped like a paddle wheel, with its two blades aligned with the illuminating holes of the front disc. The back disc thus allowed the reaction mixture to be viewed by the photomultiplier for a period between each exciting flash, but for the duration of each flash the blades of this disc protected the light-measuring equipment from the bright exciting light.

The present delayed light measurements were obtained by running the motor at two different speeds. Our decay curves from 1 to 3 msec were taken when the reaction mixture received 250 flashes of exciting light per sec, whereas the decay curves from 5 to 20 msec were taken when the reaction mixture received 40 flashes per sec. The chloroplasts received the same total energy of exciting light per sec at both disc speeds, since the duration of each individual flash of exciting light was longer at the slower speed. The flash duration at 250 flashes per sec was 0.6 msec, and at 40 flashes per sec it was 3.75 msec. Because of this situation, the decay curve is in two segments which are not continuous.

The delayed emission from the chloroplasts was detected by an RCA 7102 photomultiplier that was cooled with liquid N_2 and operated at 1400 V. The signal from the photomultiplier was fed directly into an oscilloscope (Tektronix 545A, Type D high-gain plug-in unit), so that decay of emission could be observed and photographed directly on the oscilloscope screen. Data are presented either as reproductions of the photographs or as a signal (which measures intensity of emission) read at three different times from each photograph. The signals are given on an arbitrary scale of 10.

All delayed light measurements were made with the reaction mixture at 25°. The exciting light consisted of the wavelength region 6200–12000 Å. The filament of a 1500-W tungsten bulb was focused on the cells to give an image of about 1 cm^2, and the bulb's incandescent emission was filtered through 7 cm of water and an orange Corning glass color filter CS 3-67. To eliminate the effects of dark–light transients, the reaction mixture was preilluminated for 1 min (receiving 250 flashes per sec) with 10 % of the above light (by inserting a Balzers neutral density interference filter). This preillumination at low intensity was followed by 30 sec at maximum intensity (10 % filter removed), after which the decay curve at 250 flashes per sec was photographed (0.1 sec exposure) on the face of the oscilloscope. The speed of the motor was immediately reduced to 40 flashes per sec, and within 15 sec the decay curve at 40 flashes per sec was photographed. After these control photographs were taken the motor was increased to 250 flashes per sec, and a cofactor or hydrogen acceptor was rapidly introduced into the reaction mixture by means of a hypodermic syringe. The needle of the syringe passed through a rubber stopper, which acted as a light block in the housing of the phosphoroscope, and then through a small hole in the aluminum cap of the cellulose nitrate test tube, and finally into the reaction mixture itself. We showed that complete mixing occurred by making a similar setup on the laboratory bench so that we could observe directly the injection of ink or DCIP into the reaction mixture. The effect of hydrogen acceptors and other cofactors was noted immediately on the oscilloscope face, but before the photographs were taken, 30 sec of total intensity illumination was given (as in the control photographs) to eliminate transient effects of adding cofactors. Two photographs, one at 250 flashes per sec and one at 40 flashes per sec, were then taken in the same way as the control photographs. This set of four photographs provided the raw data for the effect of any cofactor on the delayed light decay from 1 to 20 msec after the centers of repeating flashes of exciting light.

Ferredoxin was prepared according to the method of BENDALL *et al.*[21]. DCIP was purchased commercially and purified by filtering an aqueous solution and then salting out with 3 M NaCl. Pyocyanine was a gift from Dr D. A. WALKER. For brevity, the term "ascorbate" is used to indicate sodium isoascorbate.

RESULTS

Transient changes in intensity of delayed light during the first min of illumination

Leaves and chloroplast reaction mixtures show transient changes in delayed emission immediately after the onset of illumination. We did not observe strong changes in the kinetics of dark decay during these dark–light transients, but the intensity of delayed emission (measured between identical exciting flashes) took about 1 min to become stable. After the onset of illumination the decay curves increased in intensity for about 5 sec, after which they slowly decreased in intensity for about 1 min. Such delayed light transients in whole cells have been previously reported[1,22–24]. They are similar to fluorescence[25–27] and O_2 evolution[28–30] transients which have been observed at the onset of illumination. The data presented below were taken after a stable intensity of delayed light was achieved.

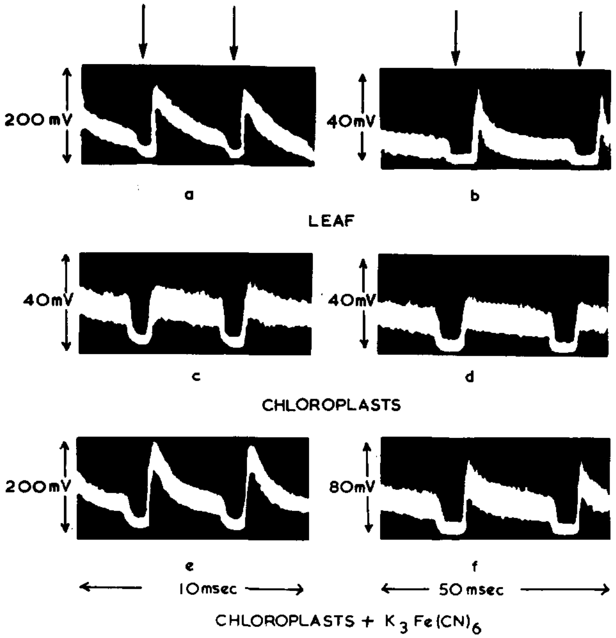

Fig. 1. Delayed light from leaves and chloroplasts. The measuring equipment was blocked from repeating flashes of exciting light which were given during the zero readings on either side of the decay curves, as indicated by arrows above photographs a and b (see text). Photographs of the oscilloscope face were taken at two different flashing rates of the exciting light: 250 flashes per sec (a, c, e) and 40 flashes per sec (b, d, f). The two photographs at the top (a, b) show the decay of delayed light from a segment of Good King Henry leaf. The decay was rapid, and different sensitivities were required to bring signals at the two flashing rates to measurable levels on the oscilloscope face. The two photographs in the middle (c, d) show the decay of delayed light from Good King Henry chloroplasts in the absence of a hydrogen acceptor. The decay was slow, and the same sensitivity was required for both flashing rates. Reaction mixture: 10 mM Tricine (pH 7.8); 3 mM $MgCl_2$; 140 μg chlorophyll/ml. The two photographs at the bottom (e,f) show the decay of delayed light from the same chloroplast reaction mixture after injection of the electron acceptor, $K_3Fe(CN)_6$, to give a final concentration of 0.5 mM. The decay was rapid, approaching the kinetics observed with leaf segments.

Comparison of delayed light decay curves from leaves to those from chloroplasts

Figs. 1a and 1b are oscilloscope photographs of the delayed light emission from a segment of a Good King Henry leaf, taken at 250 and 40 flashes per sec. Figs. 1c and 1d give the same data for chloroplasts extracted from the same sample of leaves, with no hydrogen acceptors or phosphorylation cofactors added to the reaction mixture. The absolute intensities of delayed emission from the leaf and from the chloroplasts cannot be compared because of their different optical properties. However, it is clear from Figs. 1a–1d that in the time range measured the emission from the leaves decayed much more rapidly in the dark than that from the chloroplasts. Figs. 1e and 1f show that upon injection of $K_3Fe(CN)_6$ into the chloroplast reaction mixture, the emission from the chloroplasts was increased in intensity, and the dark decay became more rapid, approaching the decay kinetics of the leaf. Similar results were

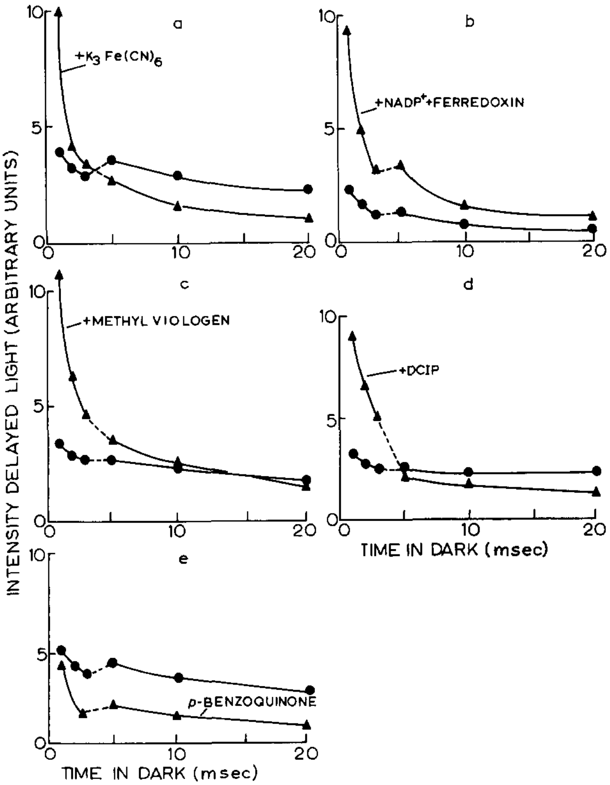

Fig. 2. Effect on delayed light of injection of electron acceptors which activate noncyclic or pseudo-cyclic electron flow. The unlabeled curves give delayed light decay from chloroplast reaction mixtures in the absence of any added electron acceptor. The labeled curves give delayed light decay after injection into the reaction mixture of the particular electron acceptor which is indicated. All of the electron acceptors caused a more rapid decay of delayed light. With the exception of *p*-benzoquinone (e), all electron acceptors caused an increased intensity of emission at 1 msec (a–d). Reaction mixtures: a, 1 mM Tris–HCl (pH 7.4); 3 mM $MgCl_2$; 0.5 mM $K_3Fe(CN)_6$; 43 μg chlorophyll/ml. b, 1 mM Tris–HCl (pH 7.4); 3 mM $MgCl_2$; 0.5 mM $NADP^+$; 50 μl ferredoxin; 43 μg chlorophyll/ml. c, 1 mM Tris–HCl (pH 7.4); 3 mM $MgCl_2$; 0.5 mM methylviologen; 43 μg chlorophyll/ml. d, 10 mM Tricine (pH 7.8); 3 mM $MgCl_2$; 4 μM DCIP; 144 μg chlorophyll/ml. e, 1 mM Tris–HCl (pH 7.4); 3 mM $MgCl_2$; 2.5 mM *p*-benzoquinone; 35 μg chlorophyll/ml.

obtained with leaves and with chloroplasts from spinach, spinach beet, and pea. We found no evidence for quantitative differences in behavior, as regards the millisecond delayed light emission, between chloroplasts extracted from the four species tested.

Effect of activation of noncyclic electron transport on delayed light

Fig. 2 gives the effect on delayed light of activating noncyclic (or pseudo-cyclic) electron flow by injecting five different compounds, $K_3Fe(CN)_6$, $NADP^+$ + ferredoxin, DCIP, methylviologen (pseudo-cyclic), and *p*-benzoquinone[31] into the reaction mixture. In all cases the dark decay of emission was more rapid on activation of noncyclic or pseudo-cyclic electron transport. With all compounds, except *p*-benzoquinone, the intensity of emission at 1 msec was 2–4 times more intense after injection of the activator. Because of the faster decay in the presence of the activators, the more intense emission at 1 msec either approached the intensity of the control at 20 msec or else crossed the control and was less intense at 20 msec. The exception to these statements, *p*-benzoquinone, resulted in a lower intensity of emission at all times of observation. No effect was produced by injection of 0.1 ml of water, or of 0.1 ml of 10 mM NaCl.

Effect of activation of cyclic electron transport on delayed light

Fig. 3 gives the effect on delayed light of activating cyclic electron flow by injection of two different cofactors: pyocyanine and phenazine methosulfate (PMS). Pyocyanine slightly increased emission at 1 msec and the decay was more rapid than in the control chloroplasts. Thus, pyocyanine had an effect similar to that of the

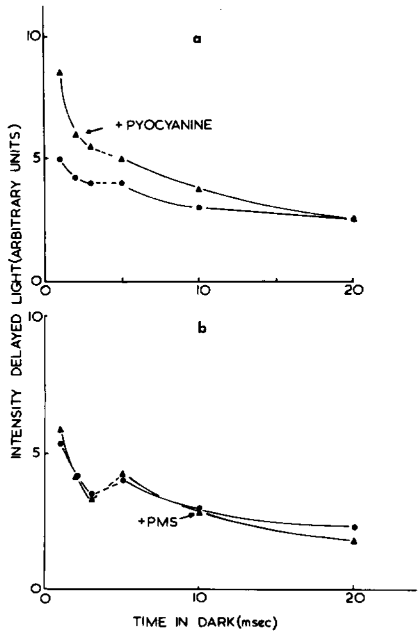

Fig. 3. Effect on delayed light of injection of cofactors of cyclic electron flow. Pyocyanine and PMS had a smaller effect on the delayed light than did the activators of noncyclic electron flow. Reaction mixtures: a, 1 mM Tris–HCl (pH 7.4); 3 mM $MgCl_2$; 30 μM pyocyanine; 35 μg chlorophyll per ml. b, 1 mM Tris–HCl (pH 7.4); 3 mM $MgCl_2$; 30 μM PMS; 58 μg chlorophyll/ml.

noncyclic activators, but reduced in magnitude. PMS, on the other hand, had no effect on the emission when added in the absence of other factors, although there was a strong interaction between PMS and ascorbate, which will be presented below.

Reduced intensity of delayed light caused by addition of ADP and P_i

Fig. 4 shows the effect of addition of ADP and P_i, added separately and added together, on the delayed light. Fig. 4a gives the effect in the absence of other cofactors, and Figs. 4b–4e give the effect in the presence of $K_3Fe(CN)_6$, pyocyanine, ascorbate, and pyocyanine *plus* ascorbate. P_i alone had no effect in any of the cases tested. ADP alone resulted in reduced emission in all cases tested, but it had little effect on the kinetics of decay. Addition of ADP and P_i together generally resulted in a further reduction in intensity over ADP alone, but this was a small effect. We assume that the small effect of adding P_i in the presence of ADP implies that our chloroplasts contained a nearly adequate endogenous phosphate supply.

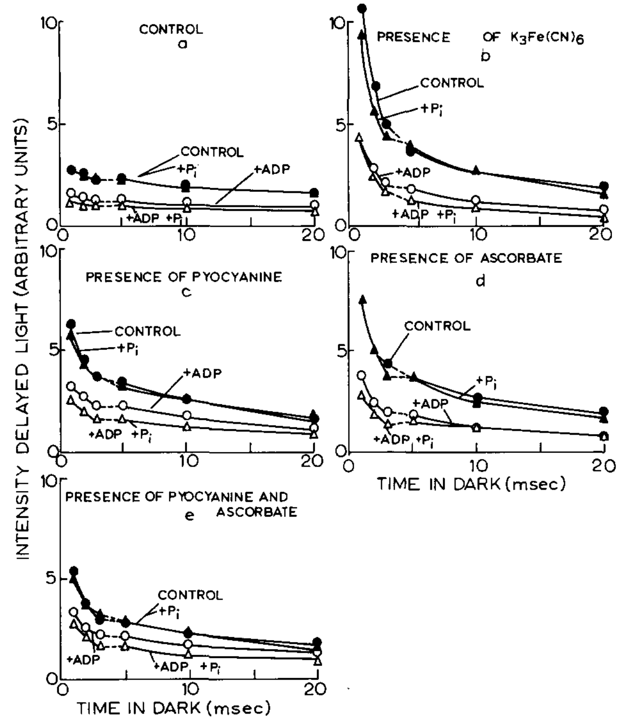

Fig. 4. Effects of ADP and P_i on delayed light. The intensity of delayed light was reduced by ADP or ADP+P_i, but P_i alone had no effect. Reaction mixtures: 10 mM Tricine (pH 7.8); 3 mM $MgCl_2$; 5 mM Na_2HPO_4–KH_2PO_4 (pH 7.8); 6 mM ADP; 41 μg chlorophyll/ml; 0.5 mM $K_3Fe(CN)_6$; 30 μM pyocyanine; 5 mM ascorbate.

Fig. 5 gives the effect of adding the combination ADP+ P_i to reaction mixtures containing $NADP^+$ + ferredoxin, methylviologen, *p*-benzoquinone, and PMS. In all cases the combination ADP + P_i reduced the intensity of delayed light without significantly affecting the kinetics of dark decay. In spite of this reduction in intensity of emission, the presence of ADP + P_i did not qualitatively change the effects of injection of $K_3Fe(CN)_6$, $NADP^+$ + ferredoxin, or pyocyanine.

Effect of addition of ascorbate on delayed light

Fig. 6 gives the effect on the delayed emission of addition of ascorbate. In the absence of other cofactors, addition of ascorbate resulted in increased intensity of emission and in a more rapid decay, as shown in Fig. 6a. This effect is similar to that of the activators of noncyclic electron flow. There were interactions between ascorbate and most other factors included in the reaction mixtures. Figs. 6b–6d give the effects of ascorbate when noncyclic electron transport was activated by $NADP^+$ + ferredoxin, methylviologen, and *p*-benzoquinone. In all these cases ascorbate slightly reduced intensity of the emission without strongly affecting the decay kinetics.

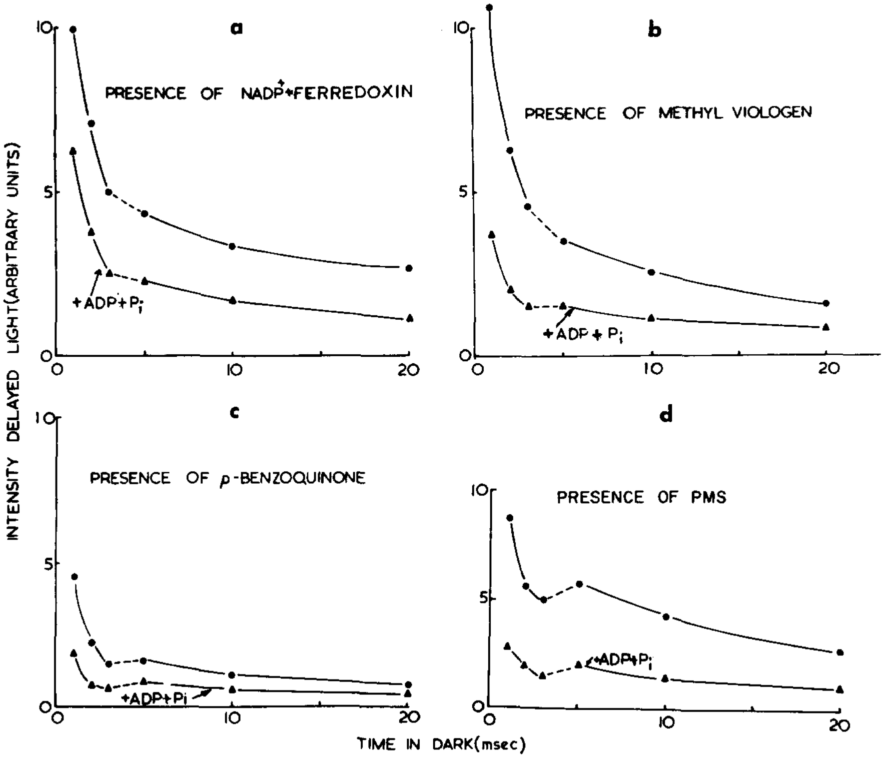

Fig. 5. Reduction of delayed light by the combination $ADP+P_i$. Addition of $ADP+P_i$ to the reaction mixture reduced the intensity of delayed light. Reaction mixtures: 1 mM Tris–HCl (pH 7.4); 3 mM $MgCl_2$; 5 mM Na_2HPO_4–KH_2PO_4 (pH 7.4); 6 mM ADP; 0.5 mM $NADP^+$; 50 µl ferredoxin; 0.5 mM methylviologen; 2.5 mM *p*-benzoquinone; 30 µM PMS. a and b, 43 µg chlorophyll/ml; c, 35 µg chlorophyll/ml; d, 58 µg chlorophyll/ml.

Although ascorbate increased the intensity of emission at 1 msec in the absence of activators of electron transport but reduced the intensity of emission in the presence of activators of noncyclic electron transport, these effects were not large enough to eliminate the effect of injecting noncyclic activators into reaction mixtures which included ascorbate. That is, injection of $NADP^+$ + ferredoxin, methylviologen, or quinone into reaction mixtures containing 5 mM ascorbate resulted in the same qualitative changes as in the absence of ascorbate. The combination $ADP+P_i$ always reduced intensity of emission regardless of the presence or absence of ascorbate, except in the case where PMS and ascorbate were included together in the reaction mixture.

The effect of ascorbate when cyclic electron flow was activated by pyocyanine is shown in Fig. 6e. Ascorbate increased the intensity of emission in the presence of

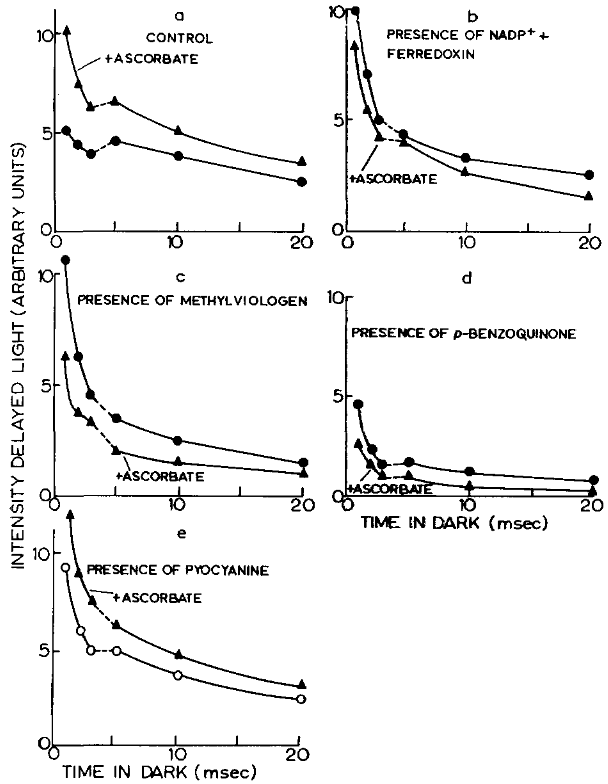

Fig. 6. Effects of ascorbate on delayed light. Reaction mixtures: 1 mM Tris–HCl (pH 7.4); 3 mM $MgCl_2$; 5 mM ascorbate; 0.5 mM $NADP^+$; 50 µg ferredoxin; 0.5 mM methylviologen; 2.5 mM *p*-benzoquinone; 30 µM pyocyanine; a–c, 43 µg chlorophyll/ml; d and e, 35 µg chlorophyll/ml.

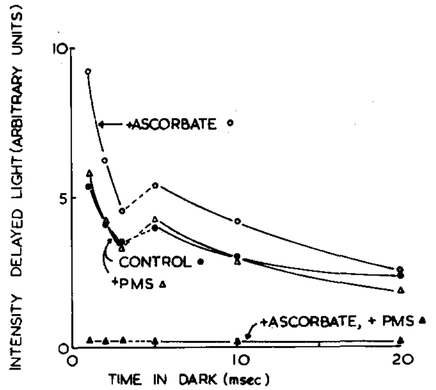

Fig. 7. Interaction between ascorbate and PMS on delayed light. Reaction mixture: 1 mM Tris–HCl (pH 7.4); 3 mM $MgCl_2$; 5 mM ascorbate; 30 µM PMS.

pyocyanine. However, Fig. 7 shows that ascorbate strongly reduced the emission in the presence of PMS. Neither ascorbate nor PMS given separately reduced the emission, but both factors together resulted in a reduction of 20–40 times in the intensity of emission.

DISCUSSION

There is a simple way of regarding the photochemical system in terms of primary reaction centers which are related to a matrix of absorbing pigments. After the original absorption act by a molecule of chlorophyll or accessory pigment in the photosynthetic apparatus, the resulting electronic excitation energy apparently migrates rapidly over many molecules of the bulk pigment system until the excitation reaches a reaction center[31]. At this site the excitation energy may be converted to chemical potential.

In less than 1 nsec (the fluorescence lifetime of chlorophyll *in vivo* is between 1 to 0.1 nsec)[8–12] the electronic excitation energy is no longer freely available to excite chlorophyll in the pigment system. There is no definitive evidence for the earliest time at which the energy may appear in enzyme systems as oxidizing and reducing power. On the other hand, at room temperature the enzymes would require somewhat more than 0.01 sec to complete their reactions and to be able to accept further energy from the pigment system. The time interval given here for completion of enzymatic processes is taken as equal to that determined by EMERSON AND ARNOLD[13,14] as the relaxation time for photosynthesis in flashing light. Once all pools are filled, the energy would be stored from approx. 1 nsec to 0.01 sec. The delayed light observed during the period from 1 to 20 msec may be the result of a low yield reexcitation of chlorophyll by the stored energy, the major portion of which is also being utilized by enzymatic reactions during this time range.

Emission spectra show that the delayed light is from chlorophyll *a*[2–4], and that delayed light is not emitted from the accessory pigments in various plant phyla[5]. From experiments with mutant algae it has been concluded that chlorophyll *a* receives delayed excitation from the energy stored by Photoreaction II (ref. 7). It is not known whether the delayed light is emitted from the bulk energy-absorbing chlorophyll *a* or from the reaction center. Nevertheless, the intensity of emission at one time must be determined by the amount of energy stored by Photoreaction II at that time.

Increase in stored energy with noncyclic electron flow

Isolated chloroplasts show decay kinetics which are slower than those given by leaf tissues (Fig. 1). Rapid decay kinetics similar to those in leaf tissue are shown by chloroplasts under conditions where stored energy can be used for reduction of a hydrogen acceptor (Figs. 1 and 2). These differences imply that the stored energy, a part of which results in delayed light, is being used for photosynthesis. This interpretation is strengthened by the earlier observations of very slow millisecond delayed light decay kinetics using whole algal cells in which electron transport was blocked by 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)[33,34] or using mutant cells in which Photoreaction I was nonfunctional[7].

Because of their different optical properties, it was difficult to compare the intensities of emission of leaves and of chloroplast reaction mixtures. However, it seems clear from the effects of $K_3Fe(CN)_6$, $NADP^+$ + ferredoxin, DCIP, and methylviologen that activation of a noncyclic or pseudo-cyclic electron flow not only produces a more rapid decay, but also increases the intensity of emission at the earliest time measured (1 msec). These results imply that activation of electron transport associated with Photoreaction II results in an increase in stored energy at times shorter than the turnover time of the enzyme systems.

The activation of electron transport by *p*-benzoquinone (Fig. 2e) gave results which were different from the other hydrogen acceptors, in that although *p*-benzoquinone caused a more rapid decay, it also reduced the intensity of emission at all times observed. The behavior of *p*-benzoquinone may result from a direct quenching of singlet emission from chlorophyll, since *p*-benzoquinone is known to quench the fluorescence of chlorophyll in solution[35–38]. This interpretation is supported by our observation that 2.5 mM *p*-benzoquinone reduced the fluorescence from spinach chloroplasts to 1/3–1/2 that of the control chloroplasts.

Effects of other cofactors

Pyocyanine and PMS, which activate cyclic electron transport, had different effects on the delayed light (Fig. 3). Pyocyanine had an effect similar to that of the noncyclic activators, but reduced in magnitude. PMS had no effect when added in the absence of the other factors. These results could be explained by assuming that pyocyanine and PMS may possibly activate different cyclic pathways of electron transport, in spite of the similarity of the two molecules. It is possible that pyocyanine catalyzes a cyclic electron transport which can utilize energy from Photoreaction II, whereas PMS catalyzes a cyclic electron transport which cannot use energy from Photoreaction II.

Addition of P_i to the chloroplast reaction mixture had no effect on the millisecond emission, but addition of either ADP alone or of ADP and P_i together reduced the intensity of the emission without strongly altering the kinetics of the dark decay for all cases tested (Figs. 4 and 5). Since electron transport in our chloroplasts was coupled to phosphorylation (O_2 evolution was enhanced by addition of either NH_4^+ or ADP + P_i, and both factors together enhanced only to the extent of NH_4^+ alone), the effects of ADP and ADP + P_i may be due to increased rates of enzymatic electron transport. This interpretation is strengthened by the observation that addition of ADP + P_i had no effect on the millisecond emission when the chloroplasts had been uncoupled by inclusion of $(NH_4)_2SO_4$ (2.5 mM) in the reaction mixture.

CONCLUSION

The present paper has been concerned with the behavior of isolated chloroplasts, as compared with the leaf, in observations of the delayed light emission. The experiments indicated that the delayed emission was closely associated with the functioning of the energy conversion mechanism of photosynthesis, and support was lent to the previous conclusion that the delayed light was emitted by Photoreaction II, the part of the mechanism closely concerned with production of O_2.

It may be argued that the chlorophyll-containing structure in the chloroplasts converts the light energy absorbed by it into a variety of chemical forms. While this process can be in no sense completely reversible, it is observed to be relatively efficient.

Therefore it might follow that any spontaneous chemical process which stored energy within the structure of the chloroplast could have a chance of transferring some energy to the chlorophyll, resulting in the form corresponding to that produced on absorption of light.

The work of MAYNE AND CLAYTON[39] has shown that changes in pH or in oxidation level, both of which result in chemical changes within the chloroplasts, will cause emission of light from chloroplasts kept in the dark. If this chemically induced chlorophyll emission results from the same energy storage state of Photoreaction II which produces the delayed emission studied here, then this storage state cannot be equated with the final high energy condition which may produce ATP (X_E). This statement is supported by the following evidence. The half-life of X_E at pH 7.4 was found to be 7 sec (ref. 40), which is about 1000 times longer than the decay of the delayed light emission here studied. DCMU inhibits the chemically induced light emission[39], but does not effect the chemically induced formation of ATP[41]. $K_3Fe(CN)_6$ diminished the chemically induced light emission[39]. The delayed light appears to be emitted only by the chlorophyll associated with Photoreaction II (ref. 7), while there is no absolute requirement for participation of Photoreaction II in photophosphorylation[42–44].

Delayed light must be related to both the initial physical processes and to the subsequent chemical processes of energy conversion by Photoreaction II. The present experiments indicate that the stored energy which results in delayed light is also utilized in electron transport. Furthermore, they imply that activation of the enzyme reactions results in an increase in energy storage at times somewhat shorter than the steady-state turnover time of the enzyme systems. This experimental result contradicts the *a priori* assumption that utilization of energy by the electron transport chain would result in less energy storage by the photoreaction at all times after excitation. In fact, the experiments imply that a transient increase in stored energy is caused by utilization of the stored energy itself. Thus this energy storage state has properties which are not analogous to such simple systems as a chemical pool, or the flow of liquid out of a container.

Two models for conversion of chlorophyll excitation into chemical potential are presented elsewhere[16]. They are the charge-transfer model and the electron-hole model. Both models have energy storage properties which are in agreement with our observations, and with other evidence. At present there seems to be no absolutely compelling evidence for choosing one model over the other.

While this research was in progress we learned that Dr. BERGER C. MAYNE of the Charles F. Kettering Research Laboratory was performing similar experiments. MAYNE also finds an increase in delayed light on activation of noncyclic electron transport[45].

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