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LOW TEMPERATURE PHOTO-INDUCED REACTIONS IN GREEN LEAVES AND CHLOROPLASTS

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SUMMARY

1. Swiss chard leaves, spinach leaves and spinach chloroplasts at liquid nitrogen temperatures exhibit photo-induced absorbance changes with maxima at 556–557, 680–682 and 703–705 nm. Experiments reported indicate that the 556 nm peak is due to the α-band of cytochrome b_{559}. The peak at 680 nm is attributed to P_{680}, the photoactive center of photosystem II, and the peak at 703 nm is attributed to P_{700}.

2. Laser-induced oxidation of cytochrome b_{559} has a half-time of 4.6 msec. Laser oxidized P_{680} has two reductive phases, one having a half-time of 30 μsec and the other of 4.5 msec. P_{700} has only one reductive phase with a half-time of approx. 30 μsec. The kinetics strongly suggest that P_{680} is the primary oxidant of cytochrome b_{559} at low temperatures.

3. The slow reductive phase of P_{680} and oxidation of cytochrome b_{559} tended to have equal and approximately constant rates between 80 and 220°K.

INTRODUCTION

CHANCE AND NISHIMURA[1] utilizing *Chromatium* D first showed cytochrome photooxidation at low temperatures (77° K). Since then the reaction has been studied extensively[2] and has been shown to involve a tunneling mechanism of electron transfer from cytochrome to bacteriochlorophyll. Low temperature cytochrome photooxidation has been shown to occur in green plants[3–5]. In addition, low-temperature photooxidation peaks in the region of 700 nm have been found[4,5].

CHANCE AND BONNER[5] and CHANCE *et al.*[6] observed the low-temperature oxidation of a pigment in intact Swiss chard leaves at 77°K. While they attributed this absorbance change to the oxidation of cytochrome *f*, they showed preliminarily[5] and subsequently conclusively[6] that the pigment oxidation was not related to P_{700}. More recently, KNAFF AND ARNON[7] have confirmed the original observations, but have attributed the 556 nm absorbance peaks to cytochrome b_{559}, which is identified with photosystem II (P680) rather than photosystem I (P700)[8,9]. Identification of P_{680} as the electron acceptor in this reaction would seem to clarify these two different interpretations of the experimental result. Our experiments now afford a definitive assignment of the peak at 556 nm, and in addition identify the electron

acceptor for the reaction to P_{680}. Temperature profiles have been obtained which demonstrate the correlation of the kinetics of b_{559} and P_{680} and furthermore provide data which are consistent with a tunneling mechanism for the cytochrome reaction in green plants.

MATERIALS AND METHODS

Swiss chard (*Beta vulgaris*) leaves, spinach leaves (*Spinacea oleracea*) and spinach chloroplasts were the experimental materials. Greenhouse-grown Swiss chard leaves were kept in distilled water in the dark and spinach leaves obtained locally were kept in a cold room (7.5°C) until use. Chloroplasts were prepared from deveined leaves by the method of CRAMER AND BUTLER[10] and AVRON[11], except that a Waring Blendor model 700 b was used to homogenize the leaves for 10 sec.

Low-temperature light *minus* dark difference spectra were obtained utilizing a dual wavelength spectrophotometer[12] with the analyzing beams usually of 3.5 $\mu W/cm^2$ intensity and half-height slit width of approximately 1 nm. When leaves were used, a 2 cm × 2 cm section of leaf was positioned between lucite windows and attached to a cuvette equipped with an aluminum cold finger to aid in rapid and prolonged thermal equilibration with the liquid nitrogen (77°K) or solid CO_2–2-propanol mixture (194.5°K) in the Dewar. Veins in the leaf sections were covered with strips of black tape. Chloroplasts were placed in a similar type cuvette having a path width of 0.5 or 1.0 mm. Spectra in the cytochrome α-band region were obtained with a continuous actinic red light (3.7 mW/cm^2) which was obtained by passing white light through a Corning 2-64 filter. The reference beam was 540 nm. Spectra in the 670–710 nm region were obtained with a 250 μsec green flash (0.45 mJ) obtained by passing a xenon flash through a Wratten-57 filter. The flashes did saturate the photomultiplier, but recovery was fast enough to yield a true representation of the absorbance change on the relatively slow dual wavelength recordings. Several flashes were required to completely light-titrate out all the components in the 670–710 region as found by CHANCE *et al.*[6]. The reference beam in this region was 635 nm.

A Q-switched ruby laser system yielding a 20 nsec, 694 nm pulse[13] was used to observe the kinetics of the photo-induced absorbance changes of the components. The ruby laser was usually used to induce oxidation of the cytochrome. However, 539 nm laser light generated by stimulated Raman effect on the ruby light focused into hydrogen at 60 atm pressure, was used as actinic light when measurements were made in the 670–710 nm region. The maximum energy obtained was 1.2 mJ per pulse. Appropriate lenses were used to obtain the desired laser intensity at the sample. When the green laser was utilized, 0.5 M $NiSO_4$, Corning 4-96 and Wratten-57 filters were used. Interference 700 nm Corning 2-64 or Wratten-24 guard filters were used in the 670–710 region and Corning 4-96 filters in the cytochrome α-band region.

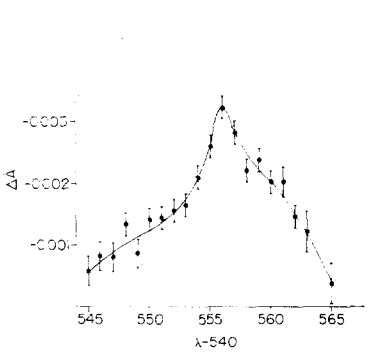
RESULTS

Fig. 1 presents the light *minus* dark difference spectra of Swiss chard leaves at liquid nitrogen temperatures. Each point is the average of two or three experiments.

There is a distinct peak at 556 nm with what appears to be a shoulder at 560 nm and 547–552 nm. KNAFF AND ARNON[7] have assigned the 556 nm peak to cytochrome b_{559}. Other than the observation by BOARDMAN AND ANDERSON[9] that the isolated cytochrome *f* difference spectrum at liquid nitrogen temperature has two peaks (552 and 548 nm) and the cytochrome b_{559} band was shifted to 557 nm, there has not been a systematic study of the migration of the peaks of cytochrome *f* and cytochrome b_{559} with decreasing temperatures. Since the 556 nm peak is nearer the room temperature peak of cytochrome *f* (554 nm) than cytochrome b_{559} (559 nm), we have done experiments that allow us to be more definitive of the 556 nm peak. These experiments were: (A) light-induced spectra in other plant material, and (B) spectra taken at temperatures intermediate to room and liquid nitrogen temperature.

Fig. 2 presents the photo-induced difference spectrum of spinach leaves and chloroplasts at liquid nitrogen temperatures. The major peak has shifted to 557 nm, making it closer to the room temperature peak of cytochrome b_{559} than cytochrome *f*. The shoulder at the long wavelength side of the major peak appears to be present in spinach leaves as in Swiss chard, but there is less evidence of it in the spinach chloroplasts. The shape of the spectra in the 547–552 region has changed considerably. There appears to be more of a peak in this region in the spinach leaves and a distinct one at 547 nm in the chloroplasts. It is not known what the 547 nm peak is due to, but it is far too large in relation to the absorbance at 552 nm for it to be considered a portion of the split α-band of cytochrome *f* (ref. 9).

Additional evidence that the 556 nm peak is due to cytochrome b_{559} is provided by the experiment in Fig. 3. At an intermediate temperature, the solid CO_2 sublimation point (194.5°K), the peak is at 558–559 nm in spinach chloroplasts. This experiment indicates there is a migration of the peak to the blue as the temperature is lowered and suggests strongly that the 556 nm peak observed at liquid nitrogen temperature in Swiss chard is due to cytochrome b_{559}.



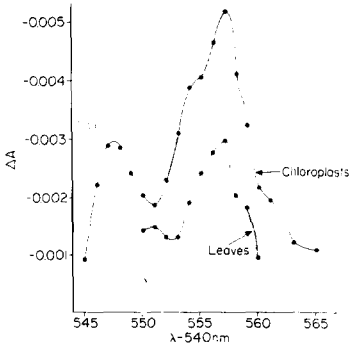


Fig. 1. Light *minus* dark difference spectra of Swiss chard leaves at liquid nitrogen temperatures. Oxidation was accomplished with a continuous light of $\lambda > 640$ nm of 3.7 mW/cm² intensity. The chlorophyll content of an average leaf section exposed to the analyzing beams was 27 nmoles. The band width was approximately 1 nm.

Fig. 2. Light *minus* dark difference spectra of spinach leaves and chloroplasts at liquid nitrogen temperatures. Actinic light was as in Fig. 1. The chlorophyll content of the chloroplast solution in the 0.5 mm wide path length cuvette was 20 nmoles. The spinach was obtained from local farms. All other conditions as in Fig. 1.

In addition to the absorbance changes occurring in the cytochrome α-band region there are photo-induced changes occurring in the 670–710 nm region. The spectrum in this region in Swiss chard leaves at liquid nitrogen temperature is shown in Fig. 4. There are absorption decreases at approximately 705 and 682 nm and an absorption increase at 690 nm. A similar type of spectrum was obtained using the green laser as an actinic source, however the absorption maxima were at 703 and 680 nm and no positive shift was observed at 690 nm. It is assumed that the peak at 703–705 nm is due to the photooxidation of P_{700}, and these changes at 680–682 nm to P_{680}.

Thus it appears that there are at least three components which show photo-induced absorbance changes at liquid nitrogen temperatures; cytochrome b_{559}, P_{680} and P_{700}. We have utilized the ruby and green laser to observe the kinetics of photo-induced absorbance changes of cytochrome b_{559}, P_{680} and P_{700}. Fig. 5 presents the kinetics of cytochrome b_{559} photooxidation in Swiss chard leaves at liquid nitrogen temperatures. Calculation of the half-times for 13 such determinations yielded an average value of 4.6 msec. On close examination it will be noted there is a small increase in absorbance (downward) $<$ 300 μsec (instrument time) after

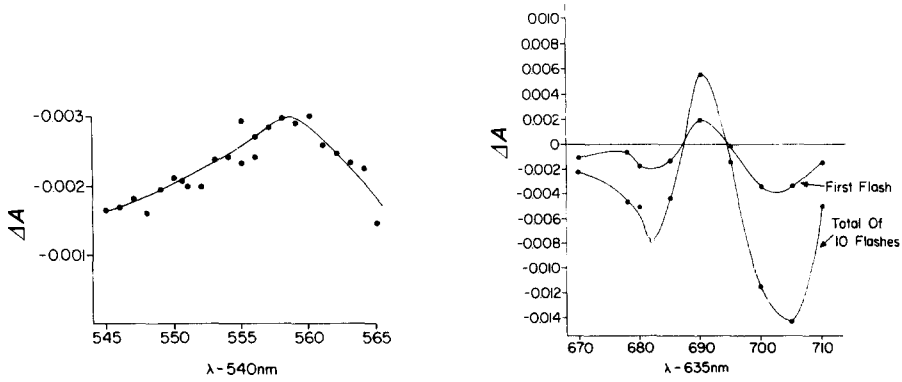


Fig. 3. Light *minus* dark difference spectra of spinach chloroplasts near 194.5°K. The spinach was obtained from the local market. All other conditions as in Fig. 2.

Fig. 4. Light *minus* dark difference spectra of Swiss chard leaves at liquid nitrogen temperatures. The actinic source was green 250 μsec flashes. All other conditions as in Fig. 1.

Fig. 5. Oxidation of cytochrome b_{559} in Swiss chard leaves at liquid nitrogen temperatures induced by the Q-switched ruby laser (130 mJ). The instrument time was 300 μsec. The band width was approximately 10 nm. All other conditions as in Fig. 1. An average of 13 such determinations yielded a half-time of 4.6 msec with a standard deviation of the mean of ± 0.13 msec.

Fig. 6. Oxidation of P_{700} in Swiss chard leaves induced by the Raman stimulated green laser (0.5 mJ). All other conditions as in Fig. 5.

the laser fired. It is not known what this transient is due to, but it was observed in most determinations and seemed to be more prevalent at higher temperatures. P_{700} and P_{680} photooxidation kinetics in Swiss chard leaves at liquid nitrogen temperatures are shown in Figs. 6 and 7, respectively. At this sweep speed it is apparent that P_{700} is oxidized by the laser and is not reduced. The slight decrease which is evident is due to the relaxation time (100 msec) of the a.c. coupling from photomultiplier preamplifier to oscilloscope. In contrast to P_{700}, P_{680} is reduced after it is oxidized by the laser. The kinetics appear to correlate well with those of cytochrome b_{559} oxidation; in fact, an average value of 4.5 msec was obtained for 9 such determinations.

It was thought that a study of the reaction rates of P_{680} and cytochrome b_{559} as a function of temperature would yield an insight into the mechanism involved. Fig. 8 presents the data obtained from four experiments in which chloroplasts prepared from market spinach were used. Regression coefficients were calculated for P_{680} reduction and cytochrome b_{559} oxidation individually; however, since the

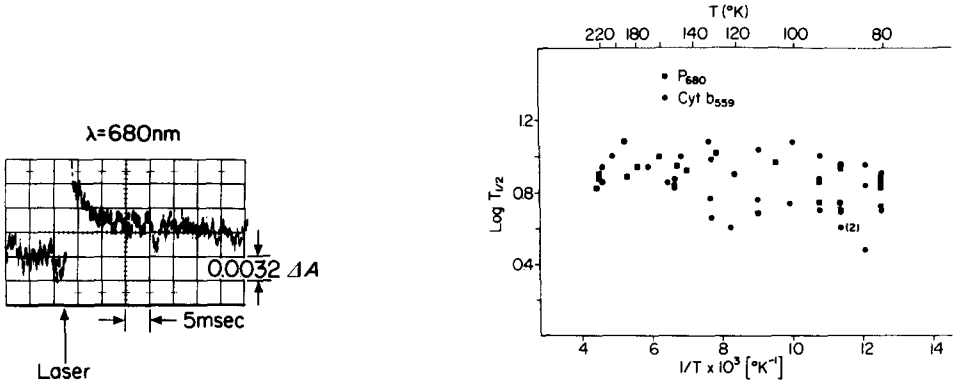


Fig. 7. P_{680} kinetics in Swiss chard leaves induced by the green laser (0.5 mJ). All other conditions as in Fig. 6. An average of 9 determinations yielded a half-time of 4.5 msec with a standard deviation of the mean of $\pm$ 0.074 msec.

Fig. 8. The slow reduction rates of green laser oxidized P_{680} and ruby laser induced oxidation rates of cytochrome b_{559} in chloroplasts from market spinach as a function of temperature. The chlorophyll content was as in Fig. 2. Band width was approximately 10 nm. Laser intensity is as in Fig. 6. The half-time is expressed in msec.

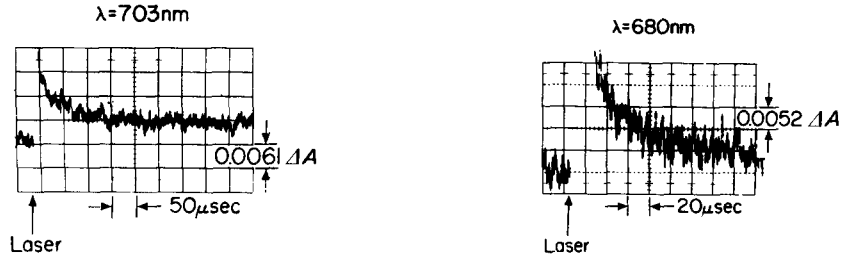


Fig. 9. The fast reductive phase of green laser oxidized P_{700} in market spinach chloroplasts at liquid nitrogen temperatures. Conditions as in Fig. 8.

Fig. 10. The fast reductive phase of green laser oxidized P_{680} in market spinach chloroplasts at liquid nitrogen temperatures. Conditions as in Fig. 8.

difference between the slopes of the two was not significant, the data were pooled and a regression line calculated. Calculation of the activation energy yielded a value of -89 ± 30 cal. There are at least three interesting points that can be derived from the data: (a) P_{680} reduction time correlates well with cytochrome b_{559} oxidation time over the entire temperature range studied. (b) The half-times of P_{680} and cytochrome b_{559} in the chloroplasts tend to be in the range of 5–10 msec, which is slightly higher than observed in the intact Swiss chard leaves. (c) This experiment does provide evidence that the reaction occurring is *via* a tunneling mechanism, as was observed in *Chromatium* D[2]. This is the first demonstration of a tunneling mechanism of electron transfer in green plants.

Even though there was no reduction of photooxidized P_{700} that correlated with the oxidation time of cytochrome b_{559}, there does appear to be a fast reduction component as shown in Fig. 9. The average of three such determinations yield a half-time value of 30 μsec. There was also a fast reduction component (30 μsec) of photooxidized P_{680} as shown in Fig. 10. It is not known what the nature of these reactions is, but the observation that both P_{680} and P_{700} are reduced with a similar half-time suggests that the two mechanisms may be closely related.

DISCUSSION

Fig. 1 demonstrates that the major peak of the difference spectra of Swiss chard leaves at liquid nitrogen temperatures is at 556 nm. The experiments presented in Figs. 2 and 3 strongly suggest that this peak is due to cytochrome b_{559}, as KNAFF AND ARNON[7] have suggested. That is, the maximum was nearer the room temperature maximum of cytochrome b_{559} in different plant material and at a temperature intermediate between room and liquid nitrogen.

A 560 nm shoulder is apparent in Swiss chard leaves (Fig. 1) and the spinach leaves, but less apparent in the spinach chloroplasts (Fig. 2). The shoulder may be due to cytochrome b_6 oxidation on the basis of the work of BOARDMAN AND ANDERSON[9]. If the 560 nm shoulder is due to cytochrome b_6 oxidation this would suggest that photosystem I is activating the oxidation since this cytochrome has been found to be closely associated with subchloroplast particles enriched in photosystem I (refs. 8 and 9).

The observation that the half-time of cytochrome b_{559} light-induced oxidation and the reduction of photooxidized P_{680} are equivalent strongly suggests that the cytochrome is the electron donor to P_{680}. The observation that the reaction rates of both had similar temperature profiles (*cf.* Fig. 8) corroborates the kinetic data. These findings necessitate an evaluation of this observation in terms of the currently accepted model of green plant photosynthesis. It should be noted that cytochrome b_{559} is tightly bound to the chloroplast lamellae[9] and its position in the electron transfer reactions of photosynthesis remains enigmatic. However, several observations provide some clues as to its function and location. BOARDMAN AND ANDERSON[9] and VERNON *et al.*[8], using subchloroplast fractions, found cytochrome b_{559} closely associated with photosystem II. Also the observations made by CRAMER AND BUTLER[10] and HIND[14] suggest that cytochrome b_{559} is far removed from the photosystem I apparatus. To explain their experimental results they postulated that there was a phosphorylation site between cytochrome b_{559} and cytochrome f, a component

usually thought to be closely associated with photosystem I. Additional evidence that cytochrome b_{559} is closely associated with photosystem II and that it is oxidized by this photosystem at low temperature is provided by the observations of KNAFF AND ARNON[7] and CHANCE AND BONNER[5] who showed an action spectrum of cytochrome oxidation typical of a photosystem II spectrum.

All of the evidence suggests that cytochrome b_{559} is closely associated with photosystem II and is, in fact, photooxidized *via* that system at low temperatures. However, it does not give clues as to whether cytochrome b_{559} is located on the reducing or oxidizing side of photosystem II in the currently accepted "Z" scheme[15] of electron flow in photosynthesis. CRAMER AND BUTLER[10] found photosystem II light more effective than photosystem I light in reducing cytochrome b_{559}. Similar conclusions were drawn from the experimental observations of HIND[14] using spinach chloroplasts and by LEVINE *et al.*[16] using *Chlamydomonas reinhardi* mutants. If cytochrome b_{559} is on the reducing side, the question remains why it is oxidized by photosystem II at low temperatures but reduced by this system at physiological temperatures. The observations that cytochrome b_{559} is closely associated with photosystem II particles[8,9] suggests that the normal electron donor to P_{680} is not operating at low temperatures and hence cytochrome b_{559} acts in its place. This has also been suggested by BUTLER (personal communication).

If it is assumed that the natural electron donor to photosystem II is inactivated at lower temperatures, the question to be answered is why would cytochrome b_{559}, which is presumably not the physiological electron donor to the system, be so efficient at lower temperatures, when cytochrome *f*, a component either the natural donor or very near the active chlorophyll P_{700} of photosystem I, is inactive at lower temperatures? The question remains to be answered but probably at least two points may be pertinent to the observations: (a) There is probably a large difference in redox potential gap between cytochrome b_{559} and the photoactive chlorophyll of photosystem II, and (b) there may be a structural rearrangement of cytochrome b_{559} and P_{680} as the temperature is lowered. The midpoint potential of cytochrome b_{559} has been reported to be 320 mV (ref. 17), 370 mV (ref. 18) and a completely different value by CRAMER AND FAN[19] of 40–120 mV. Whatever the true value is, it is certainly much lower than the value of 820 mV which is probably near the redox potential of the photoactive chlorophyll of photosystem II, presumably P_{680}, since this is the redox potential for water oxidation. Thus this large difference in redox potentials between the two may be one of the reasons that the reaction occurs at low temperatures. Another reason why the reaction occurs at low temperatures may be because there is a structural alteration such that the cytochrome is closer to P_{680} as the temperature is lowered, so that the reaction would have a greater probability of occurring. Phase changes in the membrane components, water or other components present may be responsible for the structural alterations.

Throughout this discussion it has tacitly been assumed that P_{680} is the photoactive center of photosystem II. This remains to be proven; however, the observations of WITT[20], DORING *et al.*[21,22] and GOVINDJEE *et al.*[23] do suggest that P_{680} is the active center compliment of photosystem II as P_{700} is the active center compliment of photosystem I. The evidence that DORING *et al.*[21] presented was: (a) The absorption change could be drastically reduced by heating the chloroplasts to 55°C which completely eliminated O_2 evolution capacity, and (b) 3 (3,4-

dichlorophenyl)-1,1-dimethylurea elimination of O_2 evolution followed the same concentration profile as did the relative decrease in 680 nm absorption. GOVINDJEE *et al.*[23] have also found 3 (3,4-dichlorophenyl)-1,1-dimethylurea effective in eliminating the 682 nm change in corn chloroplasts. Other supporting evidence that the light-induced 682 nm absorption change is associated with photosystem II was obtained by DORING *et al.*[22] when they showed that this change occurred in subchloroplast particles enriched in photosystem II. Thus it appears that P_{680} is the active component of photosystem II and, in fact, the present observations that P_{680} and cytochrome b_{559} react at low temperature do add support to this conclusion in view of the finding of CHANCE AND BONNER[5] and KNAFF AND ARNON[7] that cytochrome b_{559} photooxidation has a typical photosystem II action spectrum.

The observations that both P_{680} and P_{700} have a fast reductive phase, *i.e.* half-time of approx. 30 μsec (*cf.* Figs. 9 and 10), is interesting in that it implies that there is a partial fast reduction of P_{700} even though its reduction does not correspond to that of cytochrome b_{559} oxidation. The observation that the half-time of the fast phase of P_{680} and P_{700} reduction is the same suggests that the same mechanism is involved with both, or that the band is split. Evidence that the latter may be true is provided by the work of DORING *et al.*[24] who showed that in subchloroplast particles enriched in photosystem I the P_{700} photooxidation splits into two peaks in the red, one at 700 nm and the other at 682 nm. That the band is split remains to be proven and the nature of the reaction remains to be elucidated.

Whereas we believe that more work needs to be done on the temperature profile to substantiate the true activation energy, the relatively constant reaction rate,

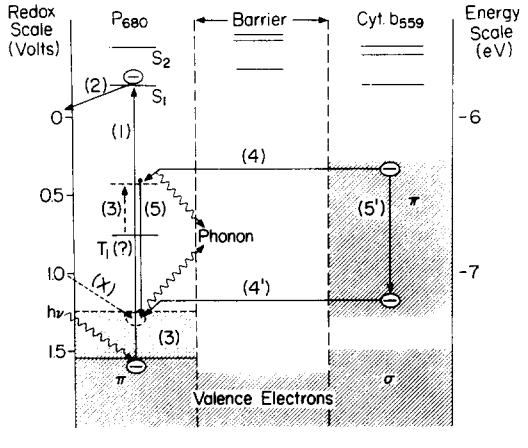


Fig. 11. Energy level diagram illustrating possible tunneling processes between cytochrome b_{559} and P_{680}. Energy levels of system II reaction center chlorophyll (P_{680}), barrier material and cytochrome b_{559} are depicted in the usual manner. The processes are: (1) excitation of P_{680} by photon absorption; (2) passage of the excited electron to the acceptor (fast); (3) relaxation of environmental charges around the ionized P_{680} raising all of its energy levels to the dotted positions (fast). At room temperature (3) would be followed by (X), the reception of an electron from H_2O or intermediates. However, this is blocked at low temperature, and we have (4) tunneling of an electron from the uppermost occupied level of b_{559} to a postulated lower excited level in P_{680}, possibly the triplet T_1, or (4') tunneling of a deep valence electron of b_{559} to the normal receptor hole in P_{680}. (4) or (4') take 4.5 msec and are followed by fast relaxation (5) or (5') respectively. One could also postulate tunneling at intermediate levels with both (5) and (5') relaxations.

in fact apparently decreasing rate as temperature is increased, leaves three phenomena to be explained: (a) the slowness (4.5 msec) of the electron transfer, (b) the mismatch of energy levels (oxidized P_{680} can ordinarily accept electrons at $>$ 800 mV redox level from water or an intermediate while the redox level of cytochrome b_{559} is at most 370 mV) and (c) the zero to negative activation energy.

The argument against a direct unhindered passage of the electron is that with the favorable "fall" from 370 mV to 800 mV it should go much more rapidly than 4.5 msec if there is no hindrance. If the passage were hindered by a barrier which is mounted by thermal agitation, the activation energy should be positive and compare appreciably to room temperature.

As was discussed earlier in the case of *Chromatium*[2] we can consider either a semiconduction mechanism by which we mean the usual band model or a tunneling process to explain the results. ELEY AND PARFITT[25] have suggested a tunneling-assisted hopping as the mechanism of electron conduction in organic solids. GLAESER AND BERRY[26] have examined these models in detail and conclude that for organic crystals like anthracene the hopping model, with tunneling, is better than the band model. We assume, therefore, that the rate limiting process is the electron tunneling.

At the moment the electron tunnels the energy levels of acceptor and donor must be matched so that the electron can transfer with no change in energy of the system (or with a small loss to phonon energy if there is phonon interaction). Fig. 11 shows two ways in which the energy matching may be done .The first (path 4) postulates a level below first excited singlet in P_{680} and which could perhaps be the first triplet. The second (path 4') was suggested by M. GOUTERMAN (personal communication). Whatever the true mechanism, the negative temperature coefficient certainly suggests an improvement in matching of energy levels as temperature decreases.

NOTE ADDED IN PROOF (Received December 17th, 1970)

The close correspondence of cytochrome b_{559} and P_{680} kinetics described here, together with the absence of C_{550} absorbance changes makes the electron transfer pathway presented by ARNON *et al.*[27] unlikely under our conditions.

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