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Gordon Tollin and Melvin Calvin

July 1957

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ABSTRACT

The luminescence of various chlorophyll-containing plant materials has been investigated under a variety of conditions. The results have been shown to be consistent with a mechanism involving the recombination of electrons and holes trapped in a quasi-crystalline lattice. Some details of such a mechanism have been proposed which suggest the mode of entry of the light energy into the photosynthetic pathway.

THE LUMINESCENCE OF CHLOROPHYLL-CONTAINING PLANT MATERIAL*

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INTRODUCTION

The concept of the chloroplast as a semiconductor-like unit, as envisaged by Katz[1] and by Bradley and Calvin,[2] has recently achieved some measure of experimental support. The studies of Arnold and Sherwood[3] on dried chloroplast films have revealed that such systems exhibit some of the properties of semiconductors. Similarly, the investigations of Commoner, Heise, and Townsend[4] and of Sogo, Pon and Calvin[5] have demonstrated unpaired electrons induced by red light in both dried and wet chloroplasts. Low temperature studies[5] suggest that the spin resonance is the result of a direct photophysical transformation. On the basis of these investigations, however, it has not been possible to decide whether the unpaired spins are associated with a triplet state, with a radical produced by the direct photodissociation of a single bond, or with trapped electrons in a quasi-crystalline lattice.

The decay of the electron spin resonance signals in the dark at room temperature in wet chloroplast preparations[4,5] suggests that at least some of the energy associated with these unpaired spins might be reemitted as light. Inasmuch as long-lived luminescences in the room-temperature range have already been reported in whole algae[6] and in chloroplasts[7], it was felt of interest to study the light emission properties of chloroplast suspensions under a wider variety of conditions in an effort to correlate such properties with the spin resonance studies,[4,5] and with the thermoluminescence effects.[3]

While the earlier results with both whole algae[6] and chloroplasts[7] showed luminescence decay constants ranging from a few seconds to minutes, the suggestion has been made[2] that time constants of the order of a tenth of a second or less might be observable in systems such as these. Thus an apparatus has been designed which is capable of detecting decay times of this order of magnitude.

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†National Science Foundation Postdoctoral Fellow, 1956-1957.

MATERIALS AND METHODS

The chloroplasts were prepared according to the method of Sogo, Pon, and Calvin.[5] The apparatus used for the measurements is diagrammed in Fig. 1. It consisted of a cylindrical brass housing having two openings at right angles to each other, each opening being fitted with an ordinary camera shutter. Shutter B opened onto a Dumont K-1292 photomultiplier tube (sensitivity range: 3000 A to 13,000 A) cooled to dry-ice temperatures. The anode current of the photomultiplier flowed through 10^9 ohms resistance to ground. A D.C. electrometer was used to measure the voltage developed across the resistance, and the electrometer output was fed into a Sanborn model-151 recorder. The time constant of the electronic system was approximately 0.01 sec.

An aluminum rod (3 cm. in diameter), which had a 2 cm. portion of its upper end cut away to provide a flat surface, was mounted inside of the housing. The sample was painted onto the flat surface, and the rod was oriented so that this surface made a 45° angle to the intersection of normal lines drawn through the centers of the two openings. The rod was fitted with a 500 watt heater, and a thermocouple was placed in the rod approximately 1 mm from the flat surface. The bottom of the rod projected out of the housing and so could be immersed in a cooling bath.

The shutters were provided with Heil and Electromatic Coil solenoid triggers. For the measurement of the luminescence decay curves, a pulse from a storage battery triggered shutter A, and the flash contacts on this shutter were used to fire a General Electric FT-503 flashtube operated from a 5000 V power supply with an output capacitance of 64 μf. The duration of the flash was approximately 2 msec. The same battery pulse was sent through a variable delay circuit and was used to open shutter B after shutter A had closed. In this manner observation of the emitted light could begin approximately 0.1 sec after the flash was completed.

For the measurement of the glow curves, a 300-watt tungsten filament lamp was used as a light source. This was focused onto the sample through a Corning No. 3480 filter in conjunction with a special water-cooled Corning infrared filter. These served to limit the incident light to those wavelengths lying between 5800 A and 8000 A. The temperature of the sample was raised at approximately 15°C per minute. A Varian G-10 recorder replaced the Sanborn recorder for the measurement of the electrometer output. Illumination times were two minutes. The heating of the samples was started approximately 5 seconds after the illumination ceased.

The absorption spectra of the chloroplast preparations were measured with a Cary model 14M recording spectrophotometer. A specially designed low temperature cell[8] was used which permitted the chloroplasts to be painted onto a flat glass surface inside of a Dewar jacket with optically flat surfaces parallel to the sample. A stream of cold nitrogen gas, generated by a small heater inside of a Dewar of liquid nitrogen, was blown over the sample in the low temperature measurement. A thermocouple was used to measure the temperature inside of the cell.

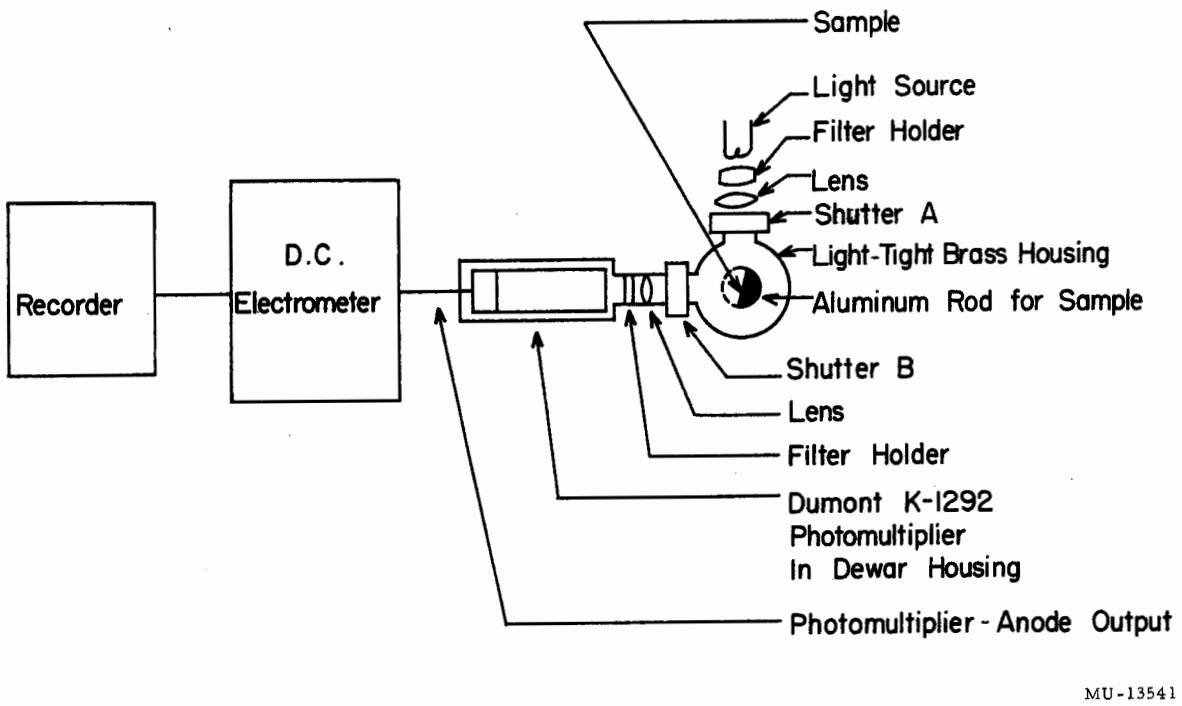

Fig. 1. Schematic diagram of apparatus used to measure luminescence decay curves of plant materials.

RESULTS

The luminescence decay curves for wet whole spinach chloroplasts at four temperatures are shown in Fig. 2. Log intensity versus time plots of these same data are given in Fig. 3.

The 23°C decay curves may be considered as being the resultant of two emissions having half lives of approximately 0.15 sec and 2 sec, respectively. Furthermore, with the use of a more sensitive detection system, it is possible to demonstrate the presence of a third emission at room temperature which has a half life of the order of 15 sec. Approximately 6% of the total integrated light intensity up to about 7 sec after the flash is due to the 0.15-sec emission.

The suggestion that the room-temperature decay curve consists of more than one component is confirmed by experiments in which the chloroplasts are cooled. The slower components diminish in intensity with decreasing temperature and have essentially vanished at about -35°C. At this temperature, the 0.15-sec component is all that remains. The slope of the decay curve at this temperature is the same as that obtained by drawing a straight line through the last few points of the 23°C curve and subtracting this line from the whole decay curve.

Upon further cooling the 0.15-sec component slowly diminishes in intensity, its decay constant remaining the same, and is gone at about -100°C. At about -90°C a fourth emission begins to grow in and gradually increases in intensity down to -196°C, which is the lowest temperature attained in these studies. This latter emission has a half life of approximately 0.3 sec.

The 0.15-sec and the 0.3-sec (curve at -140°C of Fig. 3) emissions appear to decay exponentially. We have not found it possible to definitely assess the kinetics of the slower components.

These cooling effects are completely reversible both quantitatively and qualitatively.

Both large and small spinach-chloroplast fragments[5] give room temperature decay curves much like those of whole chloroplasts. However, the decay curves of both types of fragments appear to have a somewhat smaller proportion of slower components, as compared with the 0.15-sec component, than do the whole chloroplasts.

The absorption spectra of whole spinach chloroplasts at 23°C and at -180°C are shown in Fig. 4. It is apparent that there are no changes in the low temperature spectrum which would account for the appearance of a new emission at these temperatures. The absorption spectra from 7500 A and 13,000 A was also run at both temperatures. No significant absorption could be demonstrated.

Experiments made with various Corning filters placed between the chloroplasts and the photomultiplier indicate that the room-temperature emissions are all of the same wavelength. A typical experiment, made with Corning filter No. 5970, is shown in Fig. 5. It is apparent that the

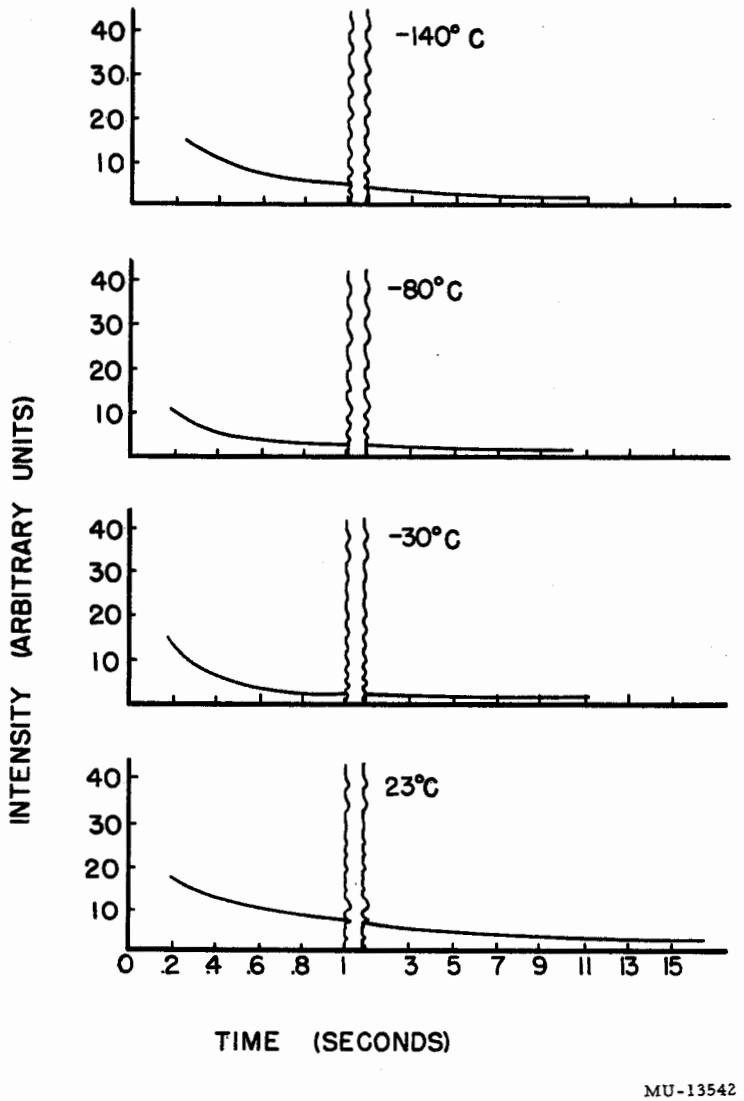

Fig. 2. Luminescence decay curves of wet, whole, spinach chloroplasts at four temperatures: intensity versus time.

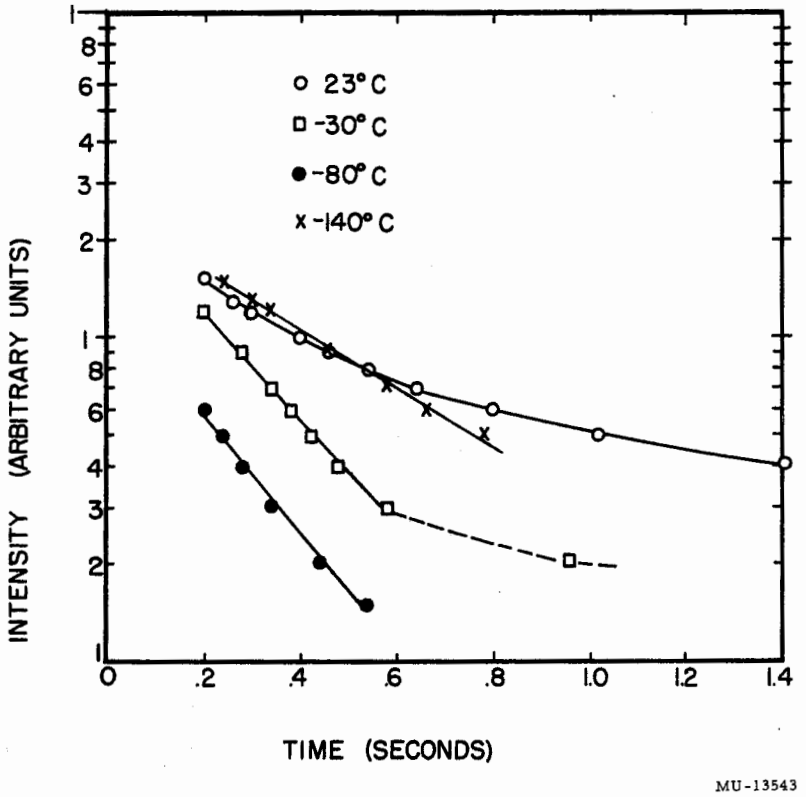

Fig. 3. Luminescence decay curves of wet whole spinach chloroplasts at four temperatures: log intensity versus time.

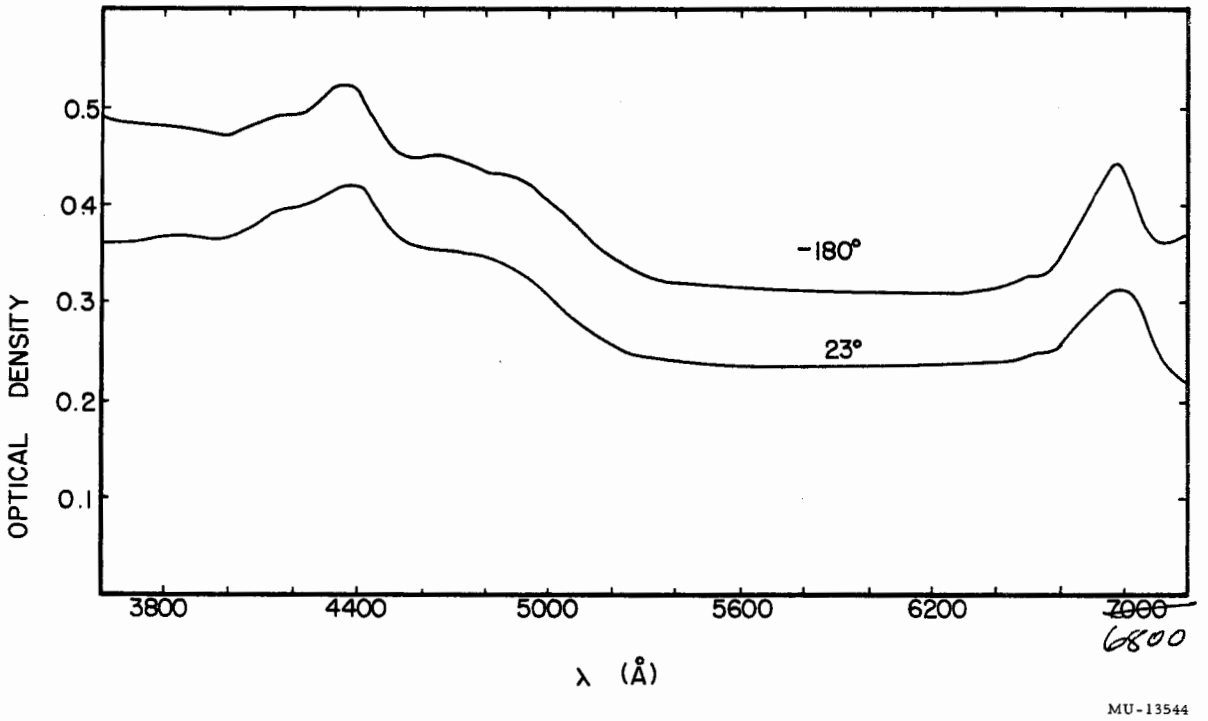

Fig. 4. Absorption spectra of films of whole spinach chloroplasts at 23°C and -180°C. The relatively high background absorption in these spectra is due to the scattering of light by the chloroplast particles.

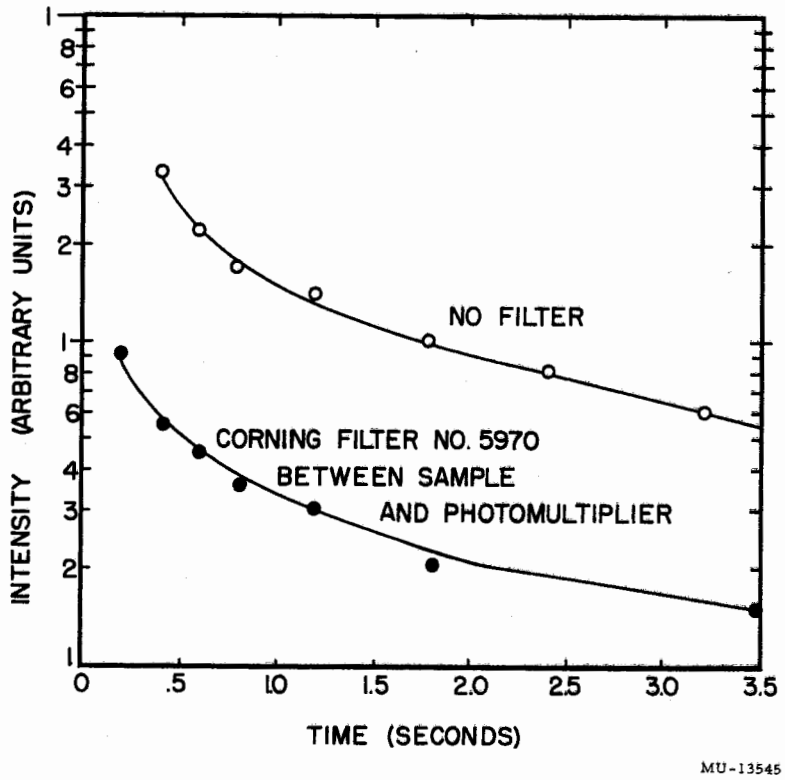

Fig. 5. Effect of Corning filter No. 5970 on the luminescence of wet, whole, spinach chloroplasts.

curve made with the filter in place is parallel to the curve in which no filter is present. An analysis of these results was made by drawing parallel straight lines through the last three points of each curve and subtracting these lines from the original curve. The points obtained in this manner were plotted and these gave parallel straight lines corresponding to half lives of about 0.15 sec. The relative areas under each of the two sets of parallel straight lines was found to be in approximately the same proportion. This indicates that both components are being depressed the same amount by the filter.

Figure 5 reveals that Corning filter No. 5970 reduces the integrated intensity of the emitted light by approximately 70%. This was also found to be true of the light emitted at -80°C. Inasmuch as this filter transmits only light of wavelengths between 7000 A and 9000 A, it is possible to assign these limits to the wavelength of the emitted light at both temperatures. The fact that only 30% of the emitted light is passed by the filter is due to its having a transmission of only 40% at 7600 A, which is its wavelength of maximum transmission. These results are corroborated by experiments made with various other Corning filters.

Similar experiments performed at -180°C lead to the conclusion that the light emitted at this temperature consists solely of wavelengths longer than 10,000 A.

Experiments performed with filters placed between the flashtube and the sample indicate that the 23°C and the -80°C emissions are excited by light composed of wavelengths between 6500 A and 7000 A. Light between 3600 A and 4200 A is also effective in exciting them. However, the -180°C emission is excited only by wavelengths between 3600 A and 4200 A. Light between 6500 A and 7000 A is completely ineffective.

Figure 6 shows the results of experiments in which freshly prepared chloroplasts are allowed to stand in the dark at room temperature over a period of time. The luminescence decay curves, measured at various time intervals, show a gradual increase in the integrated intensity of the emitted light. A maximum intensity is reached in about 6 hours. At this time the integrated intensity is about 2.7 times that of the original signal. This increase was also found to take place at 0°C, although at a much slower rate. The luminescence after 8 hours of standing at room temperature exhibits the same wavelength properties (both excitation and emission), temperature behavior, and decay curve as does the original luminescence. After about 24 hours of standing at room temperature, the emitted light has decreased to about its original intensity, although the decay curve is somewhat different. After 48 hours of standing, there is still less luminescence, and the decay curve is markedly different. After 72 hours of standing, the emission has disappeared entirely. At the end of this period, the chloroplasts appear quite dry and thermoluminescence (glow curves) can be obtained from them (see below). An examination of Fig. 6 reveals that the relative amount of the slower component appears to be decreasing after 8 hours of standing.

Upon drying with a stream of nitrogen gas for a few hours, whole spinach chloroplasts give glow curves similar to those reported by Arnold and Sherwood.[3] These are shown in Fig. 7. They differ from the earlier observations,[3] however, in that the unheated material gives a glow curve

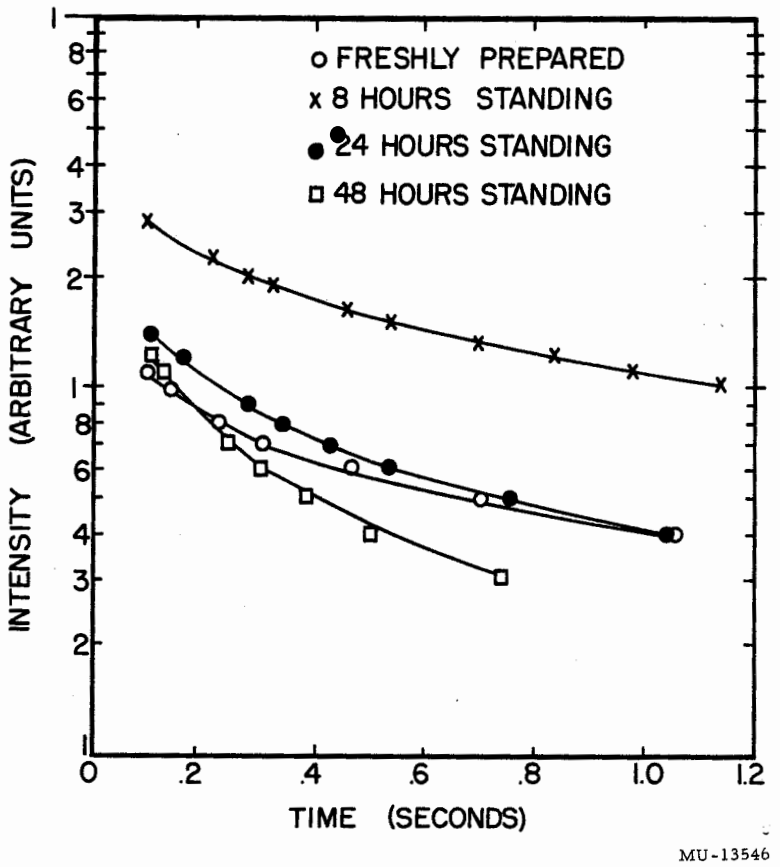

Fig. 6. Effect of standing in the dark at 23°C on the luminescence of freshly prepared, wet, whole, spinach chloroplasts.

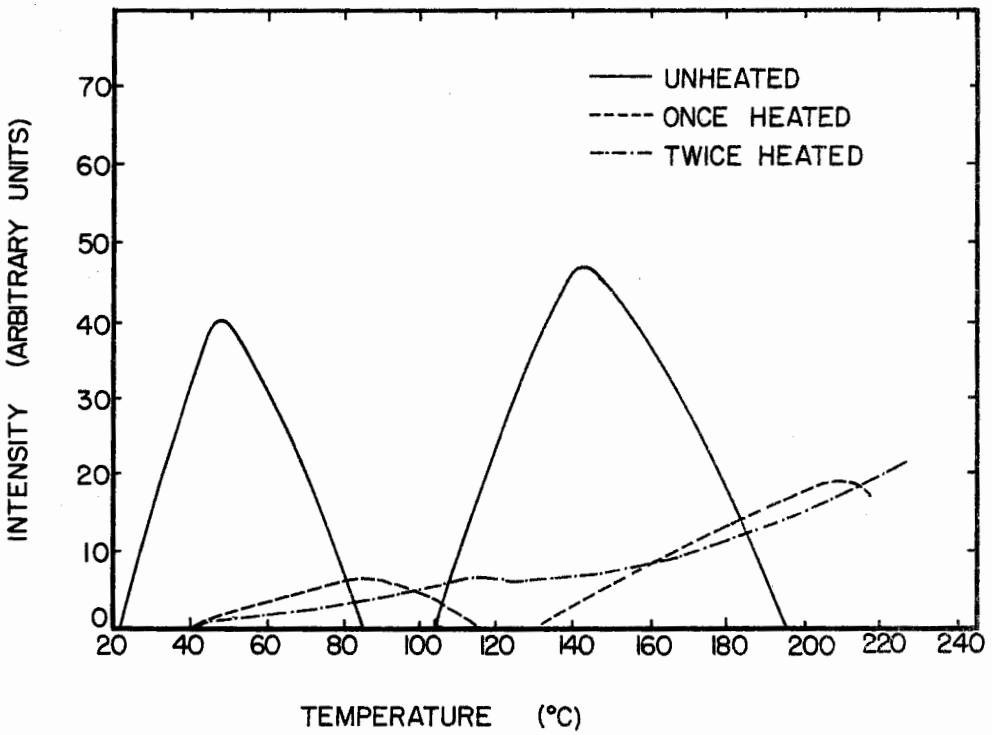

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Fig. 7. Thermoluninescence of dried, whole, spinach chloroplasts.

consisting of two well-defined peaks, one at about 50 to 60°C and the other at about 140 to 150°C. It is believed that this difference is due to a lesser degree of dryness of the chloroplasts in these studies than was the case in the earlier work. If the chloroplasts are dried for longer periods, the glow curves become similar to those of Arnold and Sherwood. Freshly prepared wet chloroplasts do not give appreciable glow curves. Before they are heated, the dried chloroplasts will emit light at room temperature.

If the once-heated chloroplasts are cooled, reilluminated, and reheated, a glow curve results in which the peaks are shifted to higher temperatures and the total emission is smaller. The chloroplasts do not give any room-temperature emission once they have been heated. A third cycle results in a curve shifted to still higher temperatures but with only a slightly smaller total intensity.

Room-temperature luminescence decay curves for whole Chlorella are shown in Fig. 8. It is seen that there is essentially only one component having a half life of about 1 sec (although there is a hint of a small amount of a faster component). When the algae are cooled to -10°C, the emission disappears entirely.

If dry nitrogen gas is blown over the Chlorella for about 15 min, the luminescence disappears. If the algae are then rewet with a drop of water, the emission returns immediately. However, this luminescence has a much different decay curve than does the original emission (see Fig. 8).

DISCUSSION

It is not possible at the present time to quantitatively compare the results reported here with the earlier spin resonance studies.[5] However, there are a number of qualitative similarities that are significant. These include:

1) Both the luminescence and the spin resonance signal are excited by the same band of wavelengths.

2) The room-temperature decay times are of the order of seconds for both the luminescence and the spin resonance absorption. The time constant of the spin resonance spectrometer was 2 sec, and thus the shorter decay times reported here could not have been detected in the earlier work.

3) At -140°C the spin resonance signals had decay times of the order of hours and the 7000 A to 9000 A luminescence had disappeared completely (a luminescence with a decay time of the order of hours would be undetectable with the apparatus used in these present studies).

4) At room temperature the decay time of the unpaired electrons in dried chloroplasts was of the order of hours. Under these same conditions, the chloroplasts did not luminesce.

5) At about 60°C the electron spin resonance of the dried chloroplasts had a decay time in the range of seconds. At approximately this same temperature there was a peak in the thermoluminescence curve of the dried chloroplasts.

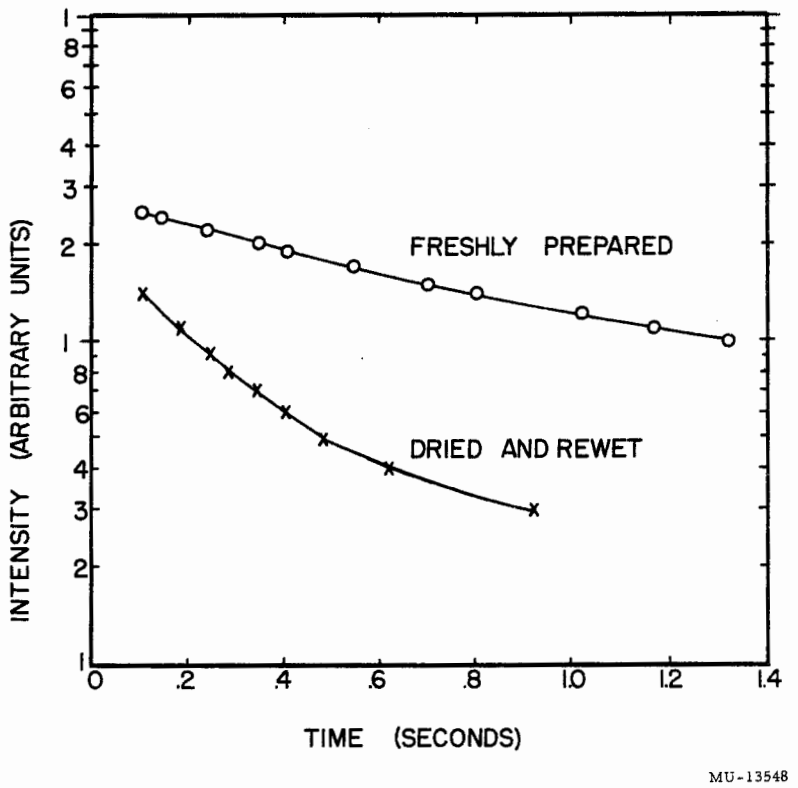

Fig. 8. Effect of drying and rewetting on the luminescence of wet whole Chlorella at 23°C.

These correlations indicate that the 7000 A to 9000 A light emission reported in the present work is, at least in part, the result of the decay of the light-induced unpaired electrons observed in the spin resonance studies. A quantitative comparison of the action spectra, quantum yields, and kinetic constants of these two phenomena is now being carried out in this laboratory. This should lead to a definitive assessment of the relationships between the two types of studies.

There are four possible mechanisms for the production of either electron spin resonance or delayed light emission in systems of the type we are concerned with here. These are:

1) T production of radicals by the direct photodissociation of a single bond, followed by their recombination in the dark.

2) The excitation and decay of a triplet state.

3) The reversible photosensitization of chemical or enzymatic processes leading to the production of free radicals.

4) The production of trapped electrons in a quasi-crystalline lattice. The thermal depopulation of these traps, with the subsequent radiative recombination of the electron and hole, would then result in light emission.

It is possible to rule out mechanism (1) by the following considerations. No known naturally occurring stable chemical bond can be dissociated by energies of wavelengths in the range used in this work (6500 A - 7000 A). In addition, the decay times (of the order of many seconds at room temperature) are not of the order of magnitude to be expected for radical recombinations at relatively high temperatures. Finally, it is difficult to reconcile such a mechanism with the existence of four separate luminescence decay times.

The fact that chloroplast luminescence shows a definite temperature dependence and that all of the luminescence excitable by 6500 A to 7000 A light eventually dissappears at -100°C, is sufficient to eliminate simple electronic transitions, such as that postulated by mechanism (2). Furthermore, the excitation of a long-lived triplet state cannot result in three emissions of the same wavelength.

While the existence of three temperature-dependent emissions of the same wavelength can be explained in terms of mechanism (3), the following considerations are incompatible with such a scheme. The rise time and the concentration of unpaired spins are the same at -140°C as at 23°C.[5] The presence of the 0.15-sec decay down to almost as low a temperature as -100°C rules out the participation of chemical or enzymatic processes in either the forward or the reverse transformation in this emission. If only the 2-sec and the 15-sec luminescences represent chemical processes, one would expect that cooling, by preventing the reaction of radical formation from taking place, would result in a greater amount of energy appearing in the form of the 0.15-sec decay. The experimental observations are not in agreement with this. Such a viewpoint is borne out by the fact that allowing the chloroplasts to stand at room temperature increases all of the emissions by the same amount.

We are thus left with only hypothesis (4), the production of trapped electrons in a quasi-crystalline lattice, as a possible mechanism for the phenomena reported here. The relationship of such a scheme to the thermoluminescence[3] and to the electron spin resonance[4,5] has already been discussed in the earlier papers. We shall now show that it is capable of accounting for the luminescence observations also.

In general,[9] at the instant the exciting light has ceased, there will be certain proportions of free electrons, trapped electrons, free holes, and trapped holes in the chloroplast. The luminescence during the first instants after cessation of excitation will then largely arise from the radiative recombination of nearby free electrons and holes. Subsequent to this, emission will be the result of the thermal excitation of electrons and holes from the shallowest traps, the decay constant being a function of the trap depth and the temperature. During the later stages the decay curve, the emitted light will come from electrons and holes in successively deeper traps. All of these emissions should be of the same wavelength. As the temperature is lowered the factor kT (where k is Boltzmann's constant and T is the absolute temperature) will become small relative to the trap depths, and light emitted as the result of thermal depopulation of traps should diminish in intensity.

The fact that the integrated intensity of the 0.15-sec component (which presumably is the result of direct recombination) decreases with decreasing temperature while the decay constant remains the same suggests that, while the rate determining step of this process is temperature independent, there exists a process whose rate increases with decreasing temperature which is competitive with the recombination. Whether this competitive process is the actual trapping of the electron or hole or is a side process is not known. The above scheme predicts that the time constants of the slower components should increase with decreasing temperature. It has not been possible to ascertain whether or not the data are consistent with this.

The increase in the intensity of the luminescence after 8 hours of standing (Fig. 6) suggests that there are enzymatic processes that compete with the recombination of the electron and hole and with the detrapping process. Inasmuch as the 8-hour decay curve is parallel to the original curve, there are apparently no changes taking place in the internal structure of the chloroplast up to that point. However, longer periods of standing do produce such changes. The appearance of a glow curve after 72 hours of standing suggests that either a deepening of the traps already present or the production of new deeper traps is occurring. The fact that quick drying of the chloroplasts will also result in thermoluninescence indicates that such deep-trap production is associated with a dehydration of the crystal.

We are now left with the question of which electronic states of chlorophyll are responsible for the 7000 A to 9000 A emissions and the longer-wavelength low-temperature emission. Figure 5 reveals that Corning filter No. 5970 reduces the integrated intensity of the room-temperature emissions by approximately 70%. Inasmuch as the red absorption maximum of the chloroplasts falls at about 6800 A (see Fig. 4), if these emissions were the result of a transition from the fluorescent level (first excited singlet) of

chlorophyll, one would expect approximately 75% to 80% of the emitted light to lie between 6800 A and 7100 A.[10] Corning filter No. 5970 should thus allow only 2% to 5% of light due to such a transition to pass. This suggests that these emissions are due to transitions between the lowest excited triplet state of chlorophyll and the ground state. The lowest triplet of chlorophyll a has never been observed.[11] Thus, it is not possible to decide which of the chlorophylls is involved. Experiments are now underway in this laboratory to obtain more accurate spectra of the chloroplast luminescence which will make possible a more definitive assignment of the transition involved.

The fact that it is not possible to excite the low-temperature infrared emission of the chloroplasts by light absorbed in the lowest excited singlet state (S^0 in Fig. 9) of chlorophyll provides strong evidence that this emission does not originate from a state of lower energy than this singlet. Therefore, one is forced to consider transitions from the second excited singlet state (S''). Inasmuch as an $S'' \rightarrow S^0$ transition (see Fig. 9) would have to compete with $S'' \rightarrow G$ and the radiationless transition between S'' and S^0, both of which would have lifetimes of the order of 10^{-8} sec, it is very unlikely that a 0.3-sec lifetime emission could be due to an $S'' \rightarrow S^0$ transition.

It is a well known fact that, in many highly conjugated organic systems, a radiationless transition between an excited singlet state and the corresponding triplet state may take place very readily.[12] This transition will be essentially independent of the temperature and of the rigidity of the environment.[13] If, then, a significant fraction of the energy absorbed in the second excited singlet state of chlorophyll finds its way into the corresponding triplet state (T''), a transition between T'' and the lowest excited triplet (T^0) would lead to an emission in the range of wavelengths observed for the low-temperature infrared emission of chloroplasts.[14] The competitive processes for such a transition would be the $T'' \rightarrow G$ transition and the radiationless transitions between T'' and T^0 and between T'' and G. Inasmuch as $T'' \rightarrow G$ is multiplicity-forbidden, it will have a relatively long lifetime and $T'' \rightarrow T^0$ would be able to compete successfully. However, the two radiationless processes will have very short time constants ($< 10^{-8}$ seconds). This suggests, therefore, that, at room temperature and down to -90°C, all of the energy acquired by T'' is dissipated thermally and emission occurs from T^0. As the temperature is lowered still further, the radiationless processes are gradually inhibited and $T'' \rightarrow T^0$ becomes an important pathway for the energy.[15]

In order to account for the relatively long lifetime (~0.3 sec) of the low-temperature emission, it is necessary to assume that the transition is highly symmetry forbidden. Theoretically, this is not an unlikely situation for a transition between two states to which transitions from the ground state are orbitally allowed. However, it is open to some question as to whether such a restriction can impart the degree of forbiddeness necessary to result in a lifetime of the order of a few tenths of a second.

The energy relationships between the electronic bands in the chloroplast and the transitions discussed above are summarized in Fig. 9. Processes a and c represent the absorption of light energy by the quasi-crystalline chlorophyll in the chloroplast. At room temperature, the radiationless processes d and h, and e and f funnel all of the energy that is not emitted as fluorescence (process b) or as heat from S^0 (process j) into the lowest triplet state (T^0). From T^0, ionization (i.e., separation of

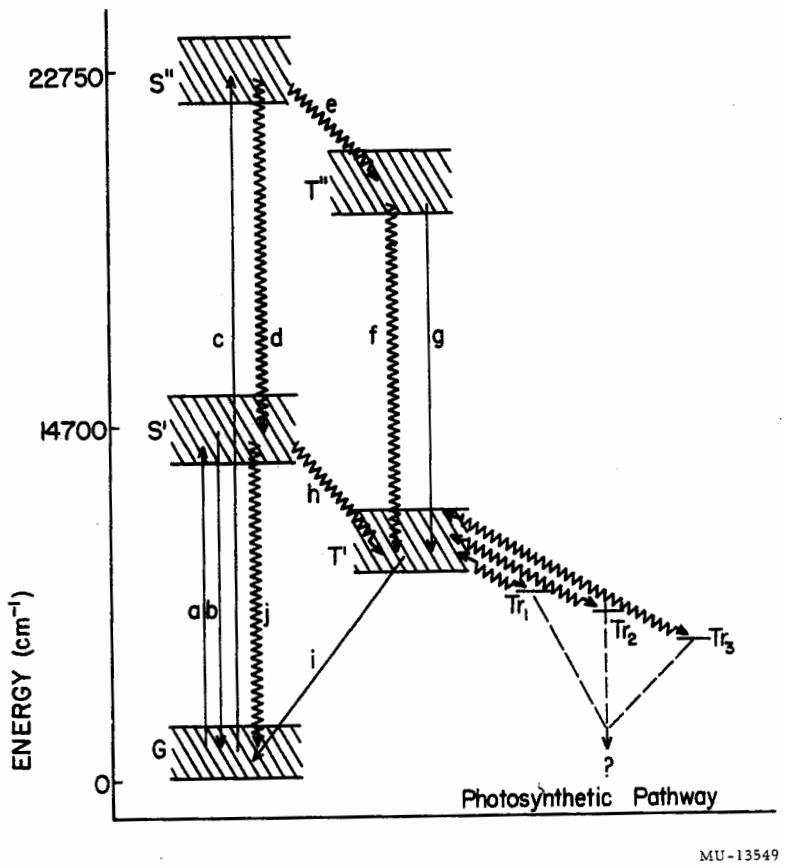

Fig. 9. Schematic representation of the electronic energy bands in spinach chloroplasts. G-ground state; S'-first excited singlet state; S"-second excited singlet state; T'-first excited triplet state; T"-second excited triplet state; Tr_1, Tr_2, Tr_3-electron traps of various depths. Straight lines represent radiative processes, zig-zag lines refer to radiationless (thermal) processes, and dashed lines symbolize chemical or enzymatic reactions.

the electron and hole) occurs, and the electron and the hole are trapped. The electron traps are designated as Tr_1, Tr_2, and Tr_3 in the figure. From the traps, the electrons and holes can be excited thermally into the conduction band where recombination takes place and energy is emitted as light (process i). Alternatively, the electrons and holes in the traps can be used up by chemical processes. Presumably about three times as much energy goes by this latter pathway in freshly prepared chloroplasts as in chloroplasts that have been aged for 8 hr, (see Fig. 6). This suggests that these processes represent the entrance of either the electron or the hole into the photosynthetic mechanism.

As the temperature is lowered, the thermal energy becomes insufficient to excite the electrons and holes out of the traps. Below -90°C, process f begins to be inhibited, and process g becomes an important pathway for chlorophyll in the state T". At these temperatures the electrons and holes formed subsequent to process g are "frozen" into the traps and no emission occurs from T".

ACKNOWLEDGMENTS

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14. In dylelike molecules such as chlorophyll the energy split between the singlet and triplet states of a given orbital configuration is of the order of 2000 cm^{-1} ± 1000 cm^{-1} [cf. R. S. Becker and M. Kasha, in The Luminescence of Biological Systems, F. H. Johnson, Ed., (American Association for the Advancement of Science, Washington, D. C. 1955) p. 34]. With an S^0-T^0 split in chlorophyll of about 3500 cm^{-1} (the actual split in chlorophyll b is 3600 cm^{-1}), an S''-T'' split of from 1800 cm^{-1} to 3800 cm^{-1} would lead to a separation between T'' and T^0 of the correct magnitude to account for the low-temperature emission. Inasmuch as the S''-S^0 separation in chloroplasts is about 8000 cm^{-1}, it would be very unlikely for the S''-T'' split to be larger than half this value. Similarly, the 1800 cm^{-1} lower limit is reasonable.

15. This temperature dependence is considered to be the result of the inhibition of the adiabatic crossing of the potential energy surfaces of the two triplet states. This may be thought of, in molecular terminology, as being due to a restriction of nuclear motion resulting in a decreased probability of a molecule assuming a configuration corresponding to an excited vibrational level of the lower electronic state. It is to be expected that such an effect would not become important until relatively low temperatures were reached. A similar picture is proposed by Porter and Windsor[13] for the effect of the viscosity of the solvent on the radiationless transition between the lowest excited triplet and the ground state of various molecules in solution.