# DETECTION OF THE TRIPLET STATES OF CHLOROPHYLLS IN VIVO

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## 1. Introduction

Although phosphorescence of chlorophylls in vitro has been observed by Becker and his co-workers [1–3] and Livingston et al. [4], the efforts to detect the triplet states of chlorophylls in vivo have failed (see [5] and [6]). However, involvement of the triplet states of chlorophylls has been implicated [6] in photochemical reactions of photosynthesis. This has been discussed in detail by Clayton [7]. Leaves at room temperature do indeed show delayed light emission. However, this is not phosphorescence since the emitted light has the same spectral quality as the chlorophyll fluorescence.

The inability to detect the triplet states of chlorophylls in vivo may be due to the low density of the triplets [6] and or due to the very short life time of the excited triplet states [5]. From his studies Witt [5] has concluded that the half-life of the chlorophyll triplets may be as short as 20 nano seconds. In view of this we thought that a procedure that has the following attributes might be useful in detecting such triplets:

(a) Preillumination of the leaf in vivo should create a pool of electrons that will remain relatively stable in the dark subsequently;

(b) It should be possible to create conditions such that these electrons will flood the triplets of chlorophylls on their way to the ground state thus producing phosphorescence;

(c) The population of the triplet states by electrons from the pool and their deactivation should last for sufficiently long period to allow detection and measurement.

Such a procedure has become available to us through our studies on the low temperature thermoluminescence (T1) of nucleic acid bases and other compounds (Tatake et al., in preparation). It is known that T1 is excited not only by the light absorbed by the compound but also by high energy radiation such as $\gamma$-rays. The resulting emission from either treatment is identical to the phosphorescence of the compound. Thus Brynda [8] from his T1 studies concluded that the appearance of T1 in a base depends on the population of the triplet state and on the phosphorescence ability of the base. Earlier Weissbluth et al. [9] working with L-tyrosine had also shown that T1 emission was identical to phosphorescence and hence indicated that the excited state from which the molecule relaxes to the ground state with the photon emission as a triplet state. The T1 method therefore detects the transitions of the detrapped electrons to the ground state through the triplet states of the compound. In our laboratory we have observed a one to one correspondence in the T1 emission and the phosphorescence of the compound in terms of both the number and the spectral spread. Therefore, the method of T1 can also be used to detect phosphorescence. In this paper we report our experiments using this technique. The data obtained lead us to suggest that one of the T1 peaks by us is indicative of the triplet to ground transitions of the chlorophylls in vivo.

## 2. Materials and methods

The spinach leaf disc was frozen in light and exposed at 77°K to white light. The leaf was heated in the dark using a cryostat [10] and the emission detected by EMI 9558 B photomultiplier. The output from the photomultiplier was recorded on a Rikadenki 3-pen

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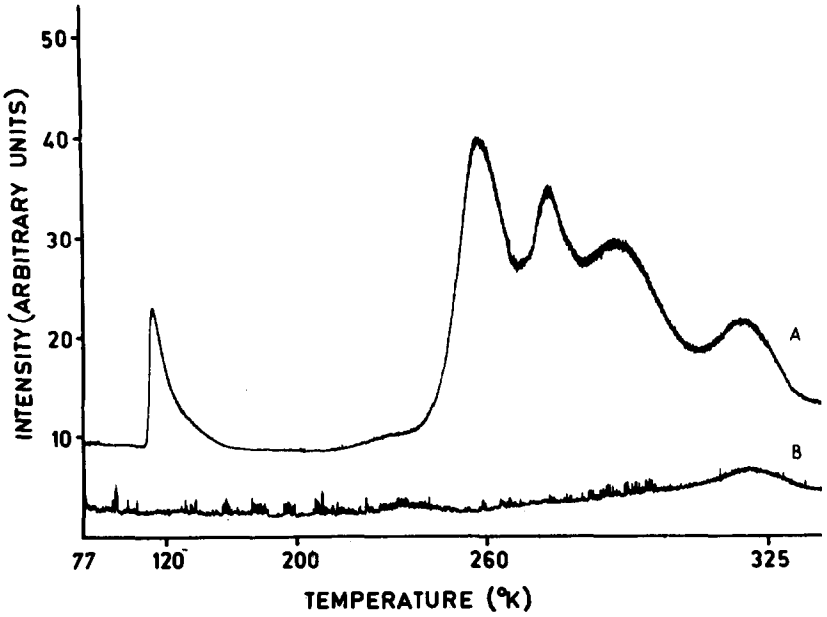


Fig. 1. Glow curves of spinach leaves frozen to 77°K. A: Leaf frozen in white light and exposed for 2 min; B: Relaxed leaf frozen in the dark.

recorder after amplification with a Keithly 610 B amplifier. The temperature of the leaf was monitored by an iron–constantan thermocouple. The leaf disc frozen in the dark was irradiated at 77°K with $\gamma$- rays. The emission spectrum of the glow curve was recorded using spectrophotofluorometer (Aminco Bowman) and R 446 photomultiplier. Chlorophylls were extracted from the spinach leaves by the procedure of Strain and Svec [11]. The extracted chlorophylls were taken up in a small volume of diethyl ether. They were dried on a planchet without further purification. The planchet containing dried chlorophylls was used for T1 studies. It was not necessary to further purify the chlorophylls because it was possible to show that the glow peaks arose out of chlorophylls.

## 3. Results and discussion

It has already been shown [12] that spinach leaves frozen in light to 77°K and illuminated subsequently with white light at that temperature give a number of glow peaks on warming (fig. 1). The first peak appearing at 118°K is not related to the photochemical reactions of photosynthesis because (a) it is not inhibited by DCMU, (b) it is present in a leaf previously heated to 90°C for 3 min to inactivate the photochemical reactions, and (c) it is present even in stored chloroplasts that are inactive in photochemical reactions. All the glow peaks except the one appearing at 118°K mentioned above are related to the delayed fluorescence and electron transport reactions of the chloroplasts [13,14]. The experiments carried out in our laboratory on these T1 peaks also support these conclusions. That the 118°K peak originates in chlorophylls is evident from the facts that it is absent in etiolated or bleached leaves devoid of chlorophylls. Further evidence in this regard is presented later. In this paper we would discuss only the peak appearing at 118°K.

The emission spectrum of this peak excited by blue light shows the emission maximum at 740 nm (fig. 2). This shows that the emission is at wavelengths longer than those of the in vivo chlorophyll fluorescence and hence indicative of its phosphorescence. Shuvalov and Litvin [14] using interference filters have shown the same emission maximum for this peak.

The excitation spectrum of the peak in the blue

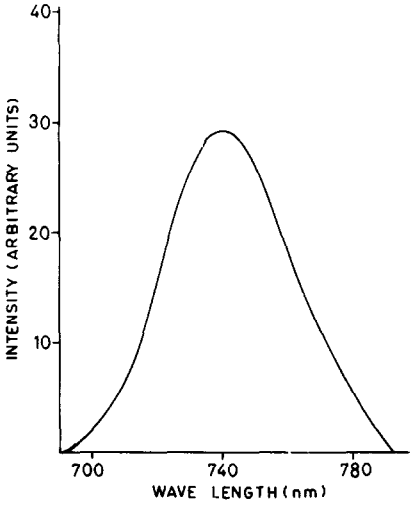


Fig. 2. Emission spectrum of the glow peak appearing at 118°K. The leaf frozen in the dark was illuminated with blue light at 77°K.

region gives 2 maxima at 440 nm and 480 nm. We have also observed that this peak can be excited by red light (maximum at 680 nm) as well. Thus the wavelengths absorbed maximally by chlorophylls in vivo are most efficient in exciting this peak. This peak, therefore, could only be attributed to chlorophylls. This is further confirmed by studying chlorophylls isolated from spinach leaves as discussed later.

As mentioned earlier in biomolecules the T1 originates in the transitions of the detrapped electrons to the ground states through the triplets giving phosphorescence type emission. Since in the present case we have observed phosphorescence type emission for T1 we feel that it is also arising as a result of transitions of electrons through the triplet states of chlorophylls. If this is true we would expect an identical glow curve and its emission when a relaxed leaf is irradiated with $\gamma$- rays at 77°K. The results of such an experiment are shown in fig. 3.

It is observed that $\gamma$- rays excite only the peak at 118°K, whose emission is at 740 nm. The figure also

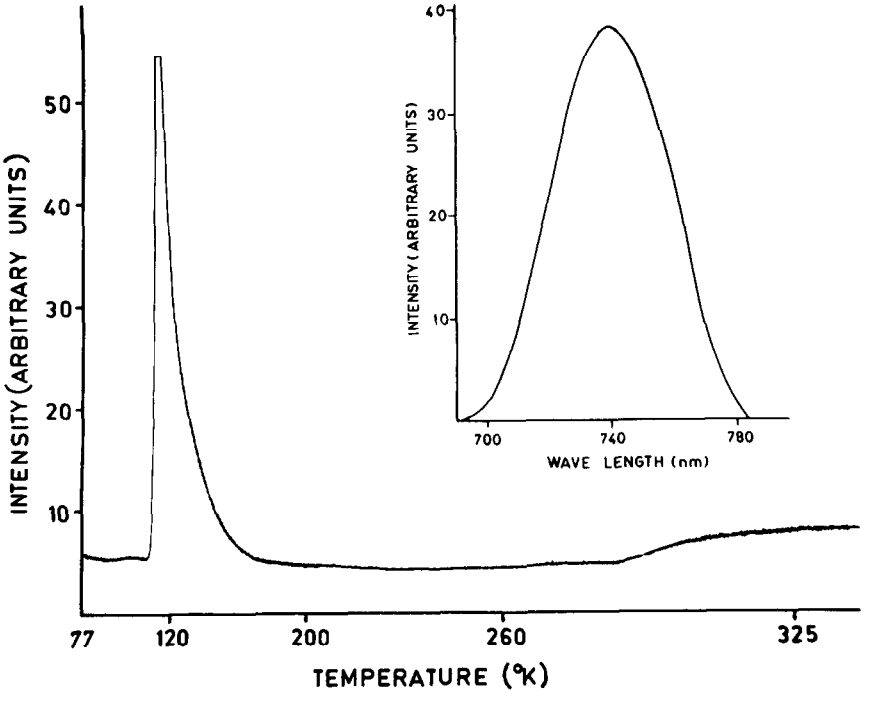


Fig. 3. The glow peak of spinach leaves frozen in the dark and irradiated with $\gamma$-rays at 77°K at a doze of 20 kr. Insert: The emission spectrum of $\gamma$-ray excited glow peak of spinach leaf.

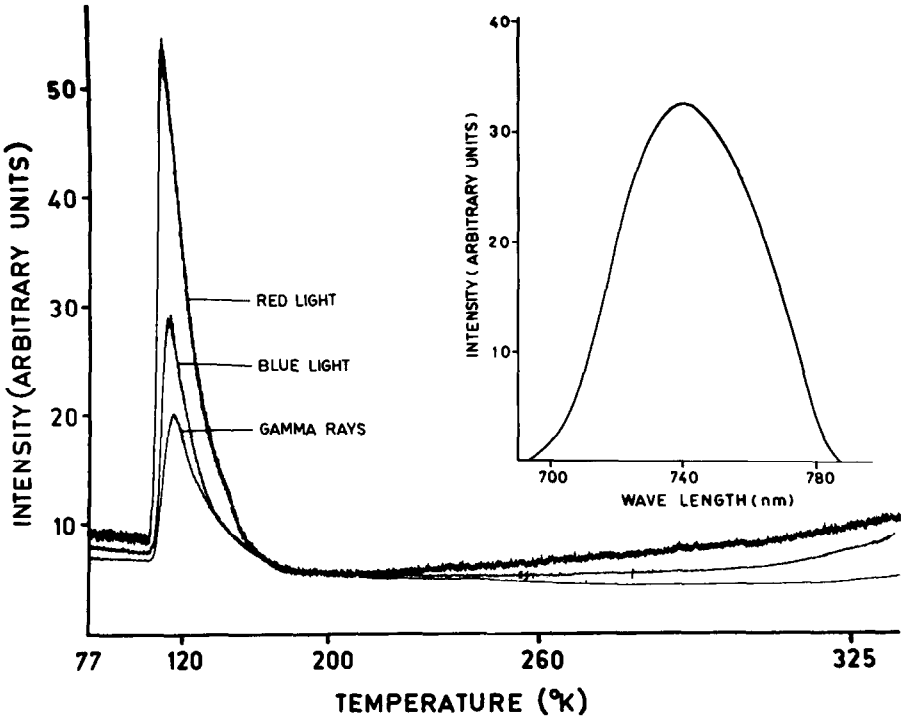


Fig. 4. The glow peaks of isolated chlorophylls frozen in the dark and illuminated at 77°K with blue or red light or irradiated with γ-rays. Amplifier gain for γ-ray excited peak : 1; for blue light excited peak : 3; and for red light excited peak : 10. Insert : Emission spectrum of the glow peak appearing at 118°K in isolated chlorophylls.

shows that other T1 peaks previously attributed to the electron transport reactions of the chloroplasts are absent. This experiment thus supports the proposal that the T1 peak at 118°K excited by visible light is indicative of triplet to ground transitions of chlorophylls in vivo. It also distinguishes the T1 peaks arising out of redox reactions of the electron transport chain and peak indicative of the phosphorescence of the molecule.

If we are indeed observing the triplet to ground transitions of electrons in chlorophyll molecules in vivo we would expect that isolated chlorophylls would also give us this peak when they are excited with γ-rays or blue or red light. Further the emission of the peak in isolated chlorophylls should be identical to the one observed for the leaf. Additionally the other peaks related to the redox reactions of the electron transport chain should be absent. Fig. 4 shows that in isolated chlorophylls also this peak appears at 118°K and that its emission is at 740 nm. Other peaks are absent as expected.

Thus (a) the peak has phosphorescence type emission, (b) it arises in chlorophylls in vivo as also in vitro as evidenced by excitation spectra, (c) it can be excited by γ-rays both in vivo and in vitro, and (d) the emissions of the T1 excited by γ-rays or blue light are identical. These facts suggest that we are observing the emission which is due to triplet to ground transitions of chlorophylls in vivo as well as in vitro. As far as we know this is the first direct evidence confirming the existence of triplet states of chlorophylls in vivo. The in vitro phosphorescence of chlorophylls *a* and *b* has been shown to have maxima at 755 and 735 nm respectively [1]. The maximum of the peak observed by us lies in this range.

The data presented above lead us to propose the following model to explain the underlying mechanism:

When the leaf is illuminated with light or irradiated

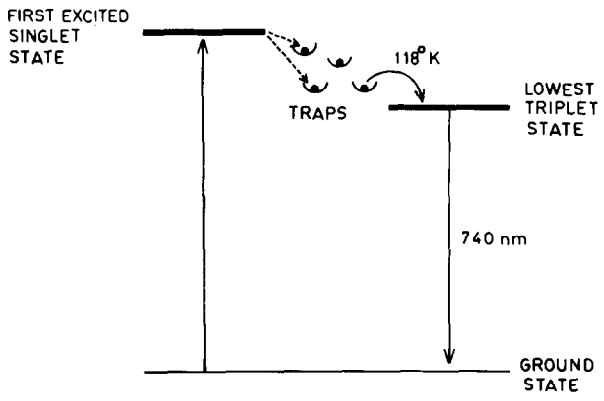


Fig. 5. Model for explaining the detection of phosphorescence of chlorophylls in vivo.

with γ-rays at 77°K some of the electrons excited to the singlet series are trapped. These traps are located somewhere in between the singlet and the triplet series on the energy scale (fig. 5). When the leaf is heated to 118°K the electrons are detrapped and flood the triplets on their way to the ground state giving rise to phosphorescence. The model proposed by us to explain the detection of chlorophyll phosphorescence would predict that if chloroplasts are irradiated at 77°K with blue or red light they would show absorption changes due to chlorophyll triplets during warming.

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