Thermoluminescence from spinach leaf without excitation by any radiation or external stimuli: Stimulatory role of thermal fluctuations

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This article argues that the sources of glow seen by the thermoluminescence (TL) technique may be from largely *in vivo* biological nanoparticles with property of quantum confinement that entails trapping of energy and delayed emission, not solely due to the charges that undergo recombination. TL in spinach leaf, following prolonged period of idling in dark at ambient temperatures but without any excitation, has been observed. Occasional loss of stringent control of heating rate revealed that fluctuations generated complex TL structures. Following the trails of this observation we discovered that ripples larger than ± 1°C gave rise to multiple small bands and spikes which were missing when the rate was controlled within ± 1°C, both with or without excitation by light. The results do not warrant the application of Randall and Wilkins' theory to interpret TL from photosynthetic materials.

WE recently reported the development of a new microprocessor-based, PC-interfaced thermoluminescence (TL) recording instrument[1] for use in the range of temperature from liquid nitrogen to + 100°C. The reason for making it fully mechanized to work as a stand-alone work-station[2] was to minimize the variations in TL characteristics that are due to human intervention in different steps involved, to which the emission pattern is sensitive. Investigators optimize different ways to elicit TL without offering a theoretical insight for doing so. While working with our old instrument[3], we surmised that non-reproducibility of TL patterns was probably due to such factors.

Sensitivity and versatility of this instrument made it possible to detect complex and reasonably well reproducible TL patterns from spinach leaves. Conversely, it was then possible to interpret variations due to factors uncontrolled or to lack of sufficient flexibility for changing relevant experimental parameters in earlier studies. More significantly, since the hard data on temperature profile and luminescence both during cooling and heating are stored, whether or not the instrument operates to ensure parameters set in the menu because of any hardware problem arising during the cooling–heating cycle, the causes of abnormalities in the TL pattern can be easily traced and analysed. This facilitated identification of a new phenomenon – stimulation of TL by thermal fluctuation – that is reported here. We propose to name this phenomenon as 'Quantum Confinement-TL', (QCTL), for reasons mentioned in the discussion, and suggested by Govindjee (pers. commun.) to distinguish it from normal TL because no excitation by photosynthetic active radiation or any other stimulus on or during cooling, as universally employed in this technique was needed to detect the emission during heating. We also distinguish this phenomenon of dark-TL (QCTL), from that generated in chloroplast by permanent electric field observed by Knox and Garab[4].

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Material and method

All the experiments were carried out when the relevant features of the new TL instrument described earlier in detail had been incorporated[1]. Briefly, the experimental operations were as follows: The sample leaf plug was placed on the sample stage at a preset temperature, chamber shut by a lid, and the cooling started by operating through menu in which the heating rate, temperature range and hold time during excitation, PMT voltage, light source and several other requisite experimental parameters were specified. In this auto mode, heating at the rate specified in the menu starts after the lower limit of the range of the cooling–heating cycle is reached. For excitation, the light source chamber moves over to sample stage when the specified temperature of the sample platform for excitation is reached. After completion of the exposure, further cooling starts until the lowest specified temperature is reached and heating begins. The luminescence intensity data are acquired at the rate of 5 points per second along with the time and temperature. More details of the facilities and options available are discussed elsewhere[1].

The samples were incised from spinach leaf bought from market. A cleaned leaf was sandwiched between a folded aluminum foil of 0.02 mm thickness, and a filter paper placed between the front side of the leaf and the top aluminum foil that completely covered it. Leaf plugs were incised by hammering a stainless steel dye with

sharp circular edge of 10 mm diameter placed on the top surface over a chosen region of the leaf. The top aluminum foil disc along with the filter paper were removed before loading the leaf plug backed by the bottom layer of the aluminum foil. For exposure to room light, the top aluminum foil and the filter paper were unfolded, and folded back if incubation in dark was needed.

Results

The persistence of dark-TL with time at ambient temperatures

TL from leaf on storage at + 30°C in the dark persists even after 20 min (Figure 1). There is no correlation between yield within the broad range of the band and time of idling in dark. This is unexpected because investigators store the experimental material in the dark generally for a few minutes[5–8] to ensure a relaxed state such that no glow would be detected on heating because of earlier exposure to light. We, however, detected signals from spinach leaf kept in dark for several days at room temperature[1]. But the samples were exposed to faint light used for loading on the stage. The experiments reported here were done to ascertain the minimum time necessary to obtain the relaxed state if exposure to light while loading was not to be avoided. The results show that even after one hour of idling in dark at near-ambient temperature, signals can be seen. However, these signals are not reproducible in all detail. For instance, Figure 2 shows that the structure of the signal varies even for short storage times up to 10 min tested at three temperatures, + 30°, + 25° and + 20°C.

There is no correlation between change of patterns or yields with time of storage in dark even for short periods up to 10 min for + 25° and + 30°C (Figure 2 *a* and *b*). Though the yield varies for the same storage time in dark, there is a gradual decrease till about 30 min. The signal persists to comparable levels of yields for 30 (Figure 3 *a*) and 60 (Figure 3 *b*) min of dark storage at + 30°C. There is considerable similarity in dark-TL profile up to at least 7 min of dark storage at + 20°C and also a gradual monotonous decrease in yield till 10 min (Figure 2 *c*). However, seven assorted samples from two young leaves of about the same size of one plant gave considerable variation on 10 min storage in dark (Figure 4 *a* and *b*).

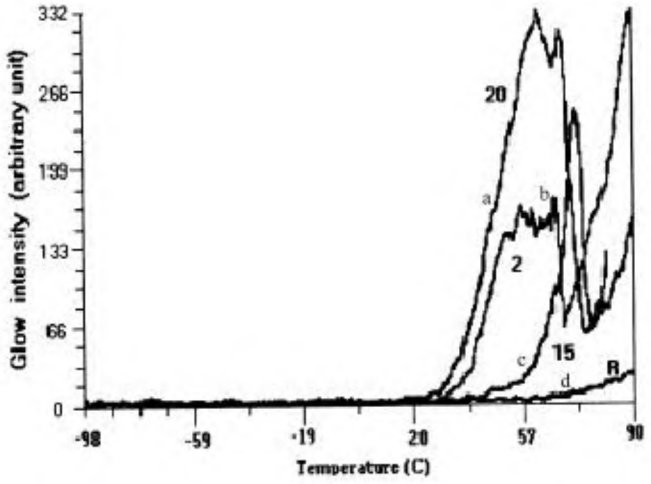

Figure 1. Dark-TL from spinach leaf for increasing periods of idling at + 30°C (± 2°C) on the sample stage in dark prior to start of the run: 2 min (a), 15 min (b), 20 min (c), on reheating the 20 min sample *in situ* in dark immediately after the first cycle (d). All the samples were incised from the same fresh leaf.

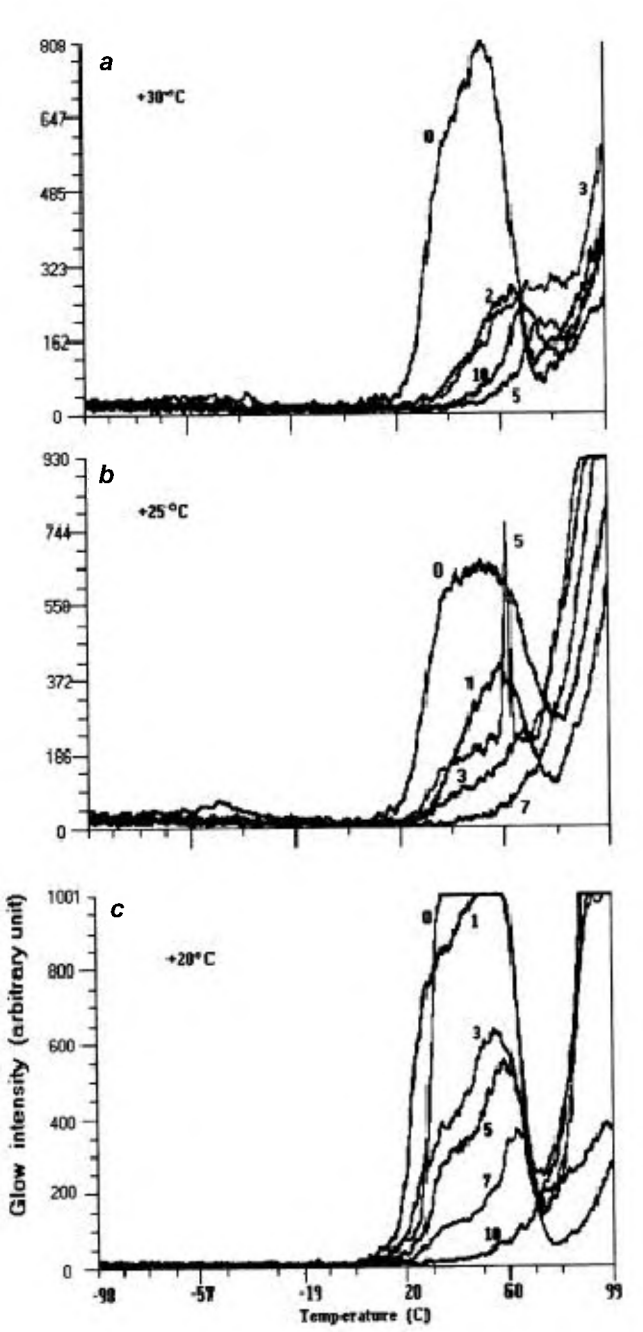

Figure 2. Dark-TL after idling at different temperatures close to ambient for varying time of idling: (*a*) + 30°C, (*b*) + 25°C, (*c*) + 20°C. Idling time in minutes is indicated on the individual curves.

Reproducibility of dark-TL structures

The non-reproducibility of dark-TL described above could be due to temperature fluctuations of the sample stage within ± 2.0°C (limit set by design[1] for controlling steady state temperature of the stage) during dark storage prior to start of cooling of the sample. The time of idling on the sample stage in dark could be reduced to a minimum of about five sec in this instrument. Reproducibility of the TL pattern could then be checked on samples with nearly the same history before loading. Since no sample standards can be devised because of uncertainties intrinsic to the technique itself, we used the simple approach of comparing QCTL from two halves of a single spinach leaf with pairs of 10 mm diameter plugs incised from approximately symmetric positions with respect to the mid-rib (Figure 5). We assume such pairs are physiologically very similar to provide a check on the reproducibility of the instrument's performance. The yield and structure of dark-TL for two different leaves shown here clearly support the assumption. The symmetric (Figures 6 *a* and 7 *a*), and even adjacent leaf plugs (Figures 6 *b* and 7 *c*) are very similar both in yield and shape. This permits the

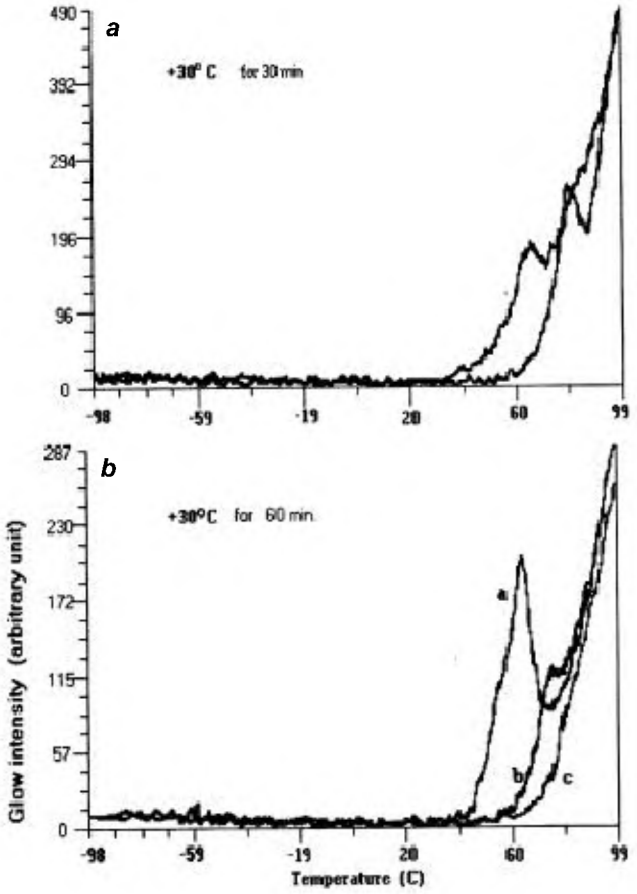

Figure 3. Dark-TL of samples from two leaves of the same plant for relatively longer idling time at + 30°C: (*a*) 30 min, (*b*) 60 min, curve (c) is recycling of 60 min sample *in situ* immediately after the first cycle (b).

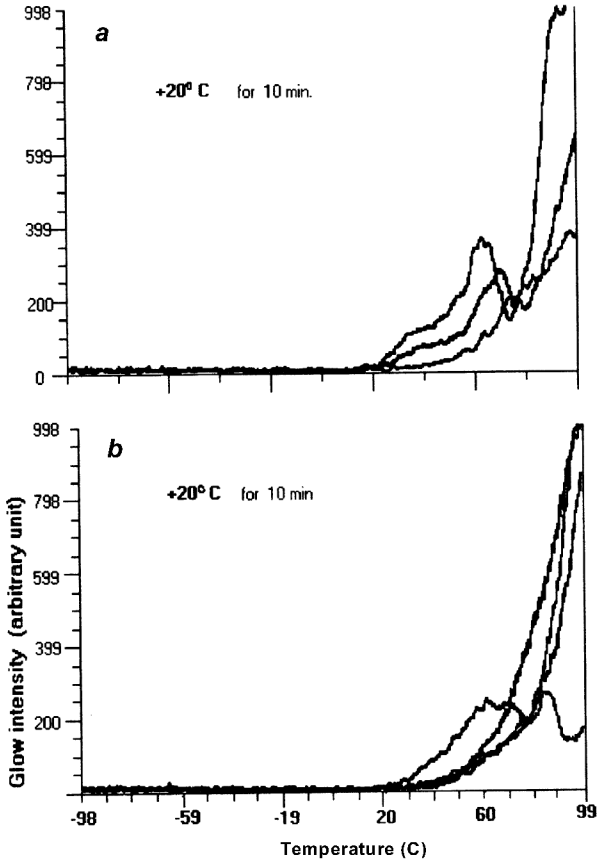

Figure 4. Dark-TL after 10 min of idling in dark at +20°C of samples from two leaves, (*a*) and (*b*), of the same plant.

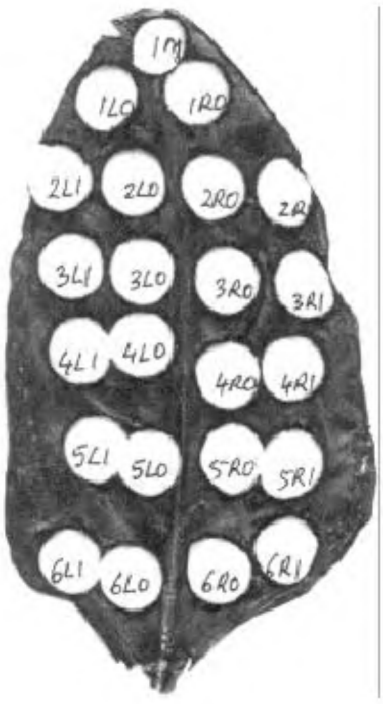

Figure 5. Scanned image of the leaf #29 after experiment with incised samples (see text). The incised positions are labelled to indicate the samples referred to in the text and figures with TL curves. The same convention of labelling a sample with respect to mid-rib of the leaf is maintained in examples when other leaves have been used.

conclusion that the grossly altered dark-TL of symmetric or adjacent pair of plugs could be due to some change in experimental parameter(s) arising from loss of control. For instance, the dark-TL structures for a symmetric pair of plugs, 1L0 and 1R0, of a leaf show a dramatic difference with three superimposed but clearly resolved bands on the falling side of the broader band only of the sample 1L0 (Figure 8 *a*). We traced this difference to transient presence of ripples in the heating rate of 1L0 that was absent in case of 1R0 (Figure 8 *b–c*).

Effect of small temperature fluctuations during heating on the complexity of dark-TL structures

The ripples were within ± 1.5°C of mean temperature during heating of 1L0 (Figure 8 *b*). Figures 9 *a* and *b* show that the grotesque structures of samples from leaf #29 also appeared when ripples were superimposed on heating rate. Fluctuations were within ± 2°C about the mean in any region of the range specified in menu. The PID-mediated control tends to repeat the previous cycle's performance unless disturbed manually. That permitted the settings to stay considerably stable, though altered from what was initially specified in the menu if the ripples were introduced manually. This behaviour of the instrument turned out to be an asset to follow-up the experiment under abnormal but interesting settings as in the present study.

In the results of experiments with lasers for excitation to study the normal TL, the same effect of the thermal fluctuation was seen without fail. The TL structures were again complex and showed peaks or small bands (Figures 10 and 11). Two samples of the same leaf that received identical exposure with He–Ne laser beam at – 50°C also show the difference. The plug 1L0 was heated with fluctuating heating rate, the fluctuation being less than ± 2.5°C about the mean and shows multiple bands compared to 3L0 that had smoother heating rate (Figure 10 *a*, *b*). Similarly, the two samples from another leaf exposed at – 79°C for 30 s with 510.5 nm line beam of copper vapour laser were heated with a fluctuating rate but one sample, 1R0 (Figure 11 *a*), shows an additional spike close to – 10°C, which had the fluctuation beginning at – 28°C. But in the heating rate of the adjacent plug 1R1 that did not give any significant signal in this region, the ripples started later at – 19°C.

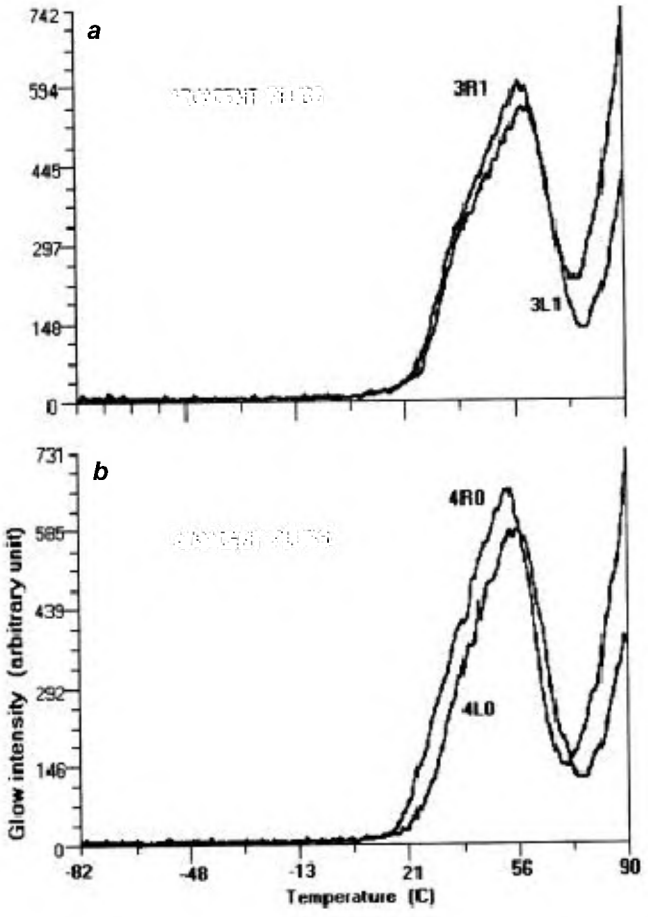

Figure 6. Dark-TL from approximately symmetric positions about mid-rib at two distant points: (***a***) nearly overlapping, (***b***) laterally shifted curves (see results). For labelling convention, see Figure 5. Leaf not shown.

Discussion

Though distorted, complex dark-TL structures were reproduced very closely as shown in Figure 7. We inferred that the ripples in temperature of the order of ± 1°C during heating could be responsible for variations in TL characteristics in normal TL obtained after excitation with light as well (Figures 10 and 11). There has been no report of such signals by earlier investigators probably because constant attempt was to improve on the stringency of control for linear heating rate. This was a theory-based constraint for correctly estimating the activation energies for de-trapping charges from data on normal TL peaks or bands by 'initial rise' method[9,10] for interpretations essentially using Randall and Wilkins' theory[11].

Our results imply that a smooth band in composite TL curve may be a consequence of artifact of measurement technique and does not necessarily represent a broadened peak that could be considered a single or aggregate of degenerate classical traps. This is because small fluctuations in heating rate, or even on heating at a stringently controlled rate after prolonged idling at a steady mean temperature in the ambient range, clearly elicit smaller bands at both high and low temperatures.

Decay kinetics of a neat glow peak of spinach thylakoid generated by a very well controlled ramp with a recently developed instrument by Townsend *et al.*[12] also detected heterogeneity that the authors attribute to two conformation states of PSII. But the detection was possible with increasing dark idling time up to nearly 15 min.

The results presented here also suggest why it has not been possible to reproduce TL pattern consistently in earlier studies and the detailed structures were presumably instrument-specific, some missing out and others observing new peaks[6–8] (also see numerous cross references in 7). The effort to smoothen the raw data to eliminate 'noise' may actually eliminate the true signal instead, as our data indicate and could partly be responsible for reported variations.

It is, therefore, very pertinent to discuss the 1957 paper of Arnold and Sherwood[5] with chloroplast that fixed the theoretical framework for exploring photosynthesis using the TL technique. They observed that light exposure at room temperature gave glow if the sample was subsequently cooled and then heated in the dark. The same sample did not give any glow if cooled and reheated. When the sample was cooled to, and re-illuminated at 20–25°C, light was emitted upon heating. It was also mentioned, without giving the data that '*each time the experiment was repeated the light emitted was lower and the curve assumed a different shape* until four or five heating when the change was insignificant'. Tollin and Calvin[13] reported such changing glow curves for two repeat heatings of chloroplasts. Arnold and Sherwood[5] considered the sample was 'stabilized' then and used such samples for their main TL studies (Figures 2 and 3 in their paper) to show that chloroplasts retained a part of the energy delivered by illumination at low temperatures with decreasing efficiency until at liquid nitrogen temperature when no glow was found on heating. Subsequent investigators detected a number of broad bands at low temperatures within the same range. But the chloroplasts used by later investigators were neither dried nor reused to 'stabilize' the way Arnold and Sherwood did. The essential physical difference in the nature of the material of chloroplasts or intact leaves which gave low temperature TL bands and the dried 'stabilized' preparation that did not, seemed to have escaped the critical attention of all the subsequent experimenters.

A new phenomenon: quantum confinement as source of TL (QCTL)

The persistence of TL stored in dark at ambient temperatures for hours suggests that charge recombination between pairs created due to absorption of light prior to

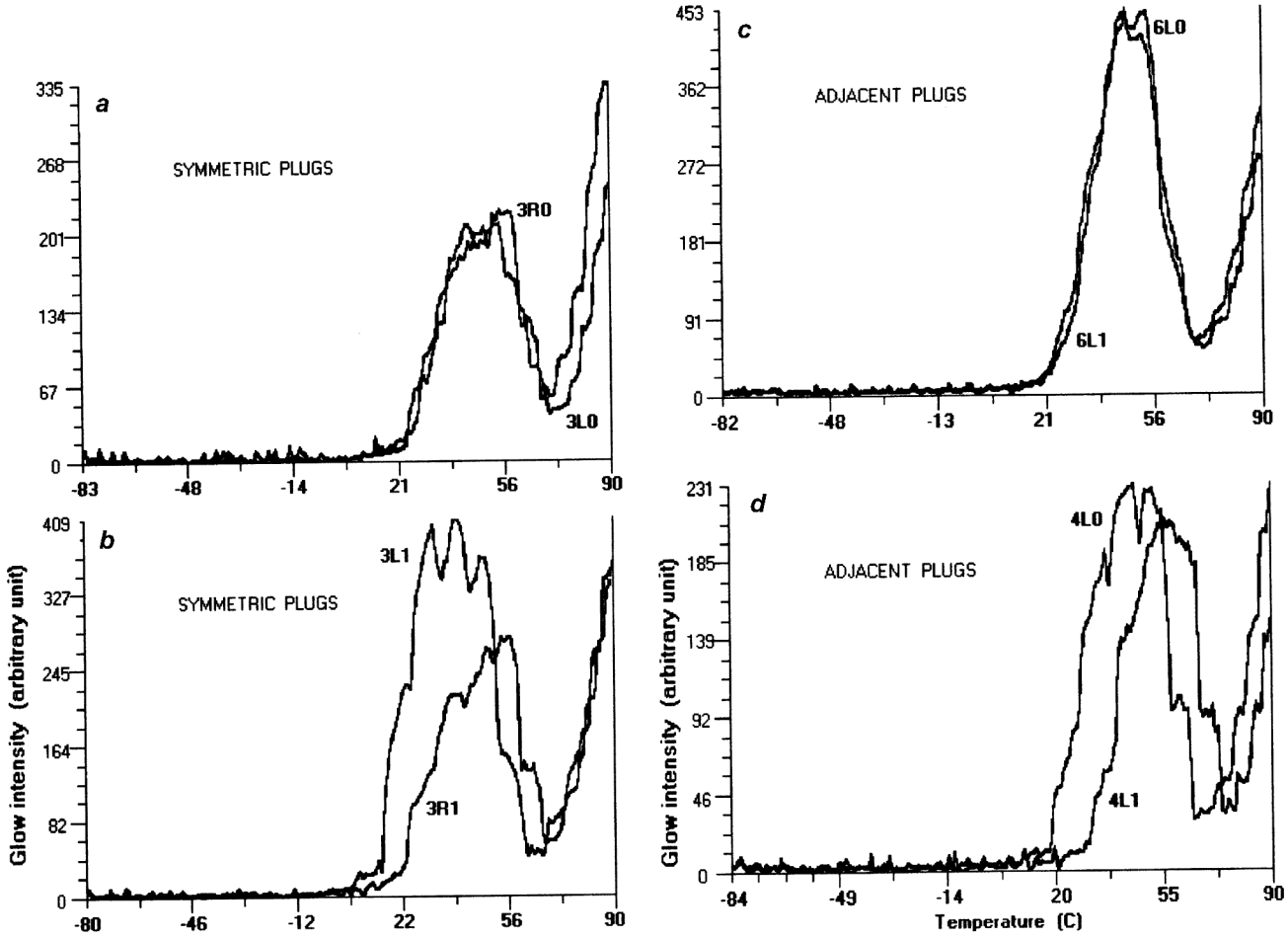

Figure 7. Dark-TL from leaf #29 shown in Figure 5. Approximately symmetric positions (*a*) and (*b*). Adjacent pairs from two distant positions along the length of the mid-rib on the same half, (*c*) and (*d*). Note that both overlapping and laterally shifted patterns are obtained from the two halves as well as the same half.

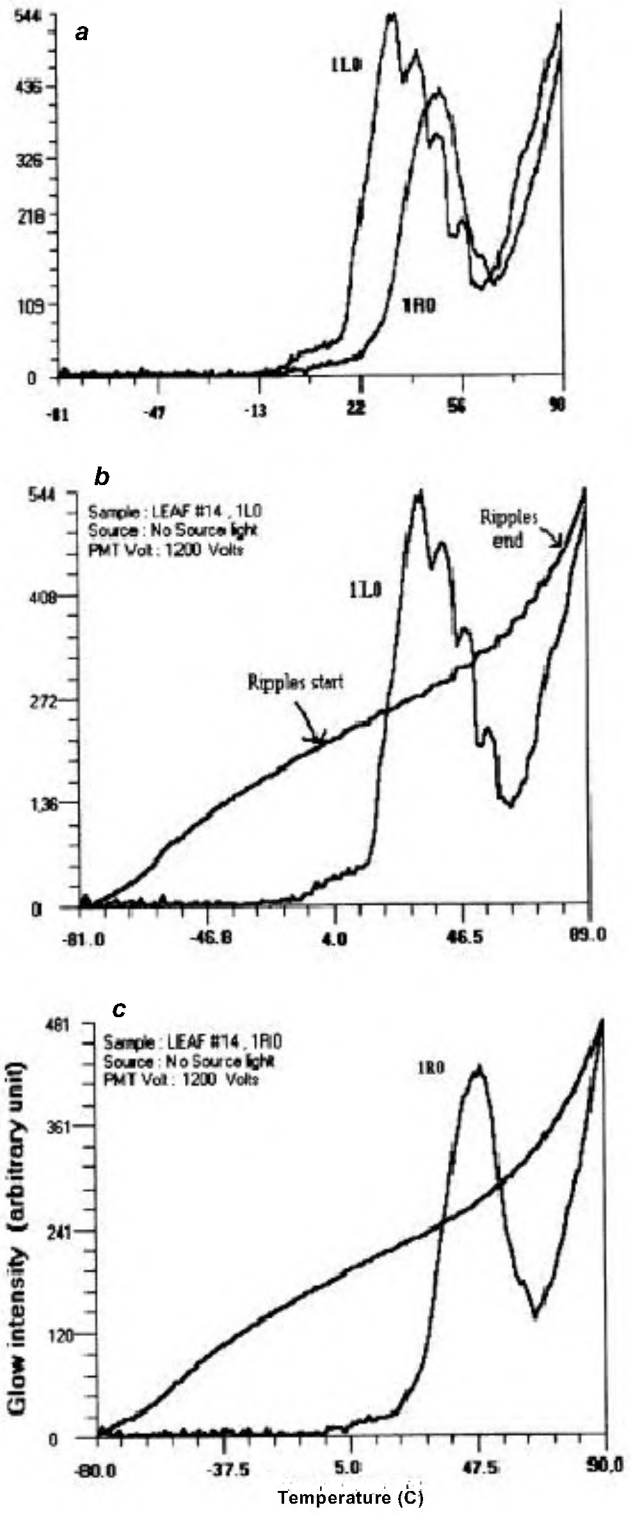

Figure 8. *a*, Dark-TL curves from symmetric positions near the tip of the same leaf (#14). *b*, Thermal profile superimposed on the glow curve for sample 1L0. The smaller bands in the glow curve on the falling side of the broader band appear in the zone of ripple in heating rate indicated. Ripple was about ± 1.5°C. *c*, The matched symmetric sample, 1R0, does not show the bands because the ripples in heating rate disappeared. Ripple was less than ± 1.0°C and within specification of control (Leaf not shown).

heating following such idling cannot be explained by equilibrium between primary charge pairs and excited chlorophyll molecules. In reaction centre core complex, decay times are of the order of a few nanoseconds or less[14–16]. However, Rutherford *et al.*[17] detected 50% charged QB that was presumably in equilibrium with other redox species estimated by flash-induced TL in spinach leaf. It may be argued that heating disturbed the equilibrium in favour of back flow of electrons to emit light via excited state of chlorophyll. Since the inference was based on TL yields from flash experiments, relative contribution of charge recombination from the pool in equilibrium prior to flash exposure and that established after the photoabsorption act cannot be resolved from their data. Moreover, absorption studies on transients at wavelengths higher than 700 nm has been partly attributed to stimulated emission following redistribution or trapping of excitation energy, not to stability of primary charged pairs in equilibrium with excited state[15].

Earlier attempts to identify the stable species to explain the origin of delayed light emission left the question open though stable EPR signals were observed after photoabsorption[18,19]. Commoner *et al.*[18] detected a light-induced EPR signal that was stable over 1 min from chloroplast preparation stored in cold for seven days, but considered their evidence inadequate to distinguish between excited triplet state of chlorophyll and primary or secondary charged radical pairs. Sogo *et al.*[19] saw EPR signal decay times of hours in wet or dry leaf, and also in whole or fragments of chloroplast but thought the signals were from trapped electrons. They favoured radical pair over chlorophyll in triplet excited state as the source of this signal. Arnold after admitting the uncertainty between stable charge pair and excited state[20] favoured charged pair because it explained the two quanta phenomenon of delayed light in the range 0.1–1.0 s following brief flash discovered by Jones[21] in algae, and also the enhancement effect[22]. Apparently, the question is open even now. In the following we suggest an alternative that reconciles luminescence associated with fast decay by charge recombination and delayed light emission.

The alternative argues for entrapment of energy, not charges as source for delayed dark-TL in photosynthetic and non-photosynthetic systems alike.

The excursion of temperature in certain ranges may help self-assemble and disassemble aggregates of structures from functional building blocks of cellular energy transduction machinery, both photosynthetic and also non-photosynthetic. The surviving (functional) complexes during the thermal cycle may, individually or collectively as small but non-communicative aggregates, throw out heat pumped in by emitting light. Thermal fluctuation would allow repeated association and dissociation of such aggregates, a fraction of which might be capable of dissipating the stored energy reserves such as potential across membranes and even of potential energy of macro-

molecular conformational states because of trapped energy. This could entail membrane vesicles of varied sizes self-assembled from a heterogeneous distribution of fragments. Kinetics of fusion and fission due to small temperature differentials in a range, probably encompassing the regions of phase transitions of various lipid constituents, may result in a net emission reflecting the complex structure of the TL. Such complexity would, consequently, be at residual level because of the history of the material, if the fluctuations were not present, thus making TL intensity a function of heating rate as well as frequency and amplitude of the ripples. The emission could be ultimately from excited chlorophyll molecule in intact PSII or *even primary charge pairs created during heating in the act of recording TL* by a reversal of non-radiative internal conversion process that follows photo-absorption.

Such reversal or direct excitation by electron tunnelling that results in quantum confinement has been demonstrated[23–26]. In silicon nanocrystals, quantum confinement increases the quantum yield of emission of visible light following absorption to 100% at 50 K or lower temperatures[23]. Chan and Nie prepared highly luminescent nanoparticles for biological detection that had time constant of emission, $t_{1/2}$, of 16 min compared to 10 s for rhodamine dye, and 20 times as bright[24]. Watanabe *et al.*[25], studying isolated molecules of CuPc in nano-scale by field electron microscopy provide clear evidence that vibrational energy can be gradually raised by strong electric field that heats up electrons in the tip and transfer the heat to a single molecule, or the emitted electron could tunnel into excite directly. Binh *et al.*[26] found that at the nano-scale, the temperature increases several hundred degrees due to field emission. Electrically stimulated delayed TL observed by Knox and Garab[4] in spinach chloroplasts could be due to such field-induced heating at the nano-scale level of complexes that emitted light even 5 min after the stimulus was removed. That the emitters were behaving as isolated quantum dots seems reasonable to assume because

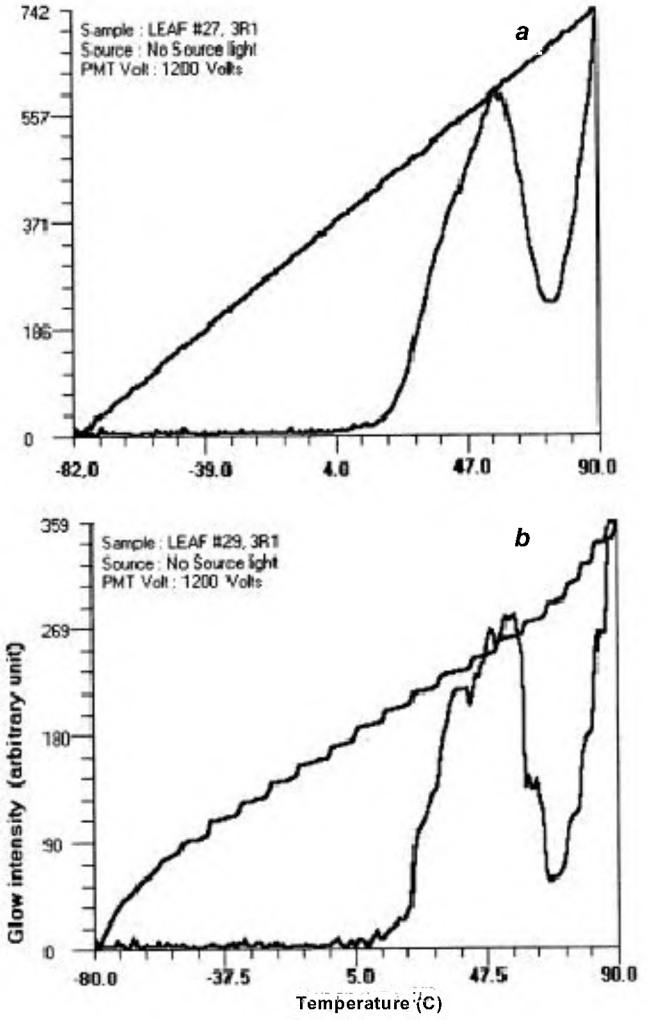

Figure 9. Difference in dark-TL from two different leaves. ***a***, Properly controlled rate (ripple less than ± 1°C) for all the 4 samples shown in Figure 6. ***b***, Abnormal, but controlled heating rate with ripples up to ± 2°C for all the samples shown in Figure 7.

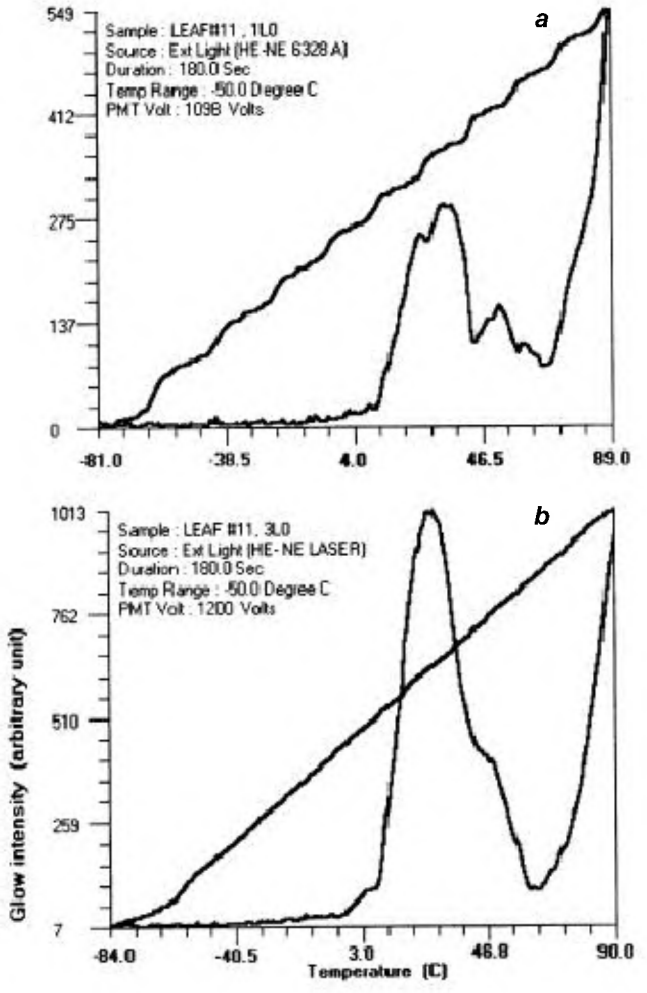

Figure 10. Normal TL after exposure to exciting light from He–Ne laser beam at – 50°C of samples from the same leaf. ***a***, Glow curve shows numerous bands. Superimposed thermal profile shows ripples about ± 2.5°C. ***b***, Glow curve missing the bands seen in (***a***). The heating rate was controlled within ± 1.0°C.

relative humidity of the samples did not show significant difference and was largely insensitive to a wide range of low temperatures at which the stimuli were given. Diffusion of charges did not seem to be involved in the process. However, Knox and Garab speculated that the stimulation of TL by electric field results from deformation of the microenvironment of the traps which in turn may facilitate charge recombination leading to emission as required by the Randal and Wilkins' theory.

TL could be a consequence of quantum confinement, the heat being supplied to the isolated nanoparticles from high voltage gradient across the electrically polarized thylakoid and/or plasma membrane housing them. Since TL emission spectra indicates chlorophyll molecules as the source in photosynthetic systems[27,28], isolated PSII/PSI core particles or parts of the light harvesting complexes adsorbed to lipid layers in the process of heating possibly behave as quantum dots that results in dark-TL.

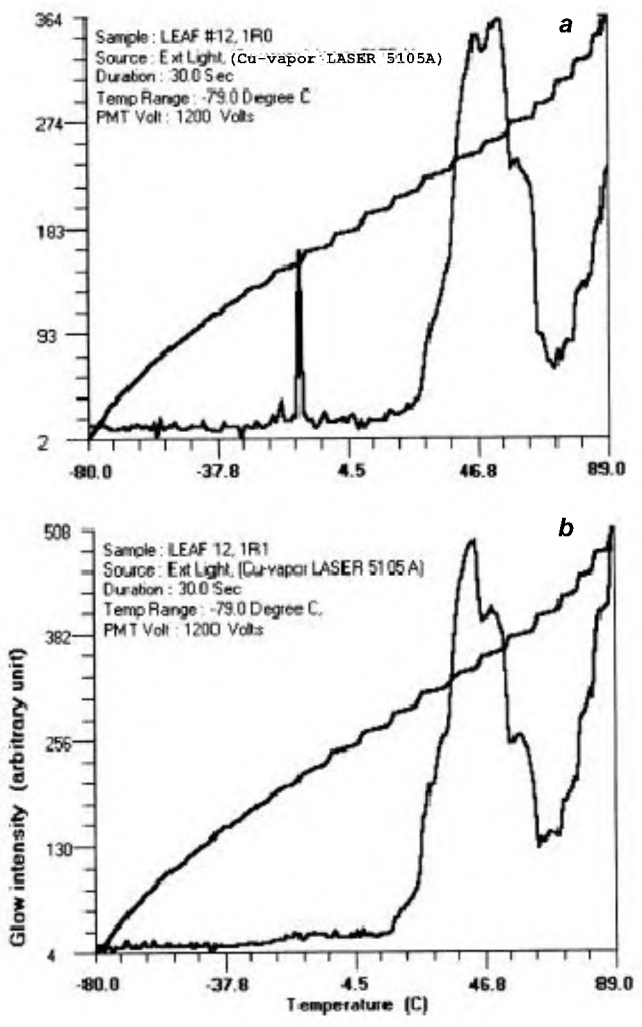

Figure 11. Normal TL after exposure to copper vapour laser at – 79°C from adjacent pair of samples from the same leaf. Both abnormally heated with ripples of about ± 2°C. ***a***, Glow curve shows a spike at – 10°C. Ripple beyond the control level started at – 28°C. ***b***, Spike missing at – 10°C. Ripples started at – 19°C.

The leaf matrix could provide the substrate for adsorption of the membrane lipids following expulsion of cellular water because of rupture by freeze-thawing that is intrinsic to TL technique applied to photosynthetic systems. In artificial system, for instance, Sastry *et al.*[29,30] could, by an ingenious simple technique, form films of colloidal nanoparticles, and even double-stranded DNA without distortion of the helical structure, by evaporation of lipid layer in the solution on a silicon matrix. The TL technique is likely to be doing precisely that by heating wet samples such as whole cells and chloroplast preparations. We have, without any kind of stimulation, detected dark-TL signals on reheating after adding water following the first thermal excursion of spinach leaf and also of cynobacterial whole cells deposited on 0.22 micron membrane filters[1]. The process may have mimicked the one generating films of nanoparticles in artificial system[29]. The alternative suggested here to visualize TL centers as quantum dots rather than charge recombination involved in Randal and Wilkins' theory[10] developed for solid material that would not go through such drastic structural changes as biological membranes during thermal excursions, makes it easier to comprehend why we were able to see dark-TL in non-photosynthetic organisms as well[1]. Genetic evidence suggests that in photosynthetic system it is the PSII complex that may function more efficiently as quantum dot than PSI. Bishop[21,22] isolated mutants of *Senedesmus* defective in PSI that gave all the TL peaks as in the wild type, but the one defective in PSII gave none.

Interestingly, in a deletion mutant of *Synechocystis* sp. PCC 6803 lacking water oxidizing 33 kDa protein that is loosely associated in the PSII complex, flash-induced oscillation of oxygen evolution was eliminated, but not of TL though the yield was less[31]. The protein presumably also serves as heat sensor associated with PSII particle that functions as quantum dot, to drain out heat from the immediate surrounding by exciting the special pair of chlorophylls of the reaction centre that finally throws out excess energy as fluorescence or phosphorescence.

To conclude, we note that Arnold and Sherwood[5] also observed the phenomenon we described here. They mention that the air-dried chloroplast preparations exposed to light and allowed to stand at room temperature in dark for several hours gave some weak glow on heating. The 'different shapes' of glow curves that were not presented in the paper (see italicized text above), marked the beginning of the future problem of non-reproducibility of TL curves from different laboratories. We could begin to investigate the neglected phenomenon, perhaps, because of greater versatility of the new instrument.

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