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CHARGE ACCUMULATION AND RECOMBINATION IN PHOTOSYSTEM II STUDIED BY THERMOLUMINESCENCE

II. OSCILLATION OF THE C BAND INDUCED BY FLASH EXCITATION

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The characteristics of the thermoluminescence band appearing at +50°C in the glow curve (C band) was investigated in maize chloroplasts. The C band, which had a half-time of 10 min, could be charged in the presence of DCMU, and its amplitude significantly increased if preilluminated chloroplasts were reexcited after DCMU addition. Inactivation of the water-splitting system by hydroxylamine- or Tris-treatment did not abolish the C band. In chloroplasts subjected to various numbers of flashes before DCMU addition, the amplitude of the C band exhibited oscillation patterns which were markedly dependent upon dark adaptation of chloroplasts. Flash excitation of chloroplasts preilluminated by continuous light for 30 s prior to 5 min dark adaptation resulted in a period-4 oscillation with maxima occurring at flash numbers 0, 4, 8, 12. After a 6-h dark-adaptation of chloroplasts the period-4 oscillation was superimposed with a period-2 oscillation. The oscillatory patterns were simulated by model calculations and the possible origin of the C band is discussed.

Introduction

In the accompanying paper [1] we have demonstrated the contribution of the S_2 and S_3 states of the water-splitting system to the generation of thermoluminescence. It was found that in agreement with delayed luminescence observations [2,3] the S_2 and S_3 states could not be distinguished by thermoluminescence measurements. This result was explained by a model proposed by Velthuys [2–4], according to which the redox midpoint potential of the water-splitting system does not change during the $S_2 \rightarrow S_3$ transition. Since the S_1 state is also a positively charged state, one would expect that it is also an oxidized substrate for luminescence. Delayed luminescence investigations could not prove unequivocally the contribution of the S_1 state to the charge recombination. Etienne and Lavorel [5] observed a weak light-emission in dark-adapted Chlorella upon the addition of DCMU which might have arisen from the S_1 state. However, this 'dark luminescence' was later attributed to the S_2 state present in very small amount in the dark-adapted material [6].

It has been published that in the glow curve of chloroplasts a thermoluminescence band appeared at about +50°C. This was labelled the C band [7,8]. The C band was first observed in DCMU treated chloroplasts and in the glow curves of etiolated leaves [8,9]. Since in etiolated leaves the

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PS, Photosystem; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

water-splitting system is not developed, it was suggested that the C band is not related to the water-splitting system.

The investigation of the oscillatory behaviour of thermoluminescence intensity in a sequence of flashes could provide a valuable contribution to the identification of electron transport components responsible for the appearance of a thermoluminescence band. In the present paper we investigate the oscillation of the thermoluminescence band at +50°C as a function of the flash number in order to obtain information concerning its origin. The results suggest that the oscillation of the C band can be directly or indirectly related to the lower S states of the water-splitting system.

Materials and Methods

Intact chloroplasts were isolated from mesophyll protoplasts prepared by enzymatic digestion of the first leaves of maize according to the method described previously [10]. The suspension of chloroplasts contained 0.4 M D-sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA and 50 mM Hepes (pH 7.5) with 100 μg Chl/ml. For Tris-treatment chloroplasts were suspended in 0.8 M Tris-HCl (pH 8.8) to yield 2 mg chlorophyll/ml and incubated for 5 min at room temperature in room light. After incubation the suspensions were diluted 40-fold with the suspension buffer to eliminate the inactivating action of Tris. Thermoluminescence was measured by using an apparatus similar to that described by Takake et al. [11]. The light emission of the samples was measured by a red-sensitive photomultiplier (EMI 9558 B) and the signal was amplified through a home-made differential amplifier and fed to an *X-Y* recorder. The temperature of the sample holder was monitored using a platinum resistor thermometer placed below the samples. Samples were illuminated with white light from a Narva halogen lamp of 650 W. The exciting light was passed through a heat-absorbing water filter (thickness 10 cm) and a Balzers neutral density filter giving an illumination intensity of 10 W/m^2. In flash experiments, samples were excited by xenon flashes (General Radio, Stroboslave, 3 μs, 0.5 J). After excitation of the samples thermoluminescence measurements were performed at a heating rate of 20°C/min.

Results and Discussion

Fig. 1 shows that in inhibitor-treated chloroplasts the recombination of the reduced primary acceptor, Q^-, with the S_2 and S_3 states does not result in a complete charge neutralization at the two sides of PS II, as the so-called C band [7,8] also appears in the glow curve at about +50°C.

In DCMU-treated chloroplasts the Q and C bands slightly overlap each other. However, it has been reported that the peak position of the Q band depends on the type of inhibitor used for blocking the electron transport chain [12,13]. Thus, in the presence of the phenolic herbicide, bromoxynil, the Q and C bands are separated more than in the case of DCMU-treated chloroplasts (Fig. 1a and b).

To clarify whether the water-splitting system is involved in the generation of the C band, chloroplasts were subjected to various numbers of flashes before DCMU addition. The amplitudes of the Q

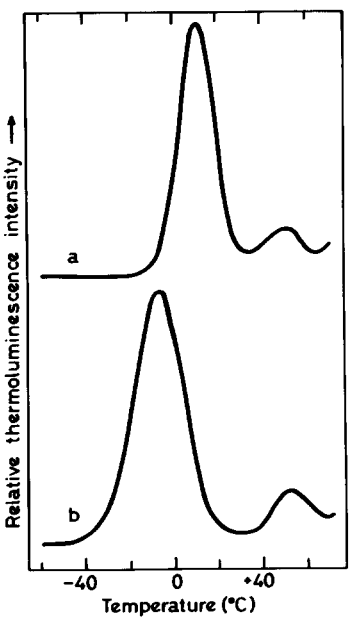

Fig. 1. The effect of DCMU and bromoxynil on the thermoluminescence of isolated chloroplasts. (a) 10 μM DCMU; (b) 180 μM bromoxynil. Chloroplasts were excited by continuous white light of 10 W/m^2 during colling from +20°C to −60°C. The suspension contained 30% glycerol to prevent the distortion of the glow curves by the solid-liquid phase transition of water.

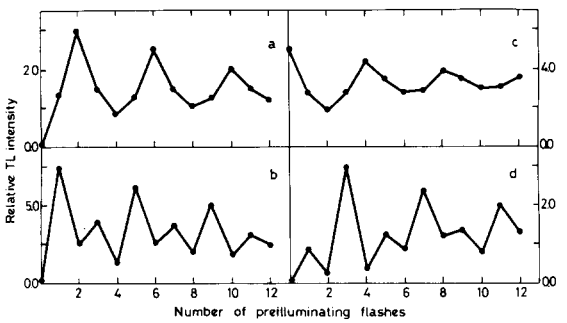

Fig. 2. Oscillation of the amplitude of the Q and C thermoluminescence bands as a function of the excitation flash number. The amplitudes of Q and C bands were measured at +10°C, curves (a) and (b) and at +50°C, curves (c) and (d), respectively. Flash excitation of the samples occurred at +1°C, and it was followed by DCMU addition. After 10 s mixing in dark the samples were cooled down to −40°C and thermoluminescence was measured. Chloroplasts were preilluminated by continuous light for 30 s at +30°C and kept in dark for 5 min before flash excitation (a) and (c), and similarly for (b) and (d) except that in the latter cases chloroplasts were stored for 6 h in dark at +6°C before flash excitation and DCMU addition.

and C bands as functions of flash numbers are shown in Fig. 2.

In chloroplasts preilluminated by continuous white light for 30 s and kept in dark for 5 min prior to flash excitation and DCMU addition the amplitude of both the Q and C bands oscillates with a periodicity of four (Fig. 2a and c). However, the oscillatory patterns of the two bands are inverted to each other. The Q band shows maxima after the 2nd, 6th and 10th flashes when the C band exhibits minima and vice versa.

After a 6-h dark adaptation of chloroplasts an oscillation of period-4 with superimposed periodicity of 2 was observed in the intensities of both the Q and C bands (Fig. 2b and d). However, while the Q band displayed the maxima of oscillations with a periodicity of 4 at 1, 5 and 9 flashes, the oscillation of the C band exhibited maxima at flash numbers 3, 7 and 11.

The high peak temperature of the C band indicates that the redox state which is responsible for this band, represents a very deep trap for the recombining charges. Furthermore, the observation that the C band can be charged in the presence of DCMU suggests that the acceptor which is related to this band is located before the DCMU block. In addition, it has been demonstrated that the S_2Q^- and S_3Q^- redox states can account for the Q band appearing at +10°C [1,14]. Consequently, the redox potential of the donor responsible for the C band should be lower than that of the S_2 state. This conclusion is corroborated by the oscillatory pattern of the C band. In preilluminated chloroplasts the B pool and thus, after DCMU addition, the Q pool is in steady-state condition ($Q : Q^- = 50 : 50$) which does not change in a series of subsequent flashes. Therefore, the oscillation of the C band is only determined by the four successive S states of the water-splitting system. Accepting that 75% of the centers are in the S_1 state in dark-adapted chloroplasts [15], the marked period-4 dependence of the emission intensity with maxima after the 0th, 4th and 8th flashes indicates that the C band can be assigned to the S_1 state of the water-splitting system.

The binary oscillation of the C band in long-term dark-adapted chloroplasts can be interpreted by the binary oscillation of the redox state of B which is reflected in the redox state of Q after DCMU addition.

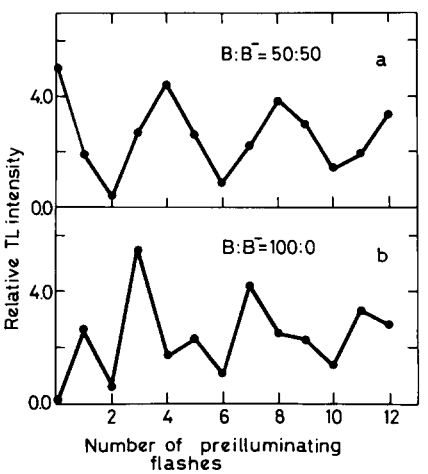

Fig. 3. Computer simulated oscillations of the C band at various redox states of the secondary acceptor pool. The intensity of the C band is determined by the sum of the centers present in S_0Q^- and S_1Q^- redox states. The miss parameter is 8%. (a) The B pool, and consequently after DCMU addition the Q pool, is 50% oxidized. The initial distribution of the reaction centers is $S_0Q : S_0Q^- : S_1Q : S_1Q^- = 15 : 15 : 35 : 35$. (b) The Q pool is 100% oxidized and the initial distribution of the centers is $S_0Q : S_0Q^- : S_1Q : S_1Q^- = 30 : 0 : 70 : 0$.

Assuming that after each flash the amplitude of the C band is determined by the amount of centers which are present in S_0Q^- and S_1Q^- states, the experimentally observed oscillations can be simulated by a similar model calculation as was applied in the preceeding paper. The results are depicted in Fig. 3.

On the basis of the oscillatory behaviour of the C band two explanations can be given concerning its origin.

It can be inferred that the C band arises from charge recombination of the S_0Q^- and S_1Q^- states. This conclusion is apparently in contradiction with the generally accepted view in the literature according to which the water-splitting system is not positively charged in the S_0 state. However, it is in agreement with Velthuys' hypothesis based on delayed luminescence measurements [2,3]. According to the Velthuys' model the oxygen evolving system can occur in addition to the five states S_0–S_4 in yet a sixth state which is denoted as the S_{-1} state [16,17]. In state S_0 the oxygen-evolving system has one positive charge [2,3,16] which may be able to react with Q^- in a radiative charge recombination reaction. Since the peak position of the C band does not change in a sequence of flashes (not shown) our conclusion implies that the redox potential of the water-splitting system remains constant in the $S_0 \rightarrow S_1$ transition. The appearance of the C band in etiolated leaves before the onset of the oxygen-evolving capacity can also be explained by assuming that during the development of the water-splitting system the S_0 and S_1 states are already functional before the higher S states begin to work.

An alternative and more probable interpretation which can explain the origin of C band is based on the comparison of our measurements with fluorescence yield results [18]. The fluorescence level measured 20 s after mixing the sample with DCMU, when the recombination of the S_2Q^- and S_3Q^- states is already completed (this corresponds to the decay of Q band in thermoluminescence measurements), exhibits the same oscillatory pattern as the C band (compare Fig. 1 in Ref. 18 and Fig. 2d). Since the fluorescence yield is determined by the amount of Q^- present (the amount of centers in states S_0Q^- and S_1Q^- which also determines the amplitude of C band) it can be inferred that the oscillation of the C band is also governed only by the redox state of the acceptor side of PS II. We can assume that after the redox state of the acceptor side of PS II has been established by the turnovers of the water-splitting system, no recombination of Q^- can take place with the less oxidized states S_0 and S_1. In this case a presently unidentified oxidized donor (D^+) should enter into a charge recombination reaction with Q^- (the oscillatory pattern does not change because it is determined by the amount of Q^-) giving rise to the appearance of the C band. Thus the C band is generated by charge recombination of the D^+Q^- state. This explains the observation that the C band peak position does not depend on the excitation flash number and also its appearance in the early phases of greening. The explanation given for the origin of C band is consistent with a recent finding of Boussac and Etienne [19]. In Tris-washed chloroplasts, where the S_2 state is inactivated [20], the reduced primary acceptor Q^- backreacts with an 'irreversible' oxidized donor of PS II showing a half-time of 10 min, a value which has been also obtained for that of the C band (Fig. 4). The long half-time as well as the low emission intensity of the C band can explain the fact that delayed luminescence attributable to the charge recombination responsible for the C band has not yet been observed.

The most likely candidates for reservoirs of positive charges responsible for the C band were the cytochrome *b*-559 and the signal II_s. However,

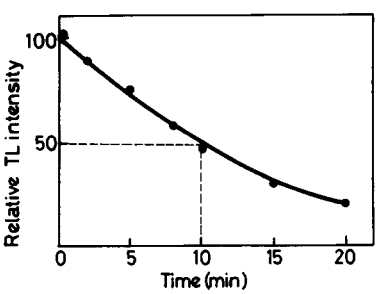

Fig. 4. The decay of the C band as a function of time. Chloroplasts treated by 10 μM DCMU were excited for 1 min by continuous white light at +25°C and stored in dark. At various times following illumination the thermoluminescence spectrum was recorded and the amplitude of the C band was measured.

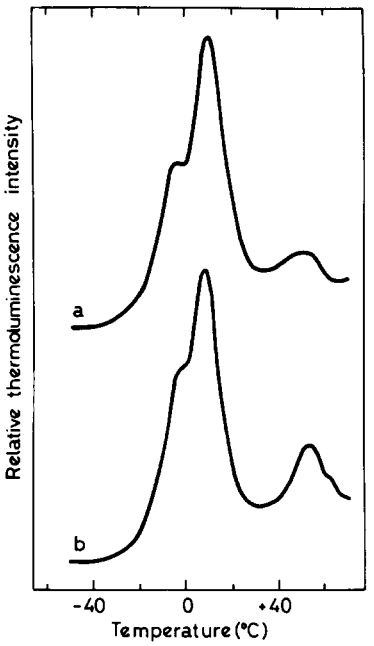

Fig. 5. Glow curves of chloroplasts in the presence of: (a) 100 μM antimycin and 10 μM DCMU; (b) 1 mM desaspidin and 10 μM DCMU. Chloroplasts were excited by continuous white light of 10 W/m^2 during cooling for +20°C to −60°C.

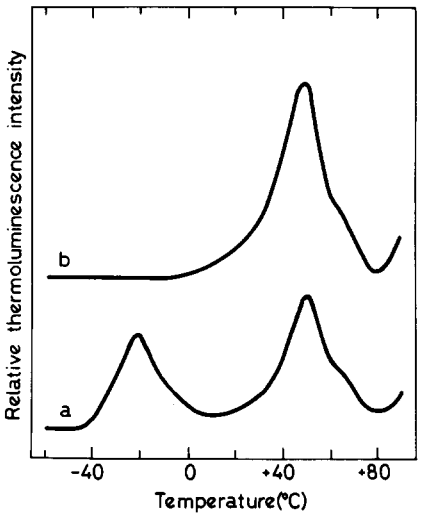

Fig. 6. Effect of hydroxylamine-treatment on the thermoluminescence of chloroplasts. Thermoluminescence was excited by continuous white light during cooling from +20°C to −60°C. (a) Chloroplasts were incubated for 10 min in dark in the presence of 50 μM NH_2OH; (b) as (a), except that 10 μM DCMU was added after NH_2OH treatment.

at room temperature the cytochrome *b*-559 cannot compete efficiently with the water-splitting system in electron transfer to the reaction-center chlorophyll [21,22]. The signal II_s can be also excluded due to various reasons. Its lifetime is several hours [23,24] while the lifetime of the C band is equal to 10 min (Fig. 4). Furthermore, in the presence of DCMU and valinomycin, as well as with DCMU and desaspidin, the signal II_s cannot be formed [23,25] however the C band can be charged in the same experimental conditions (Fig. 5).

Inactivation of the water-splitting system by hydroxylamine-treatment [26] and Tris-treatment [20] as well as by low pH [27] did not abolish the C band either (Figs. 6–8). However, after the water-splitting system had been inactivated the amplitude of the C band stopped oscillating as a function of the excitation flash number (not shown). In hydroxylamine-treated and Tris-treated chloroplasts the C band was considerably intensified when the electron flow towards PS I was blocked by DCMU addition (Fig. 6b and 7b). The appearance of the C band in the glow curve of

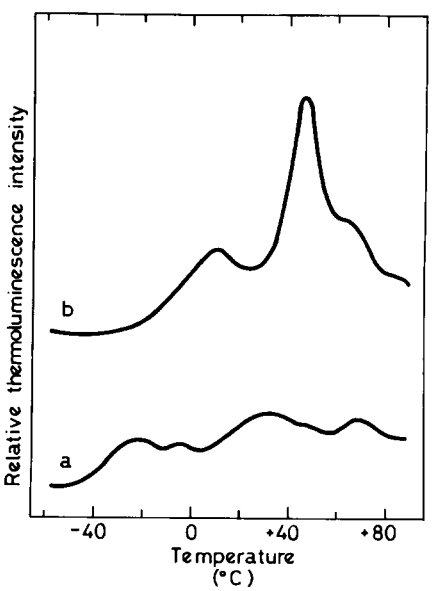

Fig. 7. Effect of Tris-treatment on the glow curve of chloroplasts. Thermoluminescence was excited by continuous light during cooling from +20°C to −60°C. (a) Chloroplasts were incubated for 5 min in 0.8 M Tris, pH 8.8; (b) as (a) except that 10 μM DCMU was added after Tris-treatment.

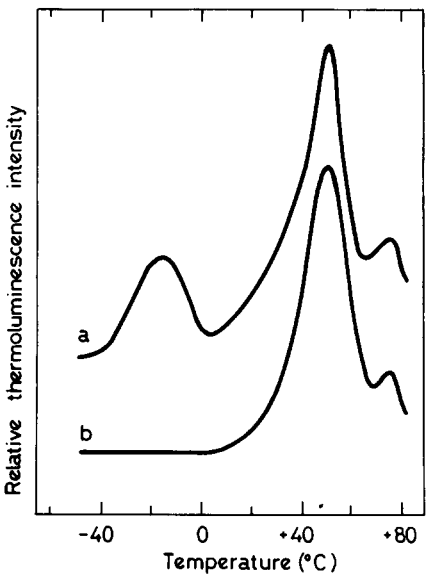

Fig. 8. Effect of low pH on the thermoluminescence of chloroplasts. Chloroplasts were suspended in a medium comprising 0.4 M D-sorbitol/10 mM NaCl/5 mM $MgCl_2$/50 mM phosphate buffer (pH 5.0). (a) Chloroplasts were excited by continuous white light for 3 min during cooling from +20°C to −60°C; (b) chloroplasts were excited for 3 min at −60°C.

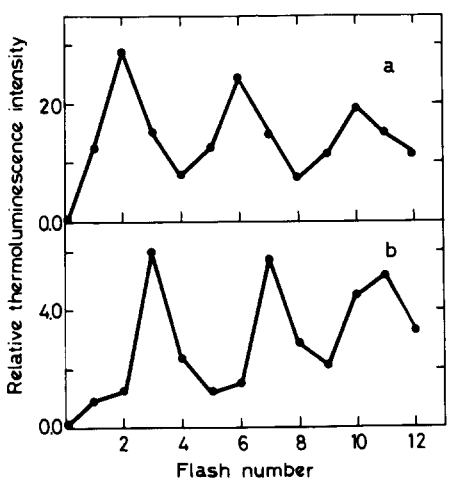

Fig. 9. Oscillation of the amplitude of the Q and C thermoluminescence bands after a variable number of flashes. Curves (a) and (b) represent the oscillation of the Q and C bands, respectively. Chloroplasts were preilluminated by continuous light for 30 s at 30°C and kept in dark for 6 h at +6°C before flash excitation. Flash excitation occurred at +1°C and it was followed by the addition of 10 μM DCMU. The suspension was mixed for 10 s in the dark and after mixing one more exciting flash was given. The sample was quickly cooled down to −40°C and thermoluminescence was measured.

chloroplasts, in which the water-splitting system is inactivated, strongly suggests that the C band originates from charge recombination of the D^+Q^- redox state. However, since it cannot be completely ruled out that the S_0 and S_1 states remain intact in an inactivated water-splitting system, even these experiments do not allow one to choose unambiguously between the two explanations concerning the origin of the C band.

If the last flash of a flash train was given after the chloroplasts had been treated by DCMU, the period-2 oscillations of the Q and C bands were cancelled and the oscillatory patterns exhibited only period-4 oscillations (Fig. 9). Concomitantly, the amplitude of the C band considerably increased. Similar intensification of the C band could also be induced if chloroplasts excited by continuous light were treated by DCMU prior to a second excitation (Fig. 10).

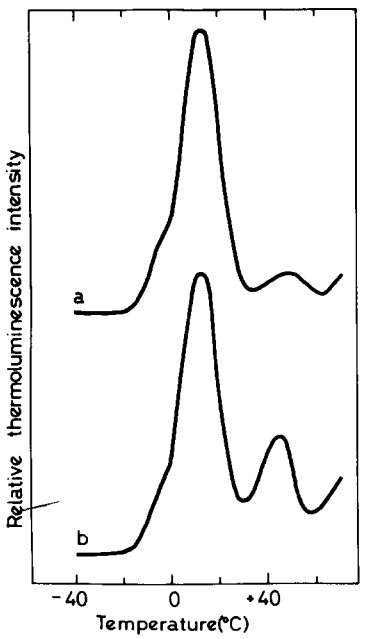

Fig. 10. The effect of illumination conditions on the thermoluminescence of DCMU treated chloroplasts. Chloroplasts were excited by continuous white light of 10 W/m^2 at +1°C. After excitation the sample was cooled down to −40°C and thermoluminescence was measured. (a) Excitation of chloroplasts occurred at +1°C for 1 min and it was followed by the addition of 10 μM DCMU; (b) the same as (a) except that the chloroplasts were preilluminated for 0.5 min before DCMU addition and the illumination was continued for an additional 0.5 min after DCMU addition.

Since continuous illumination or a flash train does not reduce the B pool completely it can be inferred that the DCMU-induced back-transfer of electrons from B^- to Q fills up the primary acceptor pool only partially. Excitation of the preilluminated chloroplasts after DCMU addition by a flash or by continuous illumination reduces the complementary part of the primary acceptor pool too, thus erasing the binary oscillation and giving rise to the intensification of the originally small C band.

A survey of the recent literature demonstrates that certain experimental results can be explained only by parallel acceptor models of PS II [19,28–30]. Thus, it cannot be precluded that a parallel acceptor is responsible for the enlargement of the C band (Fig. 10). In the presence of DCMU the light-induced electron flow is redirected resulting in a more reduced state of the parallel acceptor, which is reflected by a larger C band. However, the other characteristics of the C band (binary oscillation; quadruple oscillation; long lifetime) can hardly be reconciled by the recently proposed parallel models [18,19,28].

Summarizing our results we can say that the C band originates either from charge recombination of the S_0Q^- and S_1Q^- redox states or it is generated by recombination between an unidentified positively charged donor, D^+ and the negatively charged primary acceptor, Q^-.

However, it is obvious that further experiments are needed for a complete understanding of the origin of the C band.

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