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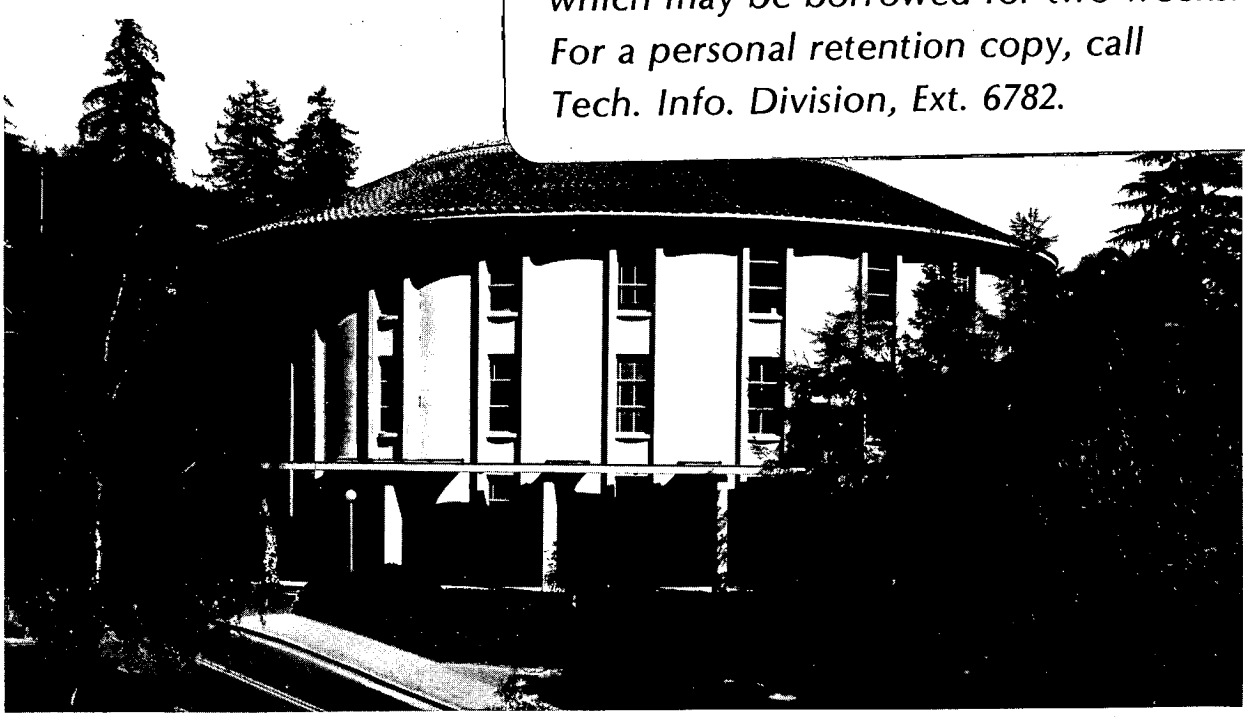

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The Effect of Temperature on the Formation and Decay of the Multiline EPR Signal Species Associated with Photosynthetic Oxygen Evolution

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Key words: Photosystem II, Oxygen evolution, EPR, (Spinach chloroplast).

Abbreviations: PS II, Photosystem II; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EPR, Electron Paramagnetic Resonance.

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SUMMARY

We have investigated the effects of temperature on the formation and decay of the light-induced multiline EPR signal species associated with photosynthetic oxygen evolution (Dismukes,G.C. and Siderer,Y.(1980)FEBS Lett.121,78-80). 1) The decay rate following illumination is temperature dependent: at 295 K the half time of decay is about 40 s, at 253 K the half time is approximately 40 min. 2) A single intense flash of light becomes progressively less effective in generating the multiline signal below about 240 K. 3) Continuous illumination is capable of generating the signal down to almost 160 K. 4) Continuous illumination after a preilluminating flash generates less signal above 200 K than at lower temperatures. Our results support the conclusion of Dismukes and Siderer that the S_2 state gives rise to this multiline signal; we find that the S_1 state can be fully advanced to the S_2 state at temperatures as low as 160 K. The S_2 state is capable of further advancement at temperatures above about 210 K, but not below that temperature.

INTRODUCTION

Photosynthetic oxygen evolution occurs through five intermediate oxidation states (for a review, see [1]). These states are denoted S_i (i=0...4), where i is the number of electrons removed from the water-splitting complex by Photosystem

II [2,3]. The states S_0 and S_1 are stable and are present in dark-adapted samples in a 1:3 ratio. The S_2 state has a lifetime of about 40 to 90 s at room temperature in broken chloroplast preparations from spinach [3,4,5]. Because S_1 is the predominant state in the dark, the reaction resulting from the first excitation of a dark PS II reaction center is the oxidation of S_1 to S_2. Samples preilluminated with a brief flash are placed mostly in the S_2 state; subsequent illumination then initially advances S_2 to S_3.

Studies at low temperatures (reviewed in [4,6]) of the mechanism of PS II reactions and of oxygen evolution have included measurements of variable fluorescence [7,8,9], absorbance changes associated with cyt b_{559} [8,9,10,11], and thermoluminescence [5,12]. At low temperatures the fluorescence rise becomes slow and cyt b_{559} is photooxidized. The threshold temperature for the observation of these effects is lower for PS II centers in states S_0 and S_1 than for those in states S_2 and S_3. Specifically, for dark-adapted samples the temperature threshold is about 170 K, while for one- and two-flash preilluminated samples no threshold is reported but it must be at least as high as 220 K. No temperature threshold based on thermoluminescence measurements is reported for the advancement of S_1 to S_2; but it has been demonstrated that the thermoluminescence band associated with S_2 can be charged with continuous light at 210 K [5]. The charging of the band

associated with S_3 by two flashes has a temperature threshold at about 240 K [5]. There is an effect of the illumination temperature near 170 K on the thermoluminescence [12], but the origin of the effect and its relation to the S states is obscure. The simplest interpretation of these results is that below the threshold temperature the rate of donation from the physiological donor becomes slow compared with that from cyt b_{559}. None of the above measurements directly monitors the S states. Thus, it is not known from those measurements whether at 200 K, for example, the water-splitting complex can advance completely to the state S_2, or just to an intermediate state S_1'.

One of the difficulties encountered in the study of photosynthetic oxygen evolution has been the spectroscopic invisibility of the S states. Recently there have been reports of a multiline EPR signal observed in samples frozen rapidly after a single flash [13,14] or frozen during illumination [15]. The conditions required for generating the signal suggest that it arises from the S_2 state of the water-splitting complex. The observation of this signal presents an opportunity to correlate previous measurements of Photosystem II reactions at low temperatures with the S state behavior.

Our results confirm the assignment of the multiline EPR signal to S_2, and we have used this signal to monitor directly the advancement of S_1 to S_2 and S_2 to S_3 as a function of temperature.

MATERIALS AND METHODS

Broken chloroplasts were prepared from market spinach. Destemmed leaves were ground at 4°C in a Waring blendor for 10 s in a medium containing 0.15 M NaCl, 4 mM $MgCl_2$, 5 mM EDTA, and 0.05 M HEPES at pH 7.5. The homogenate was strained through three layers of cheesecloth and centrifuged at 5,000 x g for 8 min. The pellet was resuspended in the same buffer and spun for 5 s at 100 x g. The supernatant from this spin was centrifuged at 5,000 x g for 8 min. The pellet was resuspended in buffer containing no EDTA and centrifuged again at 5,000 x g for 8 min. The final suspension buffer contained 50% glycerol and no EDTA. Chlorophyll concentrations were between 3 and 5 mg/ml as determined by the method of Arnon [16].

For EPR measurements samples were placed in Suprasil quartz tubes (i.d. 3mm). Samples were kept on ice in the dark for 15 to 75 min. Sometimes samples were given two brief flashes after 5 min on ice, and then allowed to dark-adapt. Flash illumination was performed with short (10 μs) intense flashes from a xenon flash lamp (14 J per flash). The oblong image was tightly focused on the sample. Continuous illumination was for 30 s from a 400 W tungsten source. Constant sample temperature was maintained with a Varian V 6040 NMR Variable Temperature Controller or with a dry ice - methanol bath. Samples were allowed to equilibrate for 3 min before illumination. With continuous illumination the sample temperature was observed to

increase 5 to 7 K. Except where indicated, samples were placed in liquid nitrogen immediately after illumination. EPR measurements were then performed within 24 hours.

EPR spectra were recorded on a Varian E 109 spectrometer equipped with an Air Products Heli Tran low temperature apparatus. All measurements were done at 10 K. Relative signal intensities were taken as the sum, after suitable baseline subtraction, of three or four of the peaks downfield from g=2, normalized by the respective sample volumes. Spectrometer conditions were:microwave power,50 mW; microwave frequency, 9.2 GHz; modulation amplitude,32 G; modulation frequency,100 kHz.

RESULTS

A single intense flash of light at about 273 K followed by freezing in liquid nitrogen generates and traps a species which produces a multiline EPR signal identical to that observed and assigned to S_2 by other workers [13,14,15]. Curiously, we find that samples which contain no glycerol do not exhibit this signal when frozen in the dark after flash illumination at 273 K. It is possible to generate the signal in samples containing no glycerol by giving continuous illumination at 195 K,but even in this case the signal intensity is significantly less than in samples containing glycerol.

By waiting a variable time between single flash illumination and freezing of the sample in liquid nitrogen, we were able to

monitor the decay of the species which gives rise to the multiline signal (Fig.1). At 295 K the decay has a halftime of 40 s. At lower temperatures the deactivation rate becomes dramatically slower; at 253 K the halftime of decay is approximately 40 min. During one hour at 243 K virtually no decrease in signal intensity is observed. We estimate an activation energy of 8 kcal mol^{-1} for the decay process above 273 K.

In a second series of experiments we studied the dependence of the signal amplitude on the sample temperature during illumination (Fig.2). For single flash illumination the maximum signal is generated at temperatures higher than 245 K; almost no signal is observed when a single flash is given at 190 K or below. To determine whether the decline in signal amplitude at lower temperature of illumination is due to competition with a back reaction, we investigated the effect of temperature on illumination with multiple flashes or with continuous light (Fig.3). The temperature range in which the signal intensity declines is lower for more extensively illuminated samples. Under continuous light the signal can be generated upon illumination at temperatures down to 160 K, but not at lower temperatures. The maximum signal is produced by continuous light at about 190 K. The signal intensities for the three curves shown in Fig.3 have been scaled to the same maximum value; a

single flash at 273 K produces only slightly more than half the signal that is formed maximally at 190 K by continuous light.

The signal intensity produced by continuous illumination begins to decrease as the illumination temperature is increased above 200 K. This may result from further reactions that deplete the multiline signal species at the higher temperatures. We attempted to investigate the temperature dependence of these reactions. To limit the observed temperature effect to donor reactions, samples were preilluminated with an intense flash at 273 K, then frozen quickly in liquid nitrogen. This process generates approximately one half the maximal amount of the multiline signal species and allows for the reoxidation of the primary acceptor. Samples were then equilibrated at a chosen temperature between 200 and 240 K for continuous illumination. For samples prepared in this manner the amplitude of the remaining multiline signal is low at illumination temperatures above 220 K, but increases sharply as the temperature is dropped from 220 K to 200 K (Fig.4). Samples illuminated below 210 K exhibited a peak at g=3.1 in the EPR spectrum, an indication of cyt b_{559} photooxidation that was not seen at higher temperatures.

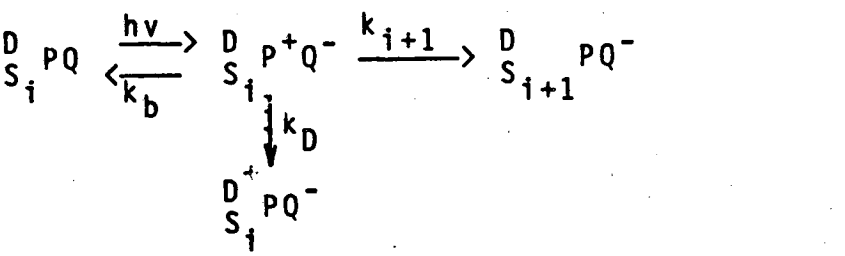
DISCUSSION

We have been able to reproduce the results of Dismukes et.al.[13,14] in generating the multiline EPR signal. We find that the presence of glycerol in the sample is important when the

sample is illuminated at 273 K. Hansson and Andréasson [15] observed the multiline signal in samples containing no glycerol by illuminating during freezing. We observe that the signal can be generated in the absence of glycerol if the sample is first frozen in the dark to 190 K and then illuminated with continuous light. That the signal can be observed in the absence of glycerol rules out a direct involvement of glycerol in the multiline signal species.

The 40 s halftime which we find for the decay of the multiline EPR signal species at 295 K provides evidence that the signal arises from a relatively stable species, and not from a transient intermediate. Previous measurements involved rapid freezing following flash illumination [13,14] or illumination with continuous light while freezing [15] and could not rule out the possibility that the signal arises from an intermediate electron donor. The decay time which we observe is very similar to that reported for the S_2 state of the PS II oxygen evolving complex [3,5]. As previously reported for a thermoluminescent species assigned to S_2 [5], the decay becomes slower at lower temperatures. In our study the temperature dependence of the decay is extended to 253 K. Below 273 K, the decay rate becomes quite slow; at 253 K the half time for deactivation is nearly 40 min.

The following model can be used to explain the effects of lowering the temperature of illumination on the generation of the multiline EPR signal.



In this diagram P and Q are the primary donor and electron acceptor, S_i represents a state S_i of the water-splitting complex, and D represents an alternative donor (possibly cyt b_{559}) which is not on the electron transfer path between the water-splitting complex and P. Since it is the S_2 state of the oxygen-evolving-complex which gives the multiline EPR signal, any configuration in which this state is present will exhibit the signal.

The results shown in Fig. 3 demonstrate that the threshold temperature for generating the multiline signal with continuous illumination is lower than the threshold temperature for generating the signal with a single flash. This result provides evidence for competition between signal formation and charge recombination in the temperature range 160 to 240 K. At the high end of this temperature range k_2 is larger than k_b while at temperatures near and below 160 K the back reaction dominates. The rate described by k_{i+1} probably involves more than one electron transfer reaction, but it is not possible to determine

from these measurements whether the rate-limiting forward reaction involves the formation of the multiline signal species itself or the oxidation of a more primary donor.

The threshold temperature for generating the multiline signal with continuous light is similar to that observed by other workers investigating fluorescence induction and cyt b_{559} oxidation [7,8,9]. The simplest explanation for the inability of continuous light to generate the multiline signal below 160 K is that there is competition between the rate of formation of the multiline signal species, determined by k_2 , and the rate of donation from an alternative donor D, determined by k_D. However, while cyt b_{559} is believed to replace the physiological donor at low temperatures, continuous illumination at 135 K produces neither the multiline signal species nor the oxidized cytochrome unless a preilluminating flash is given at 0°C (data not shown). We are currently investigating the relation between the generation of the multiline signal and the photooxidation of cyt b_{559} in this temperature region.

Further photoreactions decrease the intensity of the multiline signal at temperatures above 200 K (Fig.4). It has been reported that the reoxidation of the primary acceptor by the secondary acceptor [17] and the conversion of S_2 to S_3 [7,10,11] are strongly affected by temperature between about 210 K and 240 K. Both processes affect the signal amplitude generated upon illumination in this range. We attempted to observe the effect

of temperature on just the donor reactions by preilluminating samples with a single flash at 273 K before illuminating with continuous light at reduced temperature (Fig.4). The threshold temperature for this process is significantly higher than for the formation of the multiline signal. The pattern of the temperature threshold being higher for preilluminated samples than for dark-adapted samples, which was observed in the case of the fluorescence rise kinetics [7] and the photooxidation of cyt b_{559} [10,11], holds for the reactions involving the multiline signal species. According to the above model the rates determined by k_3 and k_D become competitive at about 220 K.

CONCLUSION

The agreement between the properties of the formation of the multiline signal which we have investigated and those of the S_2 state provides additional support for the conclusion of Dismukes and Siderer that the signal arises from the S_2 state. Further, the decay time of the multiline signal is evidence that the signal comes from the final oxidation product in the S_2 state rather than a transient species. This state is produced under continuous illumination at temperatures as low as 160 K. Between about 160 K and 240 K the formation of the S_2 state competes with a back reaction. The further oxidation of the S_2 state to S_3 occurs upon extended illumination above 200 K.

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FIGURE LEGENDS

Figure 1 Decay of the multiline signal amplitude at different temperatures. Broken chloroplasts were given one flash at the specified temperature, then placed in liquid N_2 after a variable delay. The signal amplitudes were taken as the peak to peak height of four lines downfield from g=2. The amplitudes at each temperature are normalized to the maximum value. Spectrometer conditions: microwave power, 50 mW; microwave frequency, 9.2 GHz; modulation amplitude, 32 G; modulation frequency, 100 kHz; temperature, 10 K.

Figure 2 Production of the multiline signal in broken chloroplasts by a single flash at different temperatures. Spectrometer conditions as in Figure 1.

Figure 3 Amplitude of the multiline signal produced by a single flash (□), 20 flashes (X), and 30 sec continuous light (O) at different temperatures. The signal amplitudes for different illumination schemes have been separately normalized. Spectrometer conditions and signal amplitude determinations as in Figure 1.

Figure 4 Amplitude of the multiline signal produced in broken chloroplasts by a single flash at 273 K followed by continuous illumination at different temperatures. Samples were given an intense flash at 273 K, frozen in liquid nitrogen, then warmed to the specified

temperature at which they received 30 s of continuous illumination. Spectrometer conditions and signal amplitude determinations as in Figure 1.

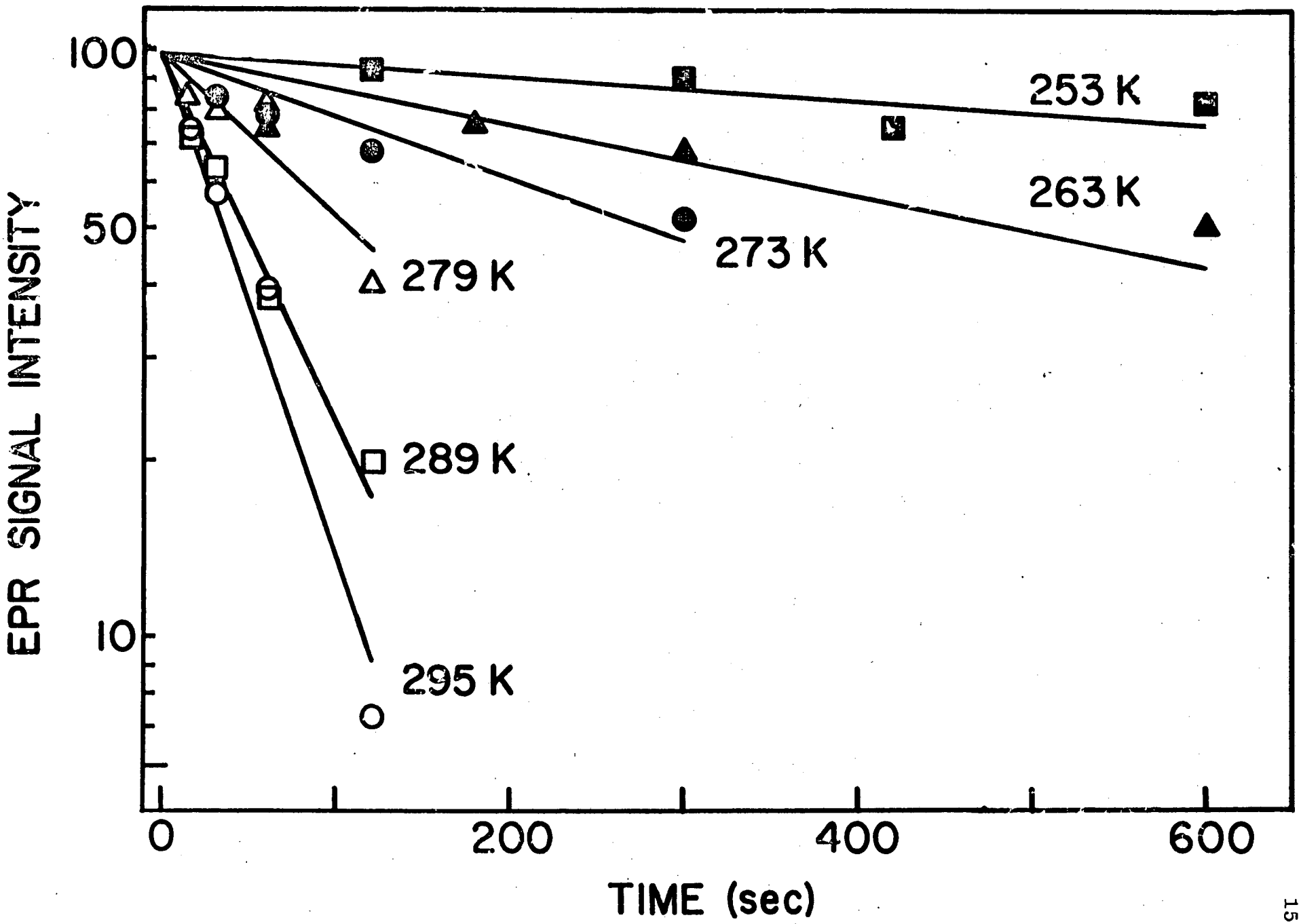
100
50
10
EPR SIGNAL INTENSITY
0
200
400
600
TIME (sec)
253 K
263 K
273 K
279 K
289 K
295 K

Fig. 1

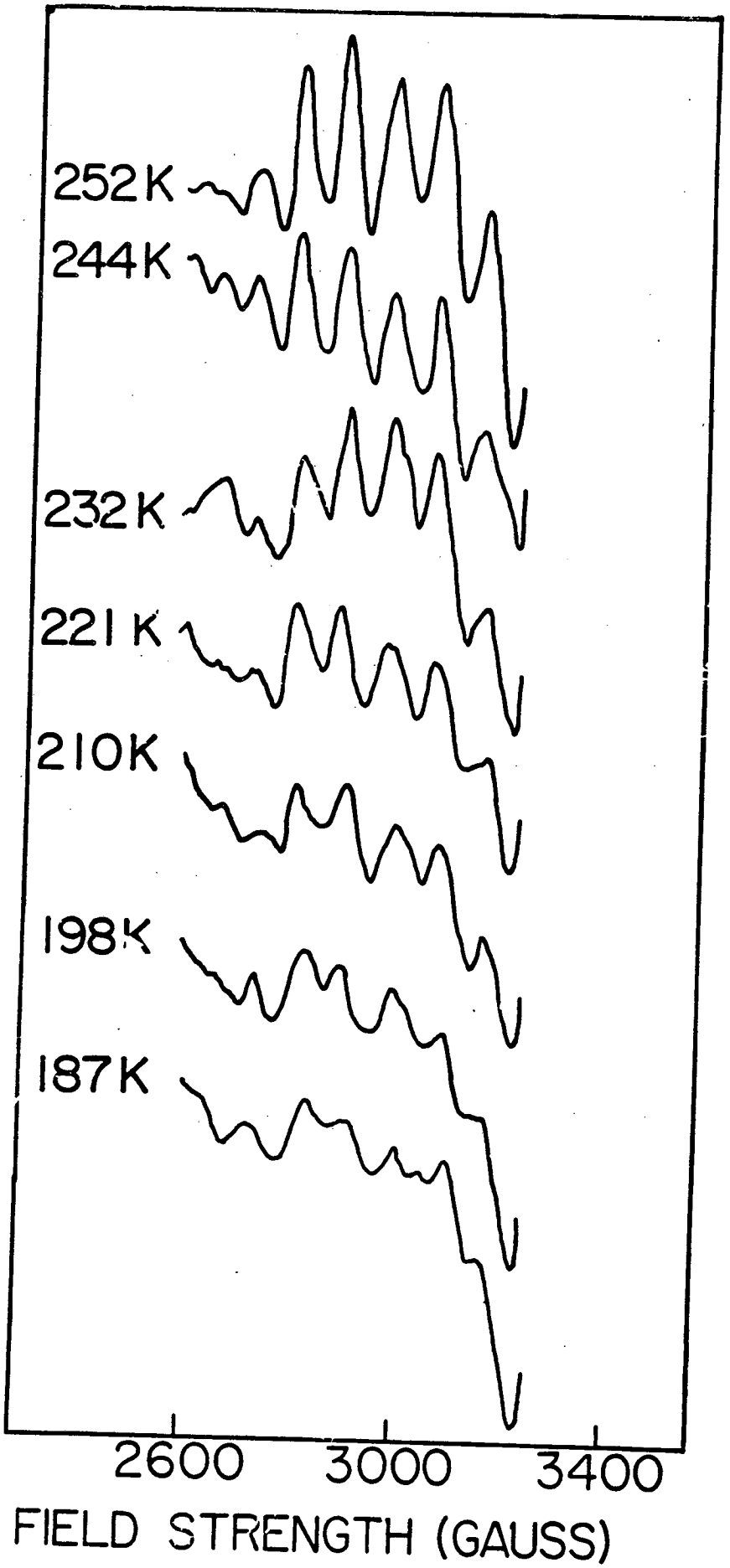
252K
244K
232K
221K
210K
198K
187K
2600
3000
3400
FIELD STRENGTH (GAUSS)

Fig. 2

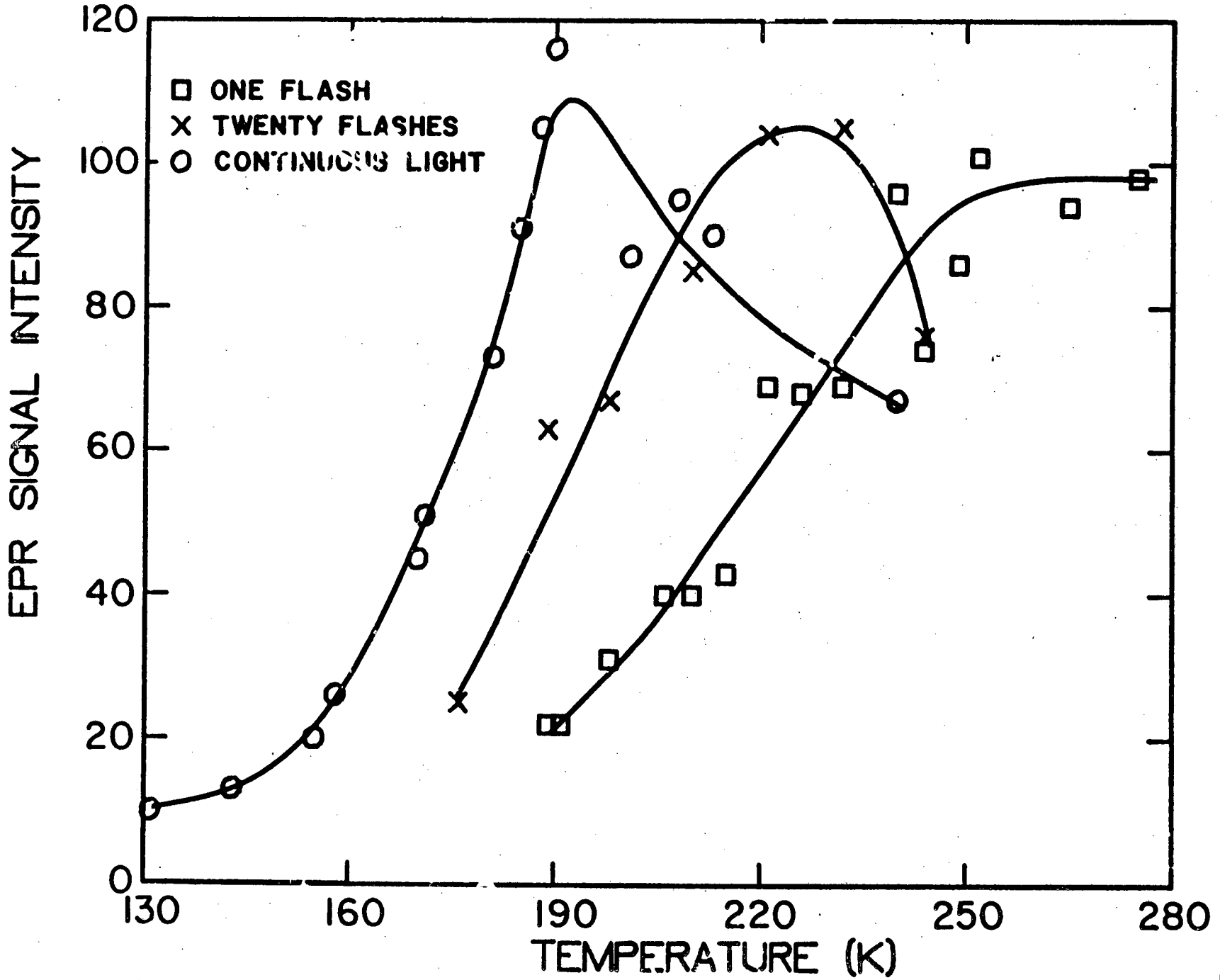
ONE FLASH
TWENTY FLASHES
CONTINUOUS LIGHT
EPR SIGNAL INTENSITY
TEMPERATURE (K)
0
20
40
60
80
100
120
130
160
190
220
250
280

Fig. 3

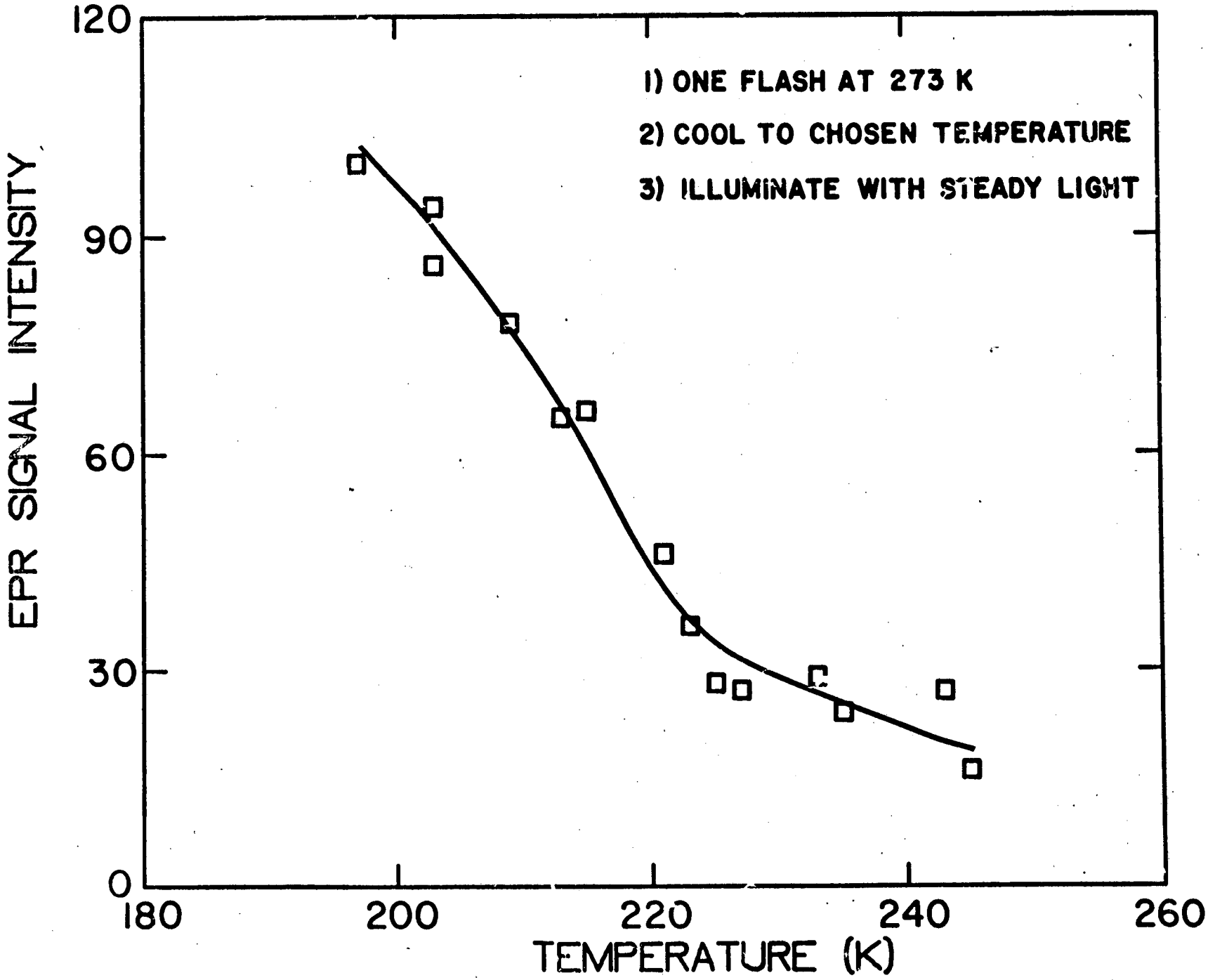
1) ONE FLASH AT 273 K
2) COOL TO CHOSEN TEMPERATURE
3) ILLUMINATE WITH STEADY LIGHT
EPR SIGNAL INTENSITY
120
90
60
30
0
180
200
220
240
260
TEMPERATURE (K)

Fig. 4

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