Title
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Publication Date
1971-04-01

Submitted to Biochimica et Biophysica Acta

UCRL-20693
Preprint c.2

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April 1971

AEC Contract No. W-7405-eng-48

LAWRENCE RADIATION LABORATORY
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UCRL-20693 c.2

DISCLAIMER

DISTRIBUTION OF VARIABLE FLUORESCENCE AMONG SUBCHLOROPLAST FRACTIONS

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Received

SUMMARY

Spinach chloroplasts were fragmented by the French press technique and were separated by fractional centrifugation. A technique for measuring variable fluorescence using the Cary 14 spectrophotometer is described. The amount of variable fluorescence was directly related to the abundance of PS 2 in the fractions. The 160K fraction which possesses negligible PS 2 activity also possesses negligible variable fluorescence. Relative to the other fractions, the yield of background fluorescence from the 160K fraction is also much less than the other fractions. The presence of inactive chlorophyll in the 160K fraction cannot be explained by damage of PS 2 during French press fragmentation.

INTRODUCTION

Stroma lamellae from chloroplasts appear to be entirely PS 1 in character, both with respect to biochemical activity and chemical composition[1]. In the previous paper we showed that while the quantum yield of $NADP^+$ reduction by stroma lamellae is 1.0 at 710 nm, the efficiency at shorter wavelengths is much less[2]. This ineffective absorption accounts for more than 50% of the chlorophyll present in stroma lamellae. Our conclusion that this inactive absorption is not due to a damaged PS 2 is based in part on the evidence presented in this paper.

The phenomenon of variable fluorescence was first reported by Duysens and Sweers[3]. They concluded that a substance, Q, lying close to the reducing side of PS 2, quenched PS 2 fluorescence when in the

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS 1 and PS 2, Photosystem 1 and Photosystem 2.

oxidized form. In the reduced form, quenching was reduced and PS 2 fluorescence increased. The component of fluorescence dependent on the oxidation state of Q is termed variable fluorescence and has been used as an assay for the presence of PS 2 in chloroplasts by Yamashita and Butler[4,5], in digitonin prepared chloroplast fragments by Boardman et al.[6] and by Vredenberg and Slooten[7], and in mechanically broken chloroplasts by Michel and Michel-Wolwertz[8,9]. In this paper we report the distribution of variable fluorescences among chloroplast fractions separated by fractional centrifugation following breakage in the French Press. These experiments were initiated to determine whether the large amount of inactive chlorophyll in our 160K (stroma lamellae) fraction resulted from a damaged PS 2.

METHODS

Chloroplast fractions were prepared from spinach using French press fragmentation followed by fractional centrifugation[1]. The fractions studied were: original chloroplasts before passage through French press, the whole homogenate obtained after passage through French press, and fractions precipitated at 1000 x g 10' (1K), 10,000 g 20' (10K), 40,000 x g 30' (40K), 70,000 x g 30' (70K). The 70K suspension was either precipitated at 160,000 x g 45' (160K) or was observed directly.

Relative fluorescence yields of the fractions at 680 nm were measured on a Perkin-Elmer MPF-2A fluorescence spectrophotometer with an R136-F photomultiplier tube and Corning 2-59 filter on the emission side of the sample. A 10 nm actinic beam at 436 nm, passing through a Corning 4-96 blue filter, excited fluorescence which was observed at 680 nm using a 10 nm band width. Samples were adjusted to 0.1 O.D. at 436 nm, and were observed in a 1 cm cuvette. Fluorescence yield was

proportional to chlorophyll content at this or lower concentrations. Addition of dithionite, as a reductant for the quencher, Q, to the original chloroplast fraction gave no increase in fluorescence signal, indicating that the exciting beam of this single beam instrument was of sufficient intensity to reduce Q and saturate variable fluorescence. An 0.8 M tris treatment of the original chloroplast fraction reduced the fluorescence yield by about 50%, indicating that variable fluorescence was contributing about half the fluorescence intensity at 680 nm. This yield of variable fluorescence is slightly less than that reported by Yamashita and Butler[4,5].

Variable fluorescence was assayed directly, using a Cary 14 spectrophotometer equipped with a % T slide wire, a high intensity light source, an RCA 6911 photomultiplier shielded from the analyzing and actinic beams by a Corning 4-77 filter overlying a 2-58 filter. A means of actinic illumination was provided through a side port[10]. The Cary 14 was operated most successfully as a single beam instrument in the reference mode. The 30 cps modulated analyzing beam from the Cary 14 monochromator was set at 436 nm and the 0 level of fluorescence was recorded in the absence of a sample. The chloroplast sample was then introduced in a 1 cm cuvette. The O.D. at 436 nm was adjusted to 0.1, which yielded fluorescence which was linear with concentration. The slit was initially adjusted by placing a sample in the reference side of the sample compartment and operating in the double beam mode. This opening was then manually set for operation in the reference mode. A continuous actinic beam at 436 nm was provided by a Bausch and Lomb 500 nm grating monochromator through a 5-58 blue filter. An increase

in signal indicated an increase in the yield of fluorescence excited by the modulated beam. Several observations were made to check the validity of this procedure. To eliminate the possibility that the modulated beam was of sufficient intensity to excite variable fluorescence its intensity was measured and the actinic beam reduced to equal the intensity of the modulated beam. Under these conditions no detectable increase in variable fluorescence could be seen upon illuminating the sample with the actinic beam. To eliminate the possibility that the actinic beam was influencing the modulated detection system, the sample was replaced by a suspension of polystyrene latex spheres. Maximum illumination with the actinic 436 nm beam yielded no measurable increase in signal. Similarly, chloroplast samples reduced with dithionite showed no increase in fluorescence when illuminated with a high intensity actinic beam at 436 nm.

Also contributing to our confidence in this technique were the observations that variable fluorescence was destroyed by tris treatment[4] and by heating[11]. It was reduced by adding $NADP^+$ and the cofactors for $NADP^+$ reduction to the sample. The reduction in variable fluorescence brought about by addition of $NADP^+$ and cofactors was reversed by 10^{-5} M DCMU.

RESULTS AND DISCUSSION

Figure 1 is a summary of data obtained for relative fluorescence yields of the fractions at 680 nm. The sample is first observed with no additions or further treatment. The fluorescence yield under this condition consists of background plus light induced fluorescence and is represented by the clear areas.

Darkened areas represent a further increase in fluorescence yield obtained upon addition of dithionite to the sample. The data show that the ability of chloroplasts to photo-reduce Q is decreased by passage through the French press. Q itself does not appear to be destroyed by the treatment since the fluorescence yield of FP upon addition of dithionite returns to the same level as that observed in the original chloroplasts.

The relative amounts of maximum fluorescence in these fractions are closely related to the abundance of PS 2. The maximum fluorescence yield of the grana lamella (10K) fraction is ten times greater than that of the stroma lamella (160K) fraction which correlates well with distribution of PS 2 in these two fractions. These data correspond well with those of Michel and Michel-Wolwertz for density gradient separated fractions. In agreement with the observations of Yamashita and Butler[4,5], tTris treatment greatly reduced the fluorescence yield of the original chloroplasts. The degree to which tris reduced the fluorescence yield in the fractions was closely related to the abundance of PS 2 in the fraction, the 10K fraction showing maximum reduction and the 160K fraction showing very little reduction.

The contribution of variable fluorescence to total fluorescence was estimated using a modulated analyzing beam as described under Methods. These results are given in Figure 2. The fluorescence excited by the low intensity modulated beam in the absence of actinic illumination or added dithionite was considered the background fluorescence. The increase in fluorescence yield of the modulated beam upon actinic illumination was considered as light induced variable fluorescence. The increase in fluorescence yield of the modulated beam upon addition of dithionite

was considered to be the total variable fluorescence. In the latter experiments fluorescence levels were recorded 1 min after dithionite addition. The actinic beam had almost no effect on fluorescence yields when dithionite was present in the reaction mixture. The data in Figure 2 show that background fluorescence is about 41-45% of the total fluorescence in the original, FP, 1K and 10K fractions. The proportion of background fluorescence rises to more than 90% in the 70K and 160K fractions which are greatly depleted in PS 2. The effect of tris treatment was studied in all the fractions. Tris treatment almost totally destroyed the ability of the chloroplast fractions to photo-reduce Q, whereas the level of background fluorescence was only slightly reduced. This effect is consistent with observation of Yamashita and Butler that tris treatment leads to inactivation of PS 2 [4,5].

While the background fluorescence can be measured directly using the Cary 14 as described here, the filter system used to shield the photomultiplier passes primarily wavelengths greater than 695 nm. Fluorescence emission spectra of the 10K and 160K fractions at room temperature show that ratio of 710 nm/680 nm emission by the 160K fraction is twice as great as that by the 10K fraction. This is in agreement with observations by Vredenberg and Slooten, who used digitonin separated sub-chloroplast fractions[7]. This fact in combination with the emphasis on long wavelength spectral response by the filter system tends to minimize the fluorescence yield differences between the 10K and 160K fractions using the Cary 14 method, both with respect to total and background fluorescence. While the Cary 14 method serves to differentiate between background and variable fluorescence, an absolute comparison of total yields is better obtained from the fluorescence spectrophotometer as shown in Figure 2.

The results given in Figures 1 and 2 demonstrate that the distribution of total variable fluorescence among the FP fractions closely follows the distribution of photosystem 2. The 160K fraction, which is totally inactive in PS 2 demonstrates negligible variable fluorescence in addition to a greatly decreased background yield. Destruction of Q during FP treatment cannot account for the absence of Q in the 160K fraction since the total abundance of Q does not appear to be reduced by FP treatment. Therefore the inactive chlorophylls in the 160K fraction[2] do not appear to have resulted from damaged PS 2. Alternatively, they may be related to sites of membrane synthesis in the chloroplast[2].

ACKNOWLEDGMENTS

The technical assistance of Albert Pfeifhofer is gratefully acknowledged. This work was supported by National Science Foundation Grant GB-255F9X and the U. S. Atomic Energy Commission.

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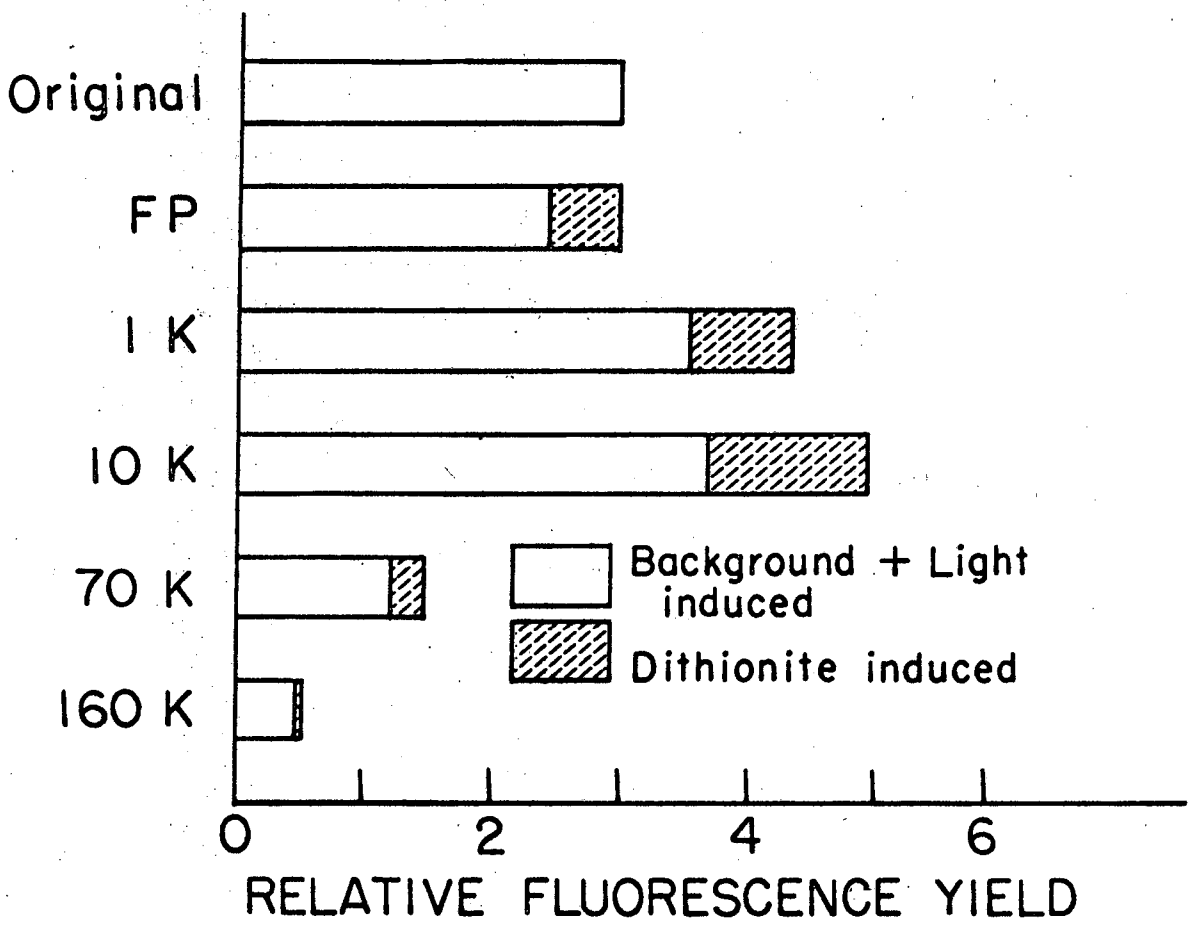
FIGURE LEGENDS

Fig. 1. Relative fluorescence yields of spinach chloroplast and chloroplast fractions measured at 680 nm. Actinic beam saturated the light-induced variable fluorescence. Increased fluorescence obtained upon addition of dithionite is represented by shaded areas.

Fig. 2. Relative fluorescence yields of spinach chloroplasts and chloroplast fractions measured using a modulated measuring beam. Background fluorescence is represented by clear areas, the light induced variable fluorescence by dotted areas and the additional fluorescence obtained upon addition of dithionite is represented by the slashed areas.


Original
FP
1 K
10 K
70 K
160 K
Background + Light induced
Dithionite induced
0
2
4
6
RELATIVE FLUORESCENCE YIELD

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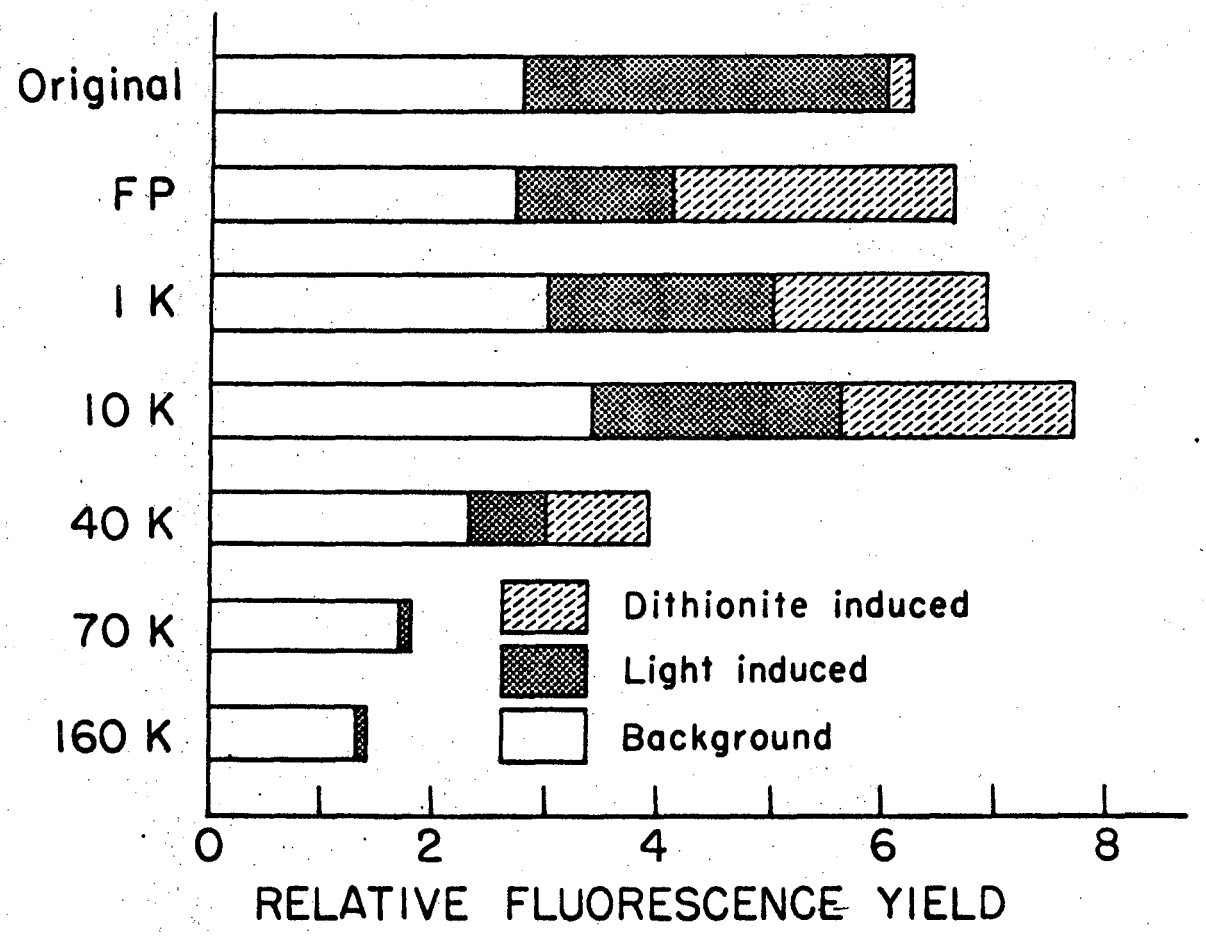

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P. L. Stanbach + Sauer Fig 2

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