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FLUORESCENCE CHARACTERISTICS OF LYOPHILIZED MAIZE CHLOROPLASTS SUSPENDED IN BUFFER

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SUMMARY

Fluorescence transients were measured in lyophilized maize chloroplasts (suspended in Tris-maleate buffer (pH 6.6)) after extraction with heptane. (The fluorescence characteristics before extraction were qualitatively similar to those in the fresh chloroplasts.) The initial fluorescence level (m) in the (dry) heptane-extracted sample remained the same as in the unextracted material, but the variable fluorescence (Δm) was drastically diminished. A portion of variable fluorescence, however, could be restored by adding $\text{Na}_2\text{S}_2\text{O}_4$. If the heptane extraction was made in the presence of water (wet), the m level was almost as high as (or higher than) the final level (M) of the unextracted sample, and Δm was reduced. The "jet" of O_2 (that measures the pool size of the intersystem intermediate A) and the "microjet" (that measures the pool size of the reaction center complex E), present in the unextracted samples, were absent in both types of extracted samples. Some of the above data may be interpreted in a hypothesis in which two quenchers (Q_1 and Q_2) control the fluorescence ($\text{O} \rightarrow \text{P}$) of chloroplasts—the reduction of Q_1 being responsible for the rapid and that of Q_2 for the slow fluorescence rise.

INTRODUCTION

LYNCH AND FRENCH¹ and BISHOP² showed that the light petroleum (b.p. 30–40°) extraction of lyophilized chloroplasts destroys the Hill activity, but the latter could be restored by adding back the extract. BISHOP² showed that the loss in activity was due to vitamin K (a quinone).

Chloroplasts contain several quinones³. There is 1 plastoquinone A (PQ-A) per 7 chlorophyll molecules and 1 plastoquinone C (PQ-C) for every 30 chlorophyll molecules⁴. The compound "Q", the electron acceptor for Pigment System II, is, however, present in smaller quantities (1/150 chlorophyll (Chl, ref. 5)). There is a

Abbreviations: CMU, 3-(*p*-chlorophenyl)-1,1'-dimethylurea; DCMU, 3-(3,4-dichlorophenyl)-1,1'-dimethylurea; Chl, chlorophyll; PQ, plastoquinone.

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possibility that a special fraction of quinones serves as the component Q. AMESZ AND FORK⁸ have shown that 70 μM of certain oxidized quinones can quench 50% of the initial fluorescence rise and 15 μM the variable fluorescence rise. They have further demonstrated that this quenching was not due to the stimulation of the electron transport because it occurred even in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). However, no definite evidence exists regarding the nature of Q.

PQ-C is a more likely candidate to be "Q" because of the closeness of its E_0' ($= 55 \text{ mV}$), to that of Q ($E_0' = -35 \text{ mV}$)⁷; the E_0' of PQ-A is 113 mV. TREBST⁸ has shown that extraction of 70% of the total PQ-A does not impair the NADP⁺ reduction in chloroplasts and WOOD *et al.*⁹ have shown that PQ-A restores only about 30% of the Hill activity when added to the extracted chloroplasts. However, the crude extract restores about 70% of the activity. Thus, other factors are involved besides PQ-A. The above results, however, do not exclude the possibility that Q is a special type of plastoquinone present in small quantities. Recently H. T. WITT (personal communication) has suggested that the primary acceptor of electrons of System II is a plastoquinone that is reduced first to a semiquinone.

In this paper we report the fluorescence characteristics of chloroplasts extracted with heptane, a universal solvent for quinones. Two methods of extraction were used. In one case, lyophilized chloroplasts were extracted with "dry" heptane. In the other case, lyophilized chloroplasts were extracted in the presence of a trace of water; this will be referred to as the "wet" heptane extraction. We present data on the time-course of fluorescence, the effects of a poison of photosynthesis 3-(*p*-chlorophenyl)-1,1'-dimethylurea (CMU), an electron acceptor ($\text{Fe}(\text{CN})_6^{3-}$) and a reductant ($\text{Na}_2\text{S}_2\text{O}_4$) on the fluorescence transient, the fluorescence yield as a function of light intensity, the fluorescence spectra of constant and variable fluorescence and the rate of O_2 exchange in these preparations.

To explain our results, we propose that two quenchers (Q_1 responsible for rapid fluorescence changes and Q_2 for slow fluorescence changes) are involved in the $\text{O} \rightarrow \text{P}$ (ref. 10) rise. The wet heptane extraction causes extraction of Q_2 . The dry heptane extraction destroys or extracts ZH (the primary donor of System II) and keeps Q_2 unavailable for reduction. In both cases the bulk of the plastoquinones are extracted. The absence of O_2 evolution suggests that in both cases some intermediate is inoperative. Attempts to reconstruct the original systems have been unsuccessful so far. Tentatively, we also entertain the hypothesis that a portion of Q may not be a real substance — it may represent a state of the matrix surrounding Chl *a*.

MATERIALS AND METHODS

Zea mays (maize) was grown in the greenhouse for 1 month. 20 g of leaves were blended in 100 ml of 0.1 M Tris-maleate, 0.4 M sucrose buffer (pH 6.6) (ref. 11). The material was filtered through cheesecloth and cotton. The filtrate was centrifuged for 2 min at $200 \times g$. The pellet was washed and suspended in 0.1 M Tris-maleate buffer (pH 6.6). The chloroplasts were then lyophilized.

For each experiment three equal quantities of lyophilized chloroplasts were weighed: (i) was suspended in 0.1 M Tris-maleate buffer (pH 6.6). (ii) was extracted with heptane in a homogenizer for 12–15 min at room temperature and then centrifuged;

the heptane was decanted and the chloroplasts dried under vacuum to remove the traces of heptane, and finally suspended in 0.1 M Tris-maleate buffer (pH 6.6). (iii) was extracted with heptane (after being wet with a trace of water) for 12–15 min and then treated as in (ii).

The amount of chlorophyll per ml of the final suspension was adjusted to be 10–12 μg Chl per ml suspension in all three samples.

Fluorescence measurements were made by the stop and flow method of LAVOREL¹². When the suspension is flowing at a fast speed, the constant fluorescence (m) is recorded; and when the flow is stopped, the fluorescence rises from m to M (the final level). In each case a record was made of the constant (m) and the variable plus constant (M) fluorescence, excited by 480-nm light obtained from a monochromator. The light source was a Xenon lamp operated at 40 A. Fluorescence was collected at right angles, passed through a Bausch and Lomb monochromator and detected by an EMI 9558A photomultiplier.

O_2 measurements were made on all samples by means of a platinum rate electrode. The chlorophyll concentration (in these measurements) was adjusted to approx. 1 mg Chl per ml. O_2 spike was measured by illuminating the chloroplasts for 10 sec with 650-nm light after 50-sec dark intervals. The microjet of O_2 was measured by using far-red light flashes of 10 μsec given every 15 sec.

RESULTS AND DISCUSSION

(1) Fluorescence induction

(A) Unextracted lyophilized chloroplasts suspended in buffer

Fluorescence induction in suspensions of fresh isolated chloroplasts^{13–19} is simpler than in whole cells (see literature citations in refs. 20 and 21). A dark-adapted suspension of chloroplasts, when exposed to bright light (approx. 10^{15} quanta $\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$) shows an instantaneous rise in fluorescence referred to here as minimum level m ($= F_0$); if the yield of m is independent of intensity, it is "O" (ref. 10). This is followed by a biphasic rise to a maximum level referred to here as M level ($= F_\infty$); when the yield of M is truly maximum, it is "P" (ref. 10).

Fig. 1 shows a plot of the time (t) it takes to obtain $(M - m)/2$ (from the time the flow of the chloroplast suspension is stopped) as a function of light intensity in fresh and in lyophilized chloroplasts suspended in buffer. In all cases, this time (t) increased with any decrease in intensity, but lyophilization did cause a slowing down of the rise from m to M . This increase in t was more pronounced when lyophilization was made by suspending chloroplasts in water rather than in buffer.

The kinetics of the rise (m to M) in lyophilized chloroplasts was similar to that in the fresh chloroplasts. First, there was a fast phase (O \rightarrow I, refs. 20–22); it was over within 1 sec. This phase was followed by a slow phase (I \rightarrow P); at certain intensities (see refs. 5 and 11) and also in the presence of certain concentrations of DCMU (or CMU) a plateau or a dip (D in ref. 20) is observed between the fast and the slow phases. The slow rise is over within 5–15 sec depending on the intensity of the exciting light and the physiological 'history' of the chloroplasts. In chloroplasts, a decline from M , although extremely slow, is present and has often been called the steady state (S).

Intensity dependence. For the purpose of ensuring that the m level was truly

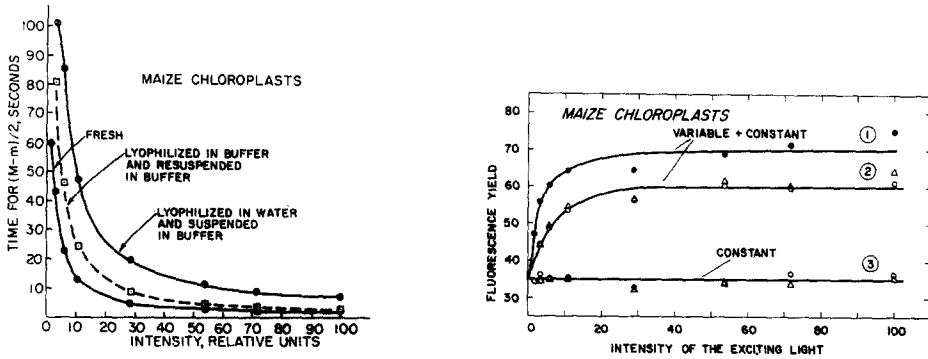


Fig. 1. Time to reach half-maximal variable fluorescence ($(M - m)/2$) as a function of the intensity of exciting light. (100 on the abscissa is equivalent to 10^{18} ergs \cdot cm $^{-2}$ \cdot sec $^{-1}$.) Buffer: 0.1 M Tris-maleate, 0.4 M sucrose (pH 6.6); 20°; 10 μ g Chl per ml suspension of maize chloroplasts.

Fig. 2. Fluorescence yield (F/I) for m (constant) and M (variable + constant) as a function of the intensity of exciting light. Curve 1, M for fresh chloroplasts in buffer; Curve 2, M for chloroplasts lyophilized in water (Δ) and in buffer (\circ), resuspended in buffer; Curve 3, m for lyophilized chloroplasts—fresh chloroplasts had the same yield (points not shown). Note that no points are plotted for very low intensities. See legend of Fig. 1.

constant fluorescence (O of LAVOREL¹⁰) and M level was indeed variable *plus* constant fluorescence, we measured the induction of fluorescence as a function of light intensity and plotted the dependence of the yield of M and m on the intensity of exciting light. Fig. 2 shows a plot of F/I versus I for m and M levels in fresh chloroplasts and in lyophilized preparations. The Curve 3 shows that the fluorescence yield of m (measured with a flowing system) in lyophilized and in fresh chloroplasts is indeed independent of light intensity. Thus, we must be monitoring true O . Curves 1 and 2 are for M of fresh and resuspended lyophilized chloroplasts. Both have the characteristics of the variable fluorescence (see refs. 20 and 23 for green plants and ref. 24 for photosynthetic bacteria) — the yield remains low and constant at low intensities, then rises and attains a high (about 2–3 times that at low intensity) and constant value. The ratio of M to m was about 1.7 for the lyophilized sample — quite close to that in fresh chloroplasts (2.0). The above results ensured that the lyophilization changed the system only slightly and quantitatively.

Effect of CMU. Lyophilized chloroplasts suspended in buffer containing 10 μ M CMU showed the same m level as the untreated sample (Fig. 3, left curves); the fast rise from m to M was slightly accelerated (16% at 0.5 sec after the flow was stopped), but the slow second phase of the rise from m to M was significantly decreased (35%, 4 sec after the flow was stopped). At a lower intensity ($I = 0.5$), the increase in the first phase was higher (37.5% at 0.5 sec) but the decrease at 4 sec was still the same as at the higher intensity.

The differential effect of CMU on the two phases of the $m \rightarrow M$ rise is not consistent with the hypothesis that the CMU simply acts as a block between Q and A (ref. 25), in which case one might expect a more rapid $m \rightarrow M$ rise (as observed) but not a decrease in M . Another possibility would be that, in chloroplasts, another "factor" different from Q controls the slow second phase of the $m \rightarrow M$ rise.

As a working hypothesis, we suggest that the chloroplast fluorescence is controlled by two quenchers (Q_1 and Q_2). The reduction of Q_1 is responsible for the rapid

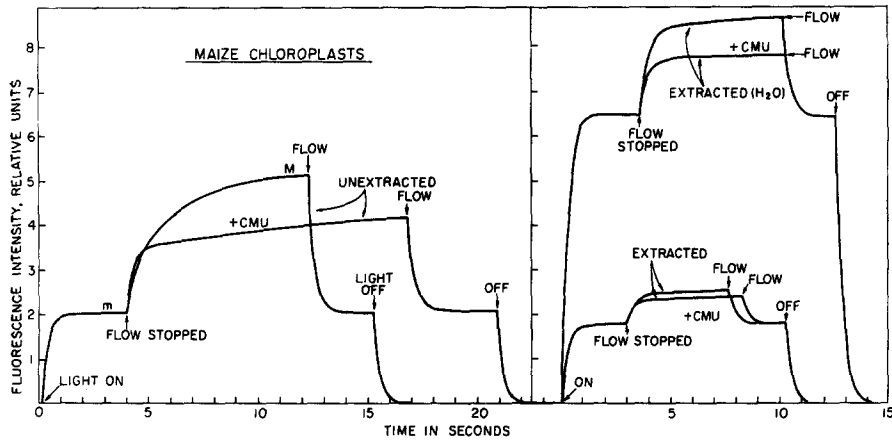


Fig. 3. Fluorescence induction in the lyophilized and resuspended chloroplasts with and without 10 μ M CMU. *m* = constant fluorescence, *M* = variable + constant fluorescence, as measured by the stop-flow method of LAVOREL. Left curves, unextracted chloroplasts; right and top curves, chloroplasts extracted with heptane (wet); right and bottom curves, chloroplasts extracted with heptane (dry). See legend of Fig. 1.

rise and the reduction of Q_2 is responsible for the slow rise; both are quenchers in their oxidized forms but not in their reduced forms. CMU keeps the Q_2 in the oxidized form, abolishing the slow rise; however, the Q_1 to Q_1H reaction is accelerated because there is no competition with Q_2 . (An explanation of the effect of DCMU on whole cells, however, meets with difficulty in this hypothesis.)

(B) *Effect of extraction with dry heptane*

The *m* level of extracted chloroplasts (dry) remained essentially the same as it is in the unextracted sample (see bottom right curves, Fig. 3), but the variable fluorescence was drastically reduced. The ratio of *M* to *m* was 1.36 in contrast to 2.22 for the unextracted sample. In several other experiments, extraction led to still lower values (1.1 and 1.2) of *M/m*. Addition of 10 μ M CMU did not affect the *m* level; the *m* \rightarrow *M* rise, that was slightly slowed down in the extracted sample, was also unaffected, but the *M* was decreased (24 %, 4 sec after the flow was stopped). Addition of an excess of $Na_2S_2O_4$ led to an increase of both *m* and *M* by about 25 % (or more) suggesting that Q_1 was still present but could not be reduced in light. The complete variable fluorescence was not restored because dry heptane extraction keeps Q_2 in the oxidized form just as the CMU does. Reduction in the variable fluorescence has been reported when chloroplasts were heated or treated with ultraviolet light^{15, 19}. These results are consistent with the hypothesis that, in addition to other effects, ZH (the primary donor of electrons for System II) is destroyed with heat and ultraviolet light and extracted (or destroyed) with heptane. YAMASHITA AND BUTLER²⁶ have shown that a block, created on the ZH side by suspending chloroplasts in 0.8 M Tris, can be bypassed by adding reduced phenylenediamine which serves as an electron donor. When 0.1 mM phenylenediamine was added to the extracted chloroplasts (dry), the small "left-over" variable fluorescence was completely quenched without affecting the *m* level. We raised the question: is it that the electron transfer is faster with phenylenediamine than without? We tested this by adding 50 μ M CMU to the system — the reduction of the variable fluorescence was still present. Thus, the effect of 0.1 mM reduced phenylenediamine, in our system, is not due to an increased electron

transfer. The reaction center complex may have been hampered by 0.1 mM phenylenediamine in our system.

(C) *Effect of extraction with heptane in the presence of a trace of water*

Lyophilized chloroplasts extracted with heptane (after the material was wetted with a trace of water) showed a very high constant fluorescence (m) and a small variable fluorescence. The m level in such preparations was equal to or higher than M level of the unextracted samples (right top curves, Fig. 3). Table I shows the variability observed in several experiments: the variable fluorescence was reduced by

TABLE I

VARIATIONS IN THE EFFECT OF EXTRACTION IN THE PRESENCE OF WATER ON THE m AND M LEVELS

Expt. No.	Conditions	m	M	Δm	$\Delta m/m$
1	Unextracted	8	22	14	1.8
	Extracted	18	25	7	0.4
2	Unextracted	15	31	16	1.1
	Extracted	31	32	1	0.3
3	Unextracted	18	35	17	0.95
	Extracted	42	51	9	0.2
4	Unextracted	10	22	12	1.2
	Extracted	21	24	3	0.14

50–95% upon extraction and the $\Delta m/m$, that ranged from 0.95 to 1.8 in the unextracted samples, were 0.13 to 0.4 in the extracted samples. In all experiments (except Expt. 3, and that plotted in Fig. 3) the increase in m upon extraction was approximately equal to the decrease in Δm . The variable fluorescence consisted of the fast component, the slow component being almost absent.

We suggest that the high m level and the decreased Δm , is due to a partial extraction (or destruction) of Q_2 ; the Q_1 reaction is almost intact. The level m in some experiments is higher than M of the unextracted sample because the latter is not a true P, since A is still present and does not allow a complete reduction of Q to QH. An alternative (or supplementary) explanation is to suggest that a change in the structure of the chloroplasts is partly responsible for the observed increase in m (in particular in Expt. 3, Table I, and Fig. 3); this is a likely interpretation because the ratios of the emission spectra for the Δm to m level showed slight fluorescence due to Chl *b* and Chl *a* 670.

We tested if the (wet) extract had more PQ-A (if it is Q_2) than the (dry) extract by assaying this plastoquinone using silica-gel chromatography²⁷. The amount of PQ-A was, however, found to be identical in the extracts obtained by the wet and dry methods of extraction. This may imply that Q_2 is not identical with PQ-A. (However, since Q_2 may be in small quantities, no definite conclusion can yet be made.)

Effect of CMU. The m level and the fast phase of $m \rightarrow M$ rise was not affected by 10 μ M CMU, but the M level was slightly reduced (38%, 4 sec after the flow was stopped at $I = 1.0$, and 26% when $I = 0.5$). For this effect, we may suggest that the "left-over" Q_2 was now kept in the oxidized form as in the unextracted case.

Effect of Fe(CN)₆³⁻. MURATA *et al.*¹⁵ and MALKIN AND KOK⁵ have shown that Fe(CN)₆³⁻ decreases the slow variable fluorescence in chloroplasts because it acts as an electron acceptor and QH is not allowed to accumulate. We tested the effect of Fe(CN)₆³⁻ on our extracted samples; the variable, but not the constant, fluorescence decreased by 50%.

Effect of reduced phenylenediamine. The addition of 60 μM reduced phenylenediamine (in dark) — like CMU — reduced the slow variable fluorescence (Table II); this may be interpreted to mean that phenylenediamine affects the reaction center just like CMU. Further addition of CMU or Fe(CN)₆³⁻ does not cause any change. At a higher concentration of reduced phenylenediamine (0.1 mM), however, the variable fluorescence was totally absent.

TABLE II
EFFECT OF REDUCED PHENYLENEDIAMINE

Expt. No.	Conditions	<i>m</i>	<i>M</i>	Δm	$\Delta m/m$
1	Extracted (in the presence of water)	29	44	15	0.52
	+ 60 μM reduced phenylenediamine (after 11 min)	31	39	8	0.26
	+ K ₃ Fe(CN) ₆	30	39	9	0.30
2	Extracted (in the presence of water)	27	35	8	0.3
	+ 0.1 mM reduced phenylenediamine	32	32	0	0
	+ 10 μM CMU	32	32	0	0

Attempts to reconstitute the system. The heptane extract was readded to the sample, and the fluorescence induction reexamined. We did not succeed in obtaining the original induction (there was only a slight decrease in *m*, and the $\Delta m/m$ ratio slightly increased from 0.3 to 0.5). Perhaps, the heptane extraction caused other changes in the photosynthetic system that could not be restored, or else the extracted intermediates could not get to the right place during the reconstitution experiment.

The failure to achieve extraction of Q₁, the ability to “extract” Q₂ and only a small effect of CMU on the variable fluorescence in the extracted samples raises the question as to whether a portion (or all) of “Q” is really a substance. An alternative concept might be that a portion (or all) of Q represents a state of matrix surrounding some special chlorophyll molecules (Chl a_{II}) where a local trap would be available for an electron photoejected from the excited molecules. Such a trap localized in the vicinity of the special chlorophyll molecules would be functionally equivalent to the hypothetical substance Q. This situation seems to be well documented for dyes embedded in solid matrix at low temperatures²⁸.

(2) Emission spectra

Fig. 4 shows the emission spectra for the constant (*m*) and variable (Δm) fluorescence of the unextracted maize chloroplasts; the two curves are arbitrarily adjusted at 660 nm and have been corrected for the transmission efficiency of the monochromator and the spectral sensitivity of the photomultiplier. Both have a peak at 682.5 nm, but the emission spectrum of the variable fluorescence is different

from that of the constant fluorescence: variable fluorescence is enriched in F685 (System II) relative to fluorescence at 700 nm.

Fig. 5 shows the emission spectra of variable and constant fluorescence in lyophilized chloroplasts extracted with heptane in the presence of water. Here, the 697-nm band in addition to the 682.5-nm band is seen very clearly in the variable fluorescence. Similar results have recently been obtained by GOVINDJEE AND LAVOREL

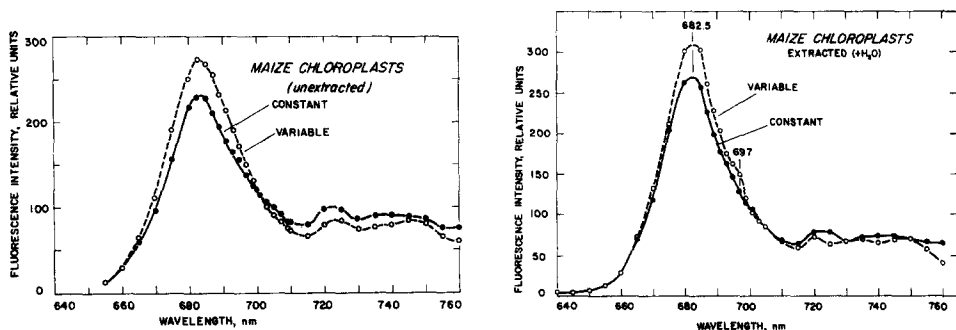


Fig. 4. Emission spectra of constant (m) and variable ($\Delta m = M - m$) fluorescence of unextracted lyophilized chloroplasts suspended in buffer. The curves were arbitrarily adjusted to give the same value at 660 nm. See legends of Figs. 1-3.

Fig. 5. Emission spectra of constant (m) and variable ($\Delta m = M - m$) fluorescence of lyophilized chloroplasts extracted in the presence of water (wet). See legends of Figs. 1-4.

(unpublished) in *Chlorella*. GOVINDJEE and co-workers^{29,30} observed a band at 693 nm in the emission spectrum of *Porphyridium cruentum* when excited with light of high intensity; they speculated that the 693-nm band was due to the trap of System II. Perhaps the 691-697-nm bands are from similar complexes in chloroplasts from maize. (For the identification of the absorption bands with the emission bands, *in vivo*, see ref. 31.)

A comparison of the emission spectra of m extracted (dry) with that of the unextracted sample showed that the changes were negligible.

A comparison of the emission spectra of Δm unextracted with that of the Δm extracted (wet) showed a slightly larger proportion of 691-697-nm fluorescence in the extracted samples; it is to be noted that the Δm extracted (wet) is mainly the fast component ($O \rightarrow I$), but Δm unextracted includes the slow rise as well. The differences in the Δm 's is a new thing; it means that the emission spectrum in the $O \rightarrow I$ phase may be different from that in the $I \rightarrow P$ phase.

(3) O_2 measurements

The unextracted samples showed the presence of a prominent O_2 spike when excited with 647-nm light (Fig. 6). The addition of 710-nm light led to an O_2 uptake and the addition of 647-nm light to 710-nm light led to increased O_2 evolution (Emerson effect). This confirms that the entire electron transport chain was intact in the lyophilized samples. These samples also showed the microjet of O_2 with far-red light flashes of 10^{-5} sec given every 15 sec. (The negative oxygen spike was also seen.) The extracted samples (both kinds) exhibited an extremely reduced O_2 spike showing that some electron carrier(s) was missing; no microjet could be detected suggesting

that the complex at the reaction center ZH Chl a_{II} Q was affected. Examination of Chl a_{II} absorbance changes showed³² that in the (dry) heptane-extracted chloroplasts these changes were absent, but they were distinctly present in the (wet) heptane-extracted chloroplasts and in the unextracted ones. It appears that the loss of ZH (but not that of Q_2) destroys the Chl a_{II} changes; perhaps ZH and Q_1 are very tightly complexed with the active Chl a_{II} molecules.

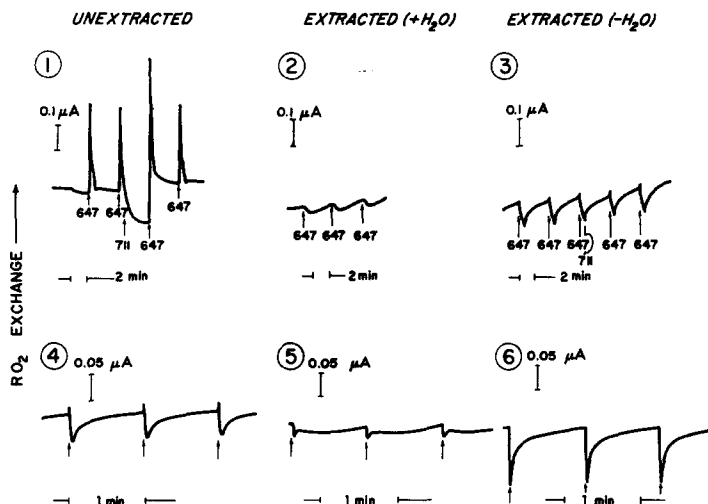


Fig. 6. Rate of O_2 exchange (measured by a platinum rate electrode) in lyophilized chloroplasts suspended in buffer. Curves 1-3, 10 sec 647-nm light, and 50 sec dark (jet of O_2 due to the pool of A); Curves 4-6, 10- μ sec far-red light flashes given every 15 sec (microjet of O_2 due to the pool of E, and negative O_2 spike). Left curves (1 and 4), unextracted chloroplasts; middle curves (2 and 5), extracted in the presence of water (wet); right curves (3 and 6), extracted in the absence of water (dry). 1 mg Chl per ml suspension; 20°; air-saturated system.

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