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ON PLASTID STATES

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1. Production of plastid states

Plastid development inside the growing leaf passes through a number of more or less definite transient states towards the final, granal chloroplast. The granal chloroplast may be defined as this steady-state that the plastid system generally reaches when any wild strain of higher plant (with wild plastids) is grown in its normal ecological environment. We may qualify it for this reason as the "normal state of the plastid".

When the plant is grown in darkness from the seed, another steady-state of the plastid system is reached,- as a result of a developmental process-, but this is no more a normal steady-state. We call it an etioplast. The etioplast becomes unstable as soon as visible light is given to it. In this case, an evolution to the granal chloroplast is seen, the transient states being not necessarily those found along the normal development. Our interest in the etioplast rests on the fact that it is a steady-state which is clearly distinct from the chloroplast and which we are able to prepare inside the leaf. We use here (and below) the expression "to prepare a plastid state inside the leaf" in a pure physical meaning, without refering to any extraction from the leaf. It means that we treat the plant in such a way that the plastids are all forced into a same state.

When we grow the plant in darkness, we simply let the light be zero in the visible range; we change but one among the wide variety of environmental parameters. The light may however be changed in many ways. Some of these have been tested, and proved to act on plastid development in a specific manner. For instance, giving repeatedly 1 msec white flashes every 15 min. from the time of sowing*, - the flash regime -, brings the plastid system inside the leaf into a steady-state characterized by primary thylakoids : we prepare primary thylakoid chloroplasts[1,2]. Giving near infrared continuously leads to another kind of primary thylakoid chloroplasts [3,4]. Giving white light for 2 min. every 100 min. leads to an agranal steady-state where some neighbour thylakoids are bound in pair[5,6].

* from a "Multiblitz Report Porba" electronic flash; electric energy 125 J; colour temperature 5800°K; discharge tube filled with xenon.

All these states are distinct from the granal chloroplast. They are easily prepared, and they allow observation, comparison and experimentation. If after the preparation inside the leaf light is reestablished as it is in the normal environment, an evolution takes place. Controlling light only, we are thus able to create specific, empirical tools for preparing within the leaf distinct steady-states of the plastid system. Comparisons between states of a single object, the plastid, are allowed without using genetic methods.

2. Static description of plastid states

Comparison between states may be static or dynamic. In the first case states are described as they have been prepared. We summarise in this section some informations on primary thylakoid chloroplasts prepared under the flash regime, or bound in pair primary thylakoid chloroplasts prepared by giving 2 min. white light every 100 min.

In primary thylakoid chloroplats, thylakoids are disposed parallel to each other (5 to 10 per plastid), the repeat distance being 170 Å. They generally follow the long axis of the plastid, and they may reach 3 to 5 μ long. A matter, which appears to consist of stroma, is inserted between neighbour thylakoids[1,2]. In some rare places this matter is replaced by some more electron dense material, and the repeat distance falls below 170 Å. Two, or sometimes three to four neighbour thylakoids are involved in these places. Grana stacks are not seen.

In bound in pair primary thylakoid chloroplasts an electron dense material fills, as a rule, the space between pairs of adjacent thylakoids. Each pair is separated from the other by stroma material[5,6]. There is again no grana stack.

Chlorophyll b is deficient in both primary and bound in pair primary thylakoids. The ratio $\left(\frac{\text{quantity of chlorophyll a}}{\text{quantity of chlorophyll b}}\right)$ is found higher than 5 and sometimes higher than 10 in these thylakoids [2,6]. In bean leaves the distribution of the carotenoid content is not the same in primary thylakoid chloroplasts as in normal chloroplasts. Specific for primary thylakoids is the high level of violaxanthine[7].

Triton X-100 or digitonin digestion of primary or bound in pair primary thylakoids, followed by a suitable separation procedure, yields two fractions : the "light" fraction I and the"heavy" fraction II [6,8]. These fractions differ spectrally from the corresponding fractions with PSI and PSII activities prepared from normal chloroplasts essentially by the absence of chlorophyll b in fraction II from primary thylakoids. On the other hand, the amount of fraction II in primary thylakoids is always low and often this fraction is not coloured at all[6,21]. Fractions I and II have been separated from primary and bound in pair primary thylakoids using sucrose gradient centrifugation, gel electrophoresis, and chromatography on hydroxylapatite column with the same result.

TABLE I

Plastid state.	Conditions for preparation inside the leaf.	Chlorophyll b content.	Membrane organisation.	Oxygen evolution on turning intense continuous light on.	First structural changes in intense continuous light.	References.
Etioplast	darkness	0	prolamellar body	0	disaggregation of prolamellar body	___
p.t.c.*	flash regime (1 msec. flash every 15 min.)	low	primary thylakoids	0	pair formation	1, 2
sensitized p.t.c.	flash regime followed by 6 min. weak continuous white light	low	?	+	pair formation	26
far red light p.t.c.	continuous far red light	low	primary thylakoids	+	pair formation	3, 4
induced p.t.c.	flash regime followed by 6-10 min. intense continuous light	low	bound in pair primary thylakoids	+	evolution to grana	18
bound in pair p.t.c.	2 min. light every 100 min.[5,6], or 2 min. light every 120 min.[21]	low	bound in pair primary thylakoids	+	evolution to grana	5, 6, 21
chloroplast	normal light continuously	normal	grana stacks	+	___	___

* primary thylakoid chloroplast.

Membrane protein analysis using SDS gel electrophoresis has shown that polypeptide 9 (Hofer's et al.[8,9]; 23.000 d; = polypeptide 8 in Remy's nomenclature[10,11]) is scarcely, - or even not -, present in primary thylakoids[8-11]. In normal chloroplasts this polypeptide is found mainly associated to the heavy fraction II. Fraction II separated from primary thylakoids is very poor in polypeptide 9 (as fraction I from these thylakoids, or fraction I from normal thylakoids). The low polypeptide 9 content of the primary thylakoid fraction II seems to correlate with the low chlorophyll b content of this fraction. Tobacco mutants poor in chlorophyll b and grana were shown to be also deficient in polypeptide 9[11], as well as unstacked membranes from barley and pea mutants, or grana-less bundle sheet cell chloroplasts of maize[12].

Primary thylakoid chloroplasts isolated from bean leaves contain very small amounts of cytochrome b-559_{HP}. C 550 is present, as well as cytochrome f, cytochrome b-563 and cytochrome b-559_{LP} (1.3 moles of cytochrome b-563 and 2.0 moles of cytochrome b-559_{LP} per mole of cytochrome f)[13].

Leaves containing primary thylakoid chloroplasts do not show any variable fluorescence emission[14,15]. The delayed fluorescence of these leaves (= delayed light emission) is also found constant for at least 30 sec., when measured with delays of 0.4 to 0.8 msec., using a single disc phosphoroscope with a frequency of 100 cycles.sec.$^{-1}$, - the ratio (duration of the illumination period/duration of the dark period) being 9/1 [16].

Isolated primary thylakoids show P_{700} photooxidation and PMS catalysed cyclic photophosphorylation, but no non-cyclic electron transport[17]. Until now it has not been possible to perform Hill reaction with isolated primary thylakoids (no oxygen evolution coupled to DCPIP or ferricyanide photoreduction)[9]. The leaves with primary thylakoid chloroplasts do not emit oxygen in the light[18]. Isolated primary thylakoid chloroplasts photoreduce however DCPIP with DPC, NH_2OH or Mn^{2+} as electron donors[11,19]. In contrast, bound in pair primary thylakoids chloroplasts perform Hill reaction when isolated in a suitable way[20,21].

Chloroplasts prepared using far red light (cycles of 12 hr far red, 12 hr darkness; far red from an incandescent lamp; light cut below 720 nm; 2 x 10^4 ergs.cm^{-2}.sec.$^{-1}$) have independant, primary thylakoids as described in the first paragraph of this section. They differ from primary thylakoid chloroplasts prepared under a flash regime by the fact that they are able to emit oxygen immediately when transfered in white light (1.75 x 10^5 ergs. cm^{-2}.sec.$^{-1}$)[3]. A systematic comparison between available states is expected in the future (Table 1).

3. Plastid states dynamics

A dynamics may be observed starting from any prepared state if one suddenly

transfers the plant from the preparation light to another, suitable light. The word "suitable" means that not all new light conditions will be able to provoke an evolution from the prepared state (= off this state).

Two classes of problems are involved here :

a. Does the system evoluate towards some new steady-state ? What is the nature of this state ?
b. What are the events in the immediate neighbourhood of the prepared state apart from which the system moves off ?

3.1 Having prepared leaves with primary thylakoid chloroplasts using a flash regime or bound in pair primary thylakoid chloroplasts, it is seen that when one transfers to normal continuous light the system evoluates through transient states towards the normal chloroplast[22,23]. Three to four hours after transfering, the structure is that of chloroplasts with grana stacks. At that time the relative amount of chlorophyll b is nearly normal (a/b = about 3.0) while chlorophyll (a + b) still accumulates[24]; thylakoid fraction II is found coloured[6]. The leaves emit normally oxygen in the light; the variable fluorescence has appeared[15,18], etc.. The carotenoid distribution is found as in normal chloroplasts in analysis made 12 hrs after switching the continuous light on (the violaxanthine content has fallen down)[7].

3.2 More elaborate is the observation of events in the near vicinity of the state from which the system moves off. Is is clear that we may require to registrate events as near this starting state as possible. Interesting points have been made in this direction, starting from primary thylakoid chloroplasts prepared under a flash regime.

3.2.1. Structural changes

Structural changes have been reported to occur in primary thylakoid chloroplasts within the leaf 4 min. after transfering from the flash regime to a continuous illumination (white light; 10^4 to 10^5 ergs.cm^{-2}.sec.$^{-1}$ at the level of the leaf)[22]. At that time the space filled with stroma material between adjacent thylakoids appears to be reduced and adjacent membranes stick in many places, the thickness of the membrane pairs being two times that of one membrane. 6 min. after the transfer, sticking is generalized while the thickness of the pairs falls often below that of two membranes. Thus, pairs of bound primary thylakoids are formed from independant, primary thylakoids within a time of the order of some minutes. Grana appear later, in the hour range (more than 30 min. after switching continuous light on).

Formation of thylakoid pairs was also observed when etiolated leaves grown under

far red light, having chloroplasts with independant primary thylakoids were transfered in continuous white light (1.1×10^4 ergs.cm^{-2}.sec^{-1}). After 3 hr of white light, fused pairs of primary thylakoids were obtained[3]. Here again, grana appear later.

3.2.2. Functions

Hill reaction becomes measurable (DCPIP photoreduction) in plastids isolated from leaves which are transfered for some minutes from the flash regime to continuous light[11]. Within the first 5 min. of illumination the Hill reaction increases almost linearly with the duration of the illumination. The maximum rate may be reached after 10 min., depending on the intensity of the continuous light. Such a photoinduction (or photoactivation) of Hill reaction occurs also when the transfer is from the original, low frequency flash regime (a 1 msec. flash every 15 min.) to a flash regime with a higher frequency (a 1 msec. flash every 5 sec.)[11].

The induction is also seen within the minute range using a leaf disc (8 mm diameter) directly in contact with a Yellow Spring Clark-type oxygen electrode[18]. Strasser[25] has devised a special equipment for registrating simultaneously several parameters during the induction process (oxygen evolution, light induced absorption changes, fluorescence kinetics, and changes in the delayed light emission). A feeble oxygen evolution is ordinarily recorded 1 min. after transfering from the flash regime to continuous light; the compensation point is reached 1 to 2 min. later. Maximum rate of oxygen production by the leaf is obtained 6 to 8 min. after switching the continuous light on.

Qualitatively the same induction process is observed if the leaf receives white, or monochromatic blue or red continuous light (10^4 to 10^5 ergs.cm^{-2}.sec.$^{-1}$ at the level of the leaf). It occurs also if the light is given as chopped light with a frequency of 150 cycles sec.$^{-1}$. It is accompanied by the appearance of a variable part of the leaf fluorescence emission[14,26]. This variable part is already noticeable after 30 to 60 sec. continuous illumination[15]; it becomes clearly evident after some minutes[26]. During the induction process, the level of the 0.4 msec. delayed fluorescence decreases markedly[16].

3.2.3. Content changes

Leaves prepared with primary thylakoid chloroplasts,using a flash regime, and induced thereafter to produce oxygen by some minutes of continuous light, contain cytochrome b-559$_{HP}$ in amounts comparable to the very small amounts of the non-induced leaves[13].

A difference absorption spectrum (primary thylakoid chloroplasts containing leaf - this leaf after induction) in the blue range is interpretable as being due to a change in some carotenoid-protein complex[27].

A particulate, olive-green to yellow fraction is detectable in the thylakoids

after induction[28]. This fraction is not seen in non-induced, primary thylakoids, but it is seen in normal green ones. It appears to be linked to the occurence of the capacity to evolve oxygen. It is a complex fraction (a particular arrangement of proteins ?) which is easily removed from the membranes. It is detected using an electrofocusing technique after partial digestion of the thylakoids by low amounts of triton X-100 mixed to the acrylamide gel.

The polypeptide pattern of non-induced primary thylakoids is similar to the pattern of the same thylakoids after induction[8]; in particular, there is no formation of polypeptide 9 during the minutes of continuous illumination which induce the capacity to evolve oxygen in leaves.

3.3 Taken alltogether the dynamical features bring the following informations :

a. Primary thylakoid chloroplasts prepared under the flash regime may evoluate under suitable light conditions to a state which differs from the normal chloroplast, but which emits photosynthetic oxygen; we call this state a "photoinduced primary thylakoid chloroplast". Under continuous light, it is a transient from the primary thylakoid to the normal chloroplast.

b. Grana formation, as already known, is a late process in the dynamics of normal chloroplast development. This process is separable from that of induction of the capacity to evolve oxygen, in that sense that, using wild strains of higher plants, it is possible to prepare a plastid which does not contain grana, but does evolve oxygen in the light.

A better knowledge of the dynamics would elicit the suspected relationships between grana formation and the content increase of some substances (chlorophyll b ?; polypeptide 9 ?;... see also ref. 29). The same is true for the suspected relationships between induction of the capacity to evolve oxygen and the appearance of the loosely bound, particulate fraction to which we have refered above; etc...

c.The time needed for inducing the capacity to evolve oxygen in primary thylakoid chloroplasts prepared under a flash regime is rather short (some minutes), compared for instance to that needed for grana formation. This does not give much informations in itself. But, refering to the ability for electron transfer from donors like DCP, NH_2OH and Mn^{2+} to DCPIP in illuminated primary thylakoid chloroplasts, and to the C-550 content of these chloroplasts, the short duration of the induction could reflect the following properties : the primary thylakoids prepared under a flash regime should have already the reaction centers of photosystem II, and the block of the oxygen evolving mechanism should be located somewhere along the way from water to photosystem II reaction centers.

d. Formation of pairs of adjacent primary thylakoids is not related in a simple manner with the development of the ability to evolve oxygen, since leaves grown

under continuous far red light, that do not have these pairs, emit oxygen immediately when transfered in intense, continuous white light for the first time. Under far red light the ability to evolve oxygen develops in the absence of any measurable actual oxygen evolution[3]; the chloroplasts are perhaps prepared in some "photosensitized" state for oxygen evolution in the sense of the section 4. Thylakoid sticking(pair formation) should be related to actual oxygen emission as it occurs during the induction process in normal white light, rather than to the development of some readiness for this emission (without actual emission; see section 4).

4. Perturbation of plastid states.

When transfering a leaf from the flash regime to continuous light, the primary thylakoid chloroplasts undergo a perturbation large enough as to make the state evoluate (= it is a suitable perturbation in the sense of section 3). The perturbation is still large enough if the transfer is from the original, low frequency flash regime (a 1 msec. flash every 15 min.)to a higher frequency flash regime (1 msec. flash every 5 sec.)[11]. But would a 1 msec. flash every 10, or 5 min. make the primary thylakoid evoluate ? Does it exist a limit between flash regimes which do not allow primary thylakoid chloroplasts to evoluate and those which do ? And if so, where is the limit ?

Experiments have shown that when the intensity of the continuous illumination is lowered much below 10^4 to 10^5 ergs.cm^{-2}.sec.$^{-1}$, an evolution takes place in the leaf off from the primary thylakoid state. Even a feeble (100 ergs.cm^{-2}.sec.$^{-1}$) green (550 nm), continuous light perturbs the original state enough as to make it evoluate[26]. Within 6-10 min. under this feeble light a state is reached which differs from the photoinduced primary thylakoid chloroplast state. This new state is ready to emit oxygen immediately at switching a high intensity white light on, but its preparation does not involve actual measurable oxygen emission. At switching intense white light on, an oxygen outburst is observed. We call this state a "photosensitized primary thylakoid chloroplast". It may be asked for the significance of the oxygen outburst from photosensitized primary thylakoids. Part of the answer to this question is possibly hidden in the fact that a "continuous" series of photosensitized states has been obtained in varying the amount of weak light during a constant 6 min. sensitization of primary thylakoid chloroplasts[30]. Empirical correlations have been found between this amount and the kinetics of oxygen evolution (including outburst), or of fluorescence emission, registrated when switching a high intensity white light on at the end of the sensitization period.

It must be stressed that it was never succeeded until now to provoke an evolution of primary thylakoids isolated from the leaf. Evolution proceeds inside the

leaf, not outside. The exchanges between plastid and surroundings are required.

In practice perturbing some plastid state means varying the environment of the plant (or of the leaf), and varying this environment appears as a tool for studying the plastid system. It is a way : a) for experimenting plastid state stability against perturbation, and b) for producing new states.

5. Mixing states

Since distinct chloroplast states may be prepared, it may be asked about what happens when thylakoids, prepared at distinct states, are isolated from the leaves and mixed together. Strasser[31] performed the following experiment :

a) Normal thylakoids were isolated from normal green leaves (= thylakoids a). The Hill reaction was inhibited by Tris-washing.

b) Primary thylakoid chloroplasts were prepared by submitting leaves to a flash regime and primary thylakoids (b)were isolated. They did not have any Hill activity.

c) Mixing thylakoids (a) and (b) restored Hill activity, the (a) and (b) thylakoids and their mixture (c) being all kept in a same medium.

What recombination occurs is at present a mystery. Although much care must be taken when dealing with such essays, they nevertheless suggest how to conceive the search for precise sites of biological activity in a biological object (the plastid) using empirical states of this object. There is no contradiction involved. Plastid states are defined by more or less arbitrarily choosen properties, as any biological species is. The environmental conditions for their preparation may however be made as precise as we wish, and this makes them perfectly reproducible.

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