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ANALYSIS OF ABSORPTION CHANGES IN THE ULTRAVIOLET RELATED TO CHARGE-ACCUMULATING ELECTRON CARRIERS IN PHOTOSYSTEM II OF CHLOROPLASTS

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SUMMARY

Spinach chloroplasts were dark adapted and then submitted to a sequence of short saturating flashes. The resulting absorption changes in the near ultraviolet were analyzed and attributed to the donor and acceptor sides of Photosystem II. Our results provide a spectroscopic support to some current models of these parts of the photosynthetic electron transport.

In Tris-treated chloroplasts (supplied with artificial donors) the absorption changes are largely due to the acceptor side. After each flash the signal decays with a fast phase ($t_{\frac{1}{2}} = 1.2$ ms at 9 °C) leaving a stationary level (on a 100-ms time scale). The fast phase has a small amplitude after odd-numbered flashes, whereas the stationary level behaves in a complementary fashion. The non-decaying signal is attributed mostly to the reduced secondary acceptor (A_2^-) and the fast phase to the simultaneous reoxidation of A_2^- and of the reduced primary acceptor (A_1^-). The effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and of redox mediators (ascorbate, ferricyanide) also support this assignment. A fraction of A_2 is shown to be reduced in dark-adapted chloroplasts, as proposed by Velthuys and Amesz (Biochim. Biophys. Acta (1974) 333, 85–94). The difference spectra support the view that A_1^- and A_2^- are plastoquinone radical anions. There are also some absorption changes that we cannot identify.

In untreated chloroplasts a non-decaying absorption change ("slow phase") occurs with a 4-flash periodicity. It is attributed to the transitions among the S states associated with the O_2-evolving complex. A fast phase ($t_{\frac{1}{2}} = 1.2$ ms) in the decay following the first two flashes behaves like in Tris-treated chloroplasts, so that the assignment is tentatively the same. After the third flash, however, the magnitude of this fast phase is too large according to the hypothesis, so that there may be some

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Abbreviations DCMU, 3-(3 4-dichlorophenyl)1,1-dimethylurea; PDA, *p*-phenylenediamine; ΔA, absorption change; PQ, plastoquinone.

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contribution from the donor side. The fast phases become slower at lower pH (5.5 instead of 7.6), although there is no evidence for a protonation A_1^- or A_2^-.

INTRODUCTION

A large part of the recent research on Photosystem II in chloroplasts has been devoted to two steps which involve charge accumulation, either on the donor or on the acceptor side of the reaction center. On the donor side, the storage of four positive equivalents has been inferred from the measurement of oxygen evolution in a sequence of short saturating flashes and also from various indirect measurements in the same conditions (see refs. 1 and 2 for a review. More specific recent observations are reported in refs. 3 and 4). The functioning of a carrier or of a complex of carriers, characterized by the so-called S state, capable of storing four positive equivalents, can be considered as well established, although its chemical identity is totally unknown. The purpose of this study is to progress towards a chemical identification using flash-induced absorption changes in the ultraviolet.

On the acceptor side, the existence of a two-electron carrier has been inferred on the basis of a periodicity of two during sequences of short flashes, in the number of electrons arriving at the reaction center of Photosystem I [5, 6] and in the light emission properties at the level of Photosystem II [7, 8]. The available evidence for a two-electron carrier is, however, not totally compelling. Electrons must be transferred from the primary electron acceptor (A_1 in Fig. 1), which is a one-electron carrier [9] and is probably a specialized plastoquinone molecule [10–13], to a pool of plastoquinone molecules [14, 15]. For that transfer two models have emerged. Stiehl and Witt [10] proposed a model ("parallel") in which Photosystem II reaction centers are

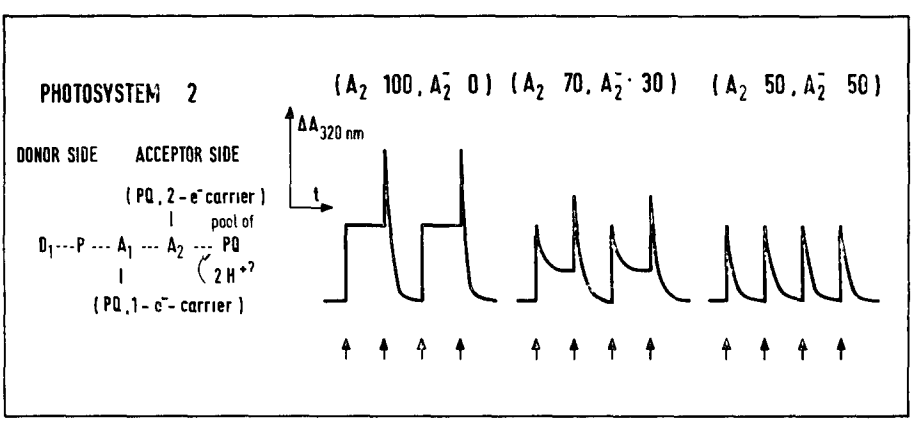

Fig. 1. A scheme of electron transfer reactions on the acceptor side of Photosystem II (P, primary donor; D_1, first secondary donor, A_1, A_2, primary and secondary acceptor) The traces represent the idealized time course of absorption changes at 320 nm due to A_1^- and A_2^-, which are supposed to be plastoquinone radical anions, in a suspension of dark-adapted chloroplasts excited by four flashes. We also suppose that a fraction of A_2 is reduced in dark-adapted chloroplasts (0, 30 or 50 %).

coupled pairwise, so that two A_1^- dismutate and yield one fully reduced plastoquinone, after uptake of two protons. In an alternative model ("series") the reaction centers are not coupled at the level of A_1, and the secondary acceptor (A_2 in Fig. 1; named B or R in refs. 5 and 7) must store two electrons before it can fully reduce a plastoquinone molecule in the pool. In this work we shall try to bring further insight on the acceptors, on the basis of the behaviour of flash-induced absorption changes during a series of flashes given to dark-adapted chloroplasts.

Absorption changes attributed to the primary acceptor of Photosystem II have been reported by Stiehl and Witt [10, 14]. They studied a species, named X-320 for an absorption maximum around 320 nm, whose difference spectrum is similar to that of the radical anion of plastoquinone (PQ^-) in vitro [16]. The species absorbing at 320 nm has a half-time of 0.6 ms (at 20 °C), as the reduced state of the primary acceptor determined by other techniques [17, 18]. At physiological temperatures, it has however been observed only by a repetitive flash technique [10, 14, 19] that probes a stationary level of the electron carriers, whereas our approach allows a better test for the two-electron carrier hypothesis. The pattern of expected absorption changes at 320 nm (due to PQ^-) at the onset of a sequence of short saturating flashes is depicted in Fig. 1, assuming that both A_1 and A_2 are plastoquinone molecules. We also assume that a fraction (0, 30 or 50 %) of the A_2 molecules is semi-reduced (A_2^-) in dark-adapted chloroplasts, as proposed by Velthuys and Amesz [7]. As an example, in the case where 30 % of A_2 is reduced after dark adaptation, the first flash will fully reduce A_1; 60 % of the absorption change will decay rapidly because of the formation of a fully reduced species, and 40 % will not decay, corresponding to the increment of A_2^- population.

Our study concerns untreated chloroplasts in which absorption changes linked to Photosystem II activity are due to both the donor and acceptor sides, and also chloroplasts treated with Tris, in which the acceptor side is normal but the donor side is inhibited, although electron transfer can be restored with artificial electron donors (refs. 20, 21 and references therein). An approach similar to ours has been used very recently by two other groups Pulles et al. [22] studied charge accumulation in Photosystem II and obtained results similar to ours except that no kinetic information was accessible by their technique. Verméglio [23] obtained very accurate information on the secondary acceptor (a specialized molecule of ubiquinone) in reaction centers from photosynthetic bacteria and its functioning as a two-electron carrier.

MATERIALS AND METHODS

Spinach leaves were ground for 10 s in 0.4 M sucrose, 0.01 M NaCl, 0.02 M Tris (pH 7.8). The juice was filtered on a nylon mesh (with 35 μm openings) and the chloroplasts pelleted by centrifugation. The pellets ("untreated chloroplasts") were kept on ice. Tris-treated chloroplasts were obtained as described by Velthuys and Amesz [7], and the resulting pellets were kept on ice. At the start of each experiment, a pellet was resuspended in 250 ml of buffer (0.35 M sucrose, 10 mM KCl, 2 mM $MgCl_2$, 50 mM Tricine, pH 7.6, unless special mention).

For the measurement of absorption changes, the suspension was kept in a reservoir and, by means of a magnetic valve, was allowed to enter the cuvette (optical path for the measuring beam: 1 cm; width: 0.4 cm; height: 3.5 cm) about 1 s before

triggering the flashes. The chloroplasts in the cuvette were excited by a train of 2–11 saturating pulses from two synchronized xenon flashes (Stroboslave, General Radio) whose light was filtered with a Schott GG 475/3 mm filter and a Balzers Calflex filter, and guided to the cuvette by two lucite light pipes. The measuring light, originating from a 800 W tungsten-iodine lamp, passed through a M-25 (Jobin-Yvon) monochromator (slits 1 mm, $\Delta\lambda = 6$ nm) and a Schott UG11/1 mm filter. The light emerging from the cuvette was measured with a photomultiplier (EMI, type 9656 QR) protected by a combination of filters (Schott UG11/2 mm, Corning CS 7-54, solution of $NiSO_4$ and $CoSO_4$ in water). The photomultiplier output was amplified with a differential amplifier whose reference input was a constant voltage (200 mV) and whose bandwidth was adjusted to the particular experiment (0.3–10 kHz). The amplified signal was recorded with a Didac (Intertechnique) multichannel analyzer. Other technical details are given in refs. 24 and 25.

The suspension obtained from one pellet served for 50 or 100 measurements whose results were added (total duration: about 15 min). The suspension was recycled, but on the average each chloroplast was flashed not more than once and its dark-adaptation time was over 3 min. The suspension was kept at 9 °C.

RESULTS AND DISCUSSION

Absorption changes in Tris-treated chloroplasts

The time course of the absorption changes at 320 nm, for two preparations of Tris-treated chloroplasts excited by trains of two or four saturating flashes, is reported in Fig. 2. The donor side was rendered functional by the addition of ascorbate and *p*-phenylenediamine. Each flash induces an absorption increase which, to a first approxi-

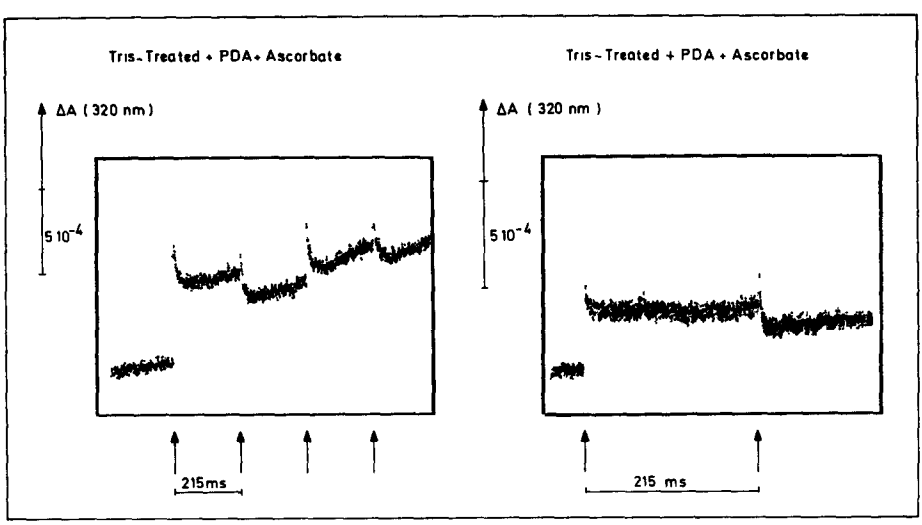

Fig. 2. Absorption changes induced at 320 nm in suspensions of Tris-treated chloroplasts supplied with 1 mM sodium ascorbate and 50 μM *p*-phenylenediamine (from a 50 mM solution in ethanol) Average of 100 measurements. The arrows indicate the times at which flashes were given Chlorophyll concentration· 2 8 10^{-5} M (left) and 1 8 · 10^{-5} M (right). Electrical bandwidth. 3 kHz

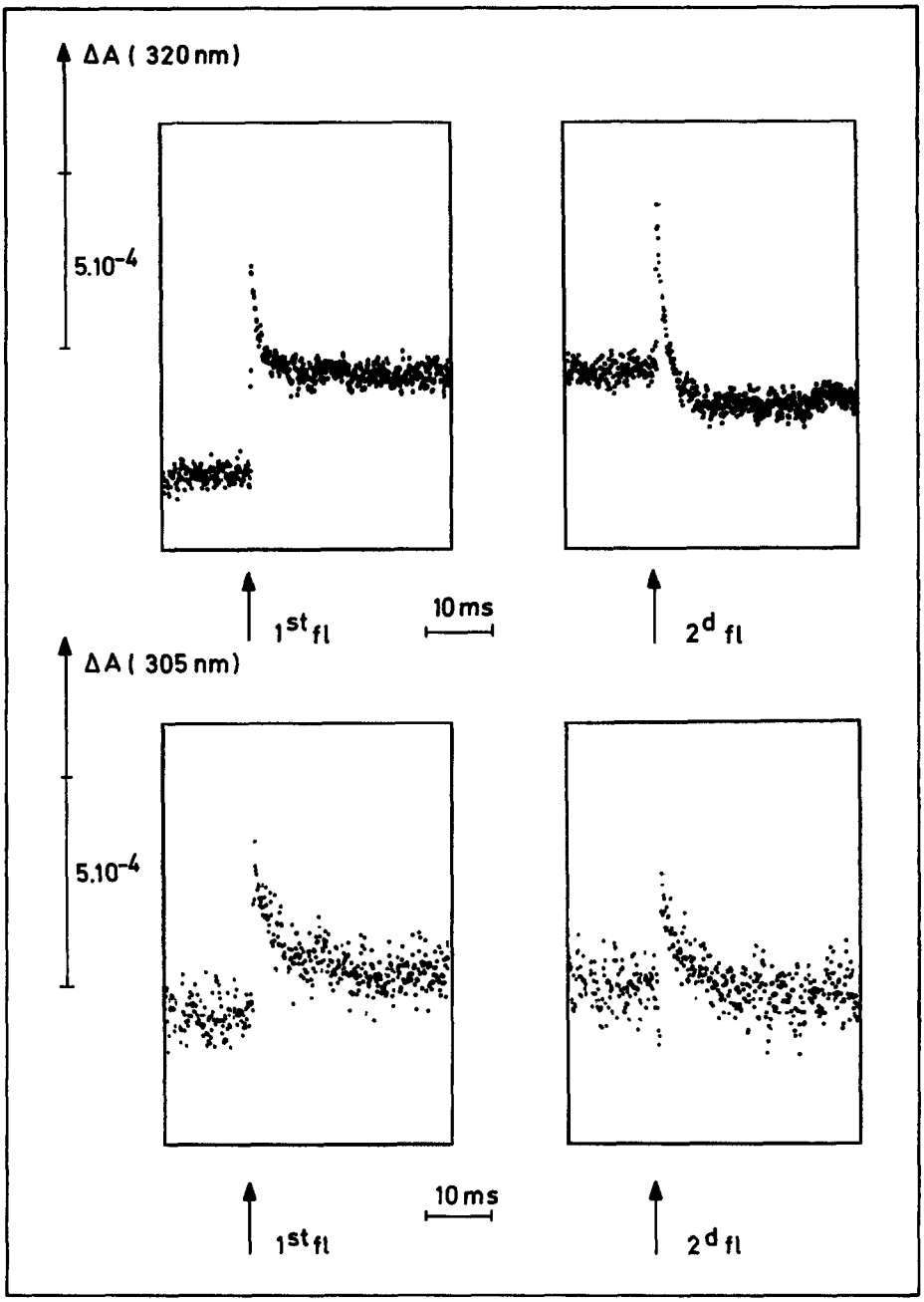

Fig 3. Absorption changes induced in suspensions of Tris-treated chloroplasts. Upper traces: enlargements of the right-hand trace of Fig. 2. Lower traces: absorption changes at 305 nm; average of 200 measurements; same addition, chlorophyll concentration $1.6 \cdot 10^{-5}$ M; experiment performed with another batch of chloroplasts. Electrical bandwidth 3 kHz.

mation, decays with a fast phase leaving a stable absorption change. By simple inspection, it appears that the magnitude of the fast phase is relatively large after the 2nd and the 4th flash, and that the stable level behaves in a complementary fashion. This behaviour is in agreement with that predicted by the model of Fig. 1 (with $A_2^- \approx 30\,\%$). The effect of the first two flashes is presented on an expanded time scale in Fig. 3. The half-time for the decay of the fast phase, averaged over several tens of experiments, is not distinguishable for different flashes in a series: $t_{\frac{1}{2}} = 1.2$ ms. There is some indication, however, of the occurrence of a minor phase of intermediate duration (about 6 ms half-time) whose contribution is more important around 300 nm (Fig. 3, bottom). The features reported in Figs. 2 and 3 were observed repeatedly. A precise measurement of stable ΔA for times longer than 20 ms was difficult because of fluctuations of the baseline, which are particularly visible in Fig. 2, left. After the first flash some variations were observed in the relative magnitude of the fast phase and of the stable ΔA; the latter fluctuated between 70 and 30 % of the total ΔA.

The difference spectra of the flash-induced ΔA have been measured for the 1st and the 2nd flash. In Fig. 4 we show the ΔA remaining 18 ms after the flash (ΔA slow) and the ΔA decaying between $t = 0$ and $t = 18$ ms (ΔA fast). To account for the phase of intermediate duration, ΔA fast has been decomposed further into a very fast phase (ΔA very fast, decaying between $t = 0$ and $t = 3.5$ ms) and a fraction which decays between 3.5 and 18 ms (ΔA (fast–very fast)). The shape of the difference spectrum of ΔA very fast is nearly the same for the 1st and the 2nd flash. It presents a maximum around 325 nm and is very similar to X-320 observed by Stiehl and Witt [14] as well as to the primary acceptor of Photosystem II observed under various conditions [11–13, 25] The difference spectra will be discussed below.

The characteristic properties of the fast phase (half-time; small amplitude after

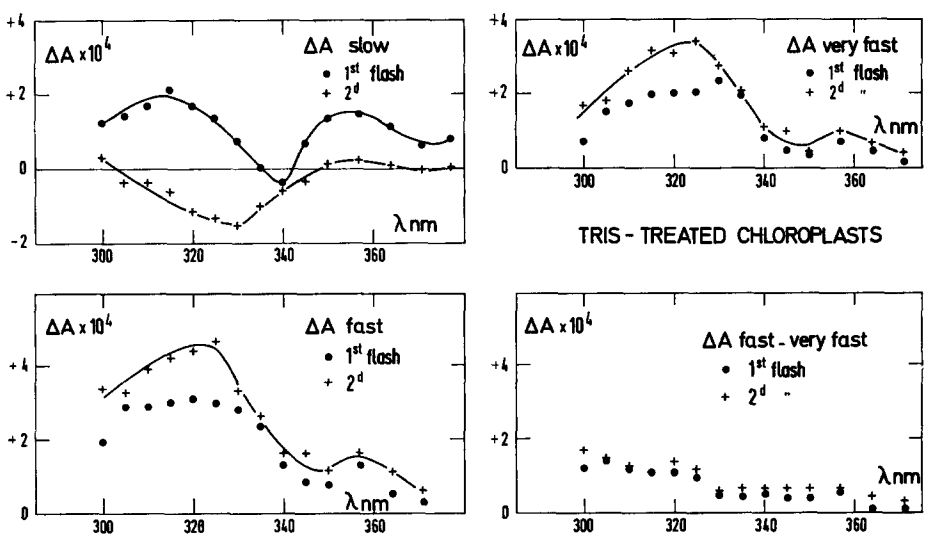

Fig. 4. Spectra of absorption changes induced in suspensions of Tris-treated chloroplasts (chlorophyll concentration: $1\,65 \cdot 10^{-5}$ M) by the 1st and by the 2nd flash. Same conditions as for Fig. 2. ΔA slow refers to the level attained 18 ms after the flash compared to the level just before the flash. For ΔA fast, ΔA very fast and ΔA (fast–very fast), see text.

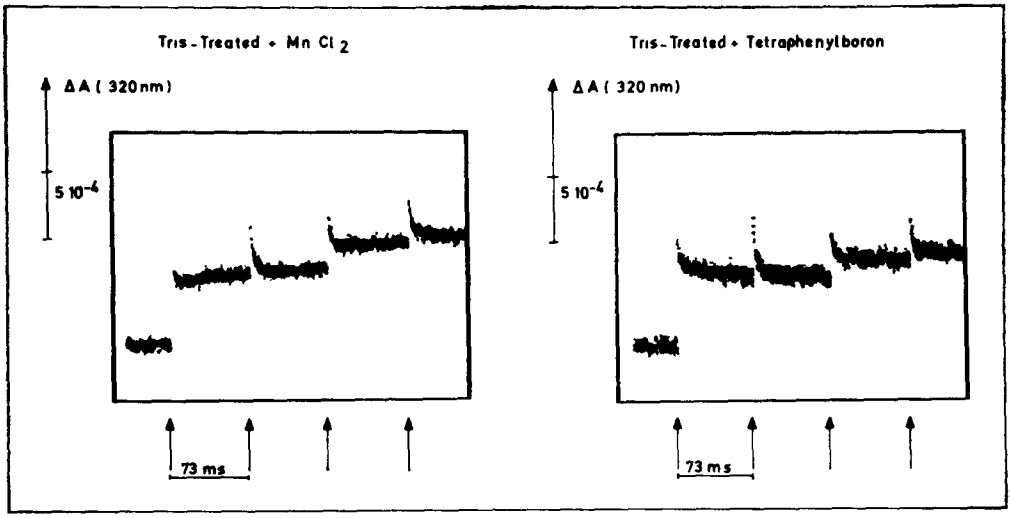

Fig. 5. Absorption changes induced at 320 nm in suspensions of Tris-treated chloroplasts supplied with 2 mM $MnCl_2$ (average of 50 measurements, chlorophyll concentration 1 8 10^{-5} M) or with 40 μM tetraphenylboron (from a 50 mM solution in ethanol) (average of 50 measurements, chlorophyll concentration: 2 2 · 10^{-5} M). Electrical bandwidth 3 kHz

the 1st and the 3rd flash, larger amplitude after the 2nd and the 4th flash) are unaffected when PDA plus ascorbate are replaced by other efficient donors to Photosystem II, like $MnCl_2$ or tetraphenylboron [21, 26, 27] (Fig. 5). For the slow phase there is also some indication of a period-2 behaviour, but probably super-imposed on a positive ΔA on each flash (Fig. 5).

In the presence of DCMU, which greatly retards the reoxidation of the primary acceptor of Photosystem II [28], the second flash (also subsequent flashes) produces no absorption change (Fig 6). The couple PDA plus ascorbate is good donor to Photosystem I [27], so that the absence of a response on the second flash in the presence of DCMU leads us to conclude that the absorption changes that we detect arise mainly in Photosystem II. We made a few measurements of ΔA at 515 nm under the conditions of Fig. 6 (+PDA+ascorbate+DCMU), but exciting with red light. A rather large ΔA was observed on the second flash, indicating that Photosystem I was functional. As the difference spectrum of the pigment absorption shifts due to the transmembrane electric field is the same for Photosystem I and Photosystem II [29], we conclude that these electrochromic shifts do not contribute significantly in the spectral range under study (300–350 nm). In the presence of DCMU the fast phase is totally absent after the first flash. The phase of intermediate duration is still present, and the slow phase is nearly as large as in the control (Fig. 6).

Taken together, our results are best interpreted according to the scheme of Fig. 1, accepting that the fastest phase in the decay of ΔA is due to the simultaneous disappearance of A_1^- and A_2^-. The spectrum of these species (Fig. 4, ΔA very fast) corresponds closely to that of the radical anion of plastoquinone [16]. At 325 nm we estimate a $\Delta\varepsilon$ of 9000 (average of the ΔA due to the 1st and the 2nd flash; we assumed 400 chlorophyll molecules per reaction center). This value may be somewhat under-

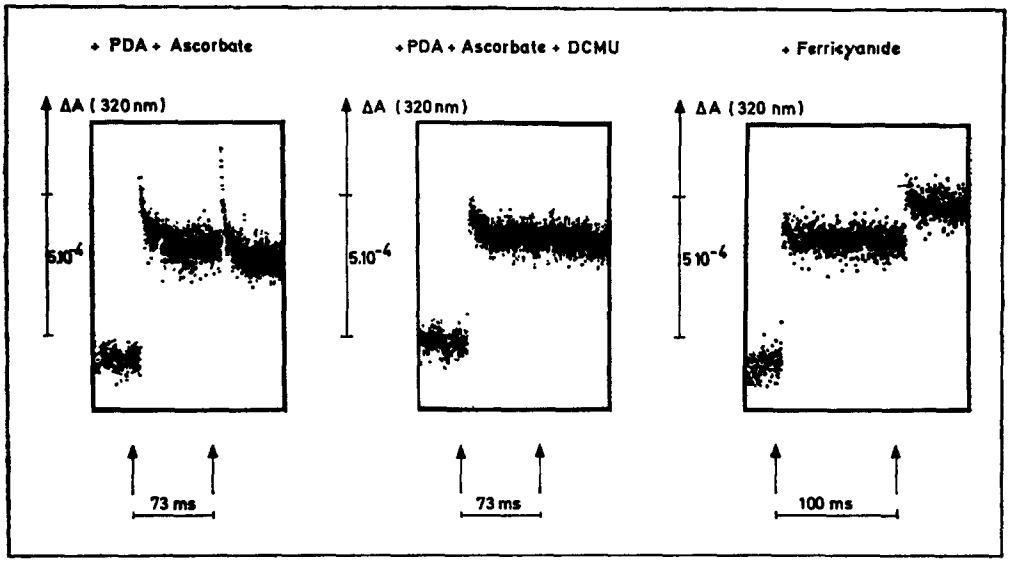

Fig. 6. Absorption changes induced at 320 nm in suspensions of Tris-treated chloroplasts supplied with 50 μM phenylenediamine plus 1 mM ascorbate (chlorophyll concentration· 1.9 10^{-5} M) or with the same chemicals plus 5 μM DCMU (from a 5 mM solution in ethanol) (chlorophyll concentration: $1.7 \cdot 10^{-5}$ M) or with 0.1 mM $K_3Fe(CN)_6$ (chlorophyll concentration $1.6 \cdot 10^{-5}$ M). Average of 50 measurements in each case Electrical bandwidth 3 kHz.

estimated (less than 20 %) because of an insufficient electronic bandpass in the results of Fig. 4. A distorsion of the spectra by sieve effect may also have to be considered (see ref. 30). Stable absorption changes (ΔA slow) are probably difficult to interpret unambiguously. There may be some contribution of the oxidation products of the artificial donors, as suggested by the difference in the pattern of ΔA slow observed with PDA plus ascorbate, $MnCl_2$ or tetraphenylboron (Figs. 2 and 5). The negative ΔA slow observed with PDA plus ascorbate after the 2nd and the 4th flash is, however, in good agreement with the model of Fig. 1. It should correspond to the disappearance of A_2^- remaining after the 1st or the 3rd flash. The spectrum of ΔA slow after the 2nd flash (Fig. 4) is indeed similar to that of ΔA very fast.

Our interpretation requires that part of A_2 is reduced in dark-adapted chloroplasts, as originally proposed by Velthuys and Amesz [7], because a very fast phase is observed after the first flash. This partial reduction is understandable, because we add electron donors to Photosystem II but no Photosystem I acceptor. In support of that interpretation we did not observe any fast phase after the first flash in the presence of ferricyanide, which may cause a complete oxidation of A_2 in dark. In that case the second flash produces a small signal, probably because there is no donor available, and we expect a fast back reaction ($t_{\frac{1}{2}} \approx 150$ μs; it would not be detected in the experiments of Fig. 6) between P^+ and A_1^- (see refs. 24 and 25).

At the present time we cannot interpret the phase of intermediate duration (about 6 ms, see Fig. 2), whose difference spectrum (approximated by ΔA fast–very fast) is rather featureless, but clearly different from other ΔA values. It is not suppressed (after the first flash) in the presence of DCMU, but it is not observed in the presence

of ferricyanide, probably because it is retarded. We cannot exclude the possibility that this change is a scattering change rather than a true absorption change.

Absorption changes in untreated chloroplasts

Flash excitation of dark-adapted untreated chloroplasts leads to absorption changes which can be described similarly to those obtained with Tris-treated chloroplasts. The magnitude of the stable ΔA at 320 nm is presented in Fig. 7 (a fast phase is not time resolved in that experiment). In a long series of flashes, it presents a period-4 behaviour. The stable level of absorption is high after the 1st and the 2nd flash, as well as after the 5th and the 6th flash; it is low after the 3rd and the 4th flash, as after the 7th and the 8th. The damping appears to be rather fast. It is even faster without gramicidin, which has been added for this particular experiment. A period-4 behaviour is totally absent in Tris-treated chloroplasts. In untreated chloroplasts, it is also absent in case of addition of tetraphenylboron (Fig. 8), which is a powerful donor to Photosystem II [26]. In the presence of DCMU, only the first flash induces an absorption change (Fig. 8). The difference spectra of these slowly reversible ΔA values, measured 18 ms after the 1st, the 2nd or the 3rd flash, are presented in Fig. 9 (ΔA slow). In view of the preceding properties and of the stability of the S_1 state in chloroplasts [2], we propose that ΔA slow represents mostly the absorption changes due to the transition from the S_1 to the S_2 state (1st flash), S_2 to S_3 (2nd flash) and S_3 to S_0 (3rd flash). As for our interpretations with Tris-treated chloroplasts, this is only a first-order interpretation since other species may contribute to the absorption changes, as for instance A_2^- or cytochrome *f*. In some cases also the negative ΔA after the 3rd flash (see e.g. Fig. 8, bottom right) was absent although a period-4 behaviour was always observable

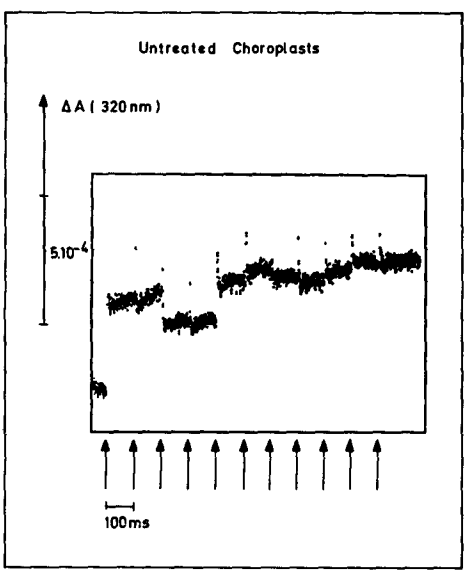

Fig. 7 Absorption changes induced at 320 nm by a sequence of 11 flashes given to untreated chloroplasts. Addition of 10^{-7} M gramicidin D. Average of 200 measurements. Chlorophyll concentration: 1.6 10^{-5} M. Electrical bandwidth. 0.3 kHz.

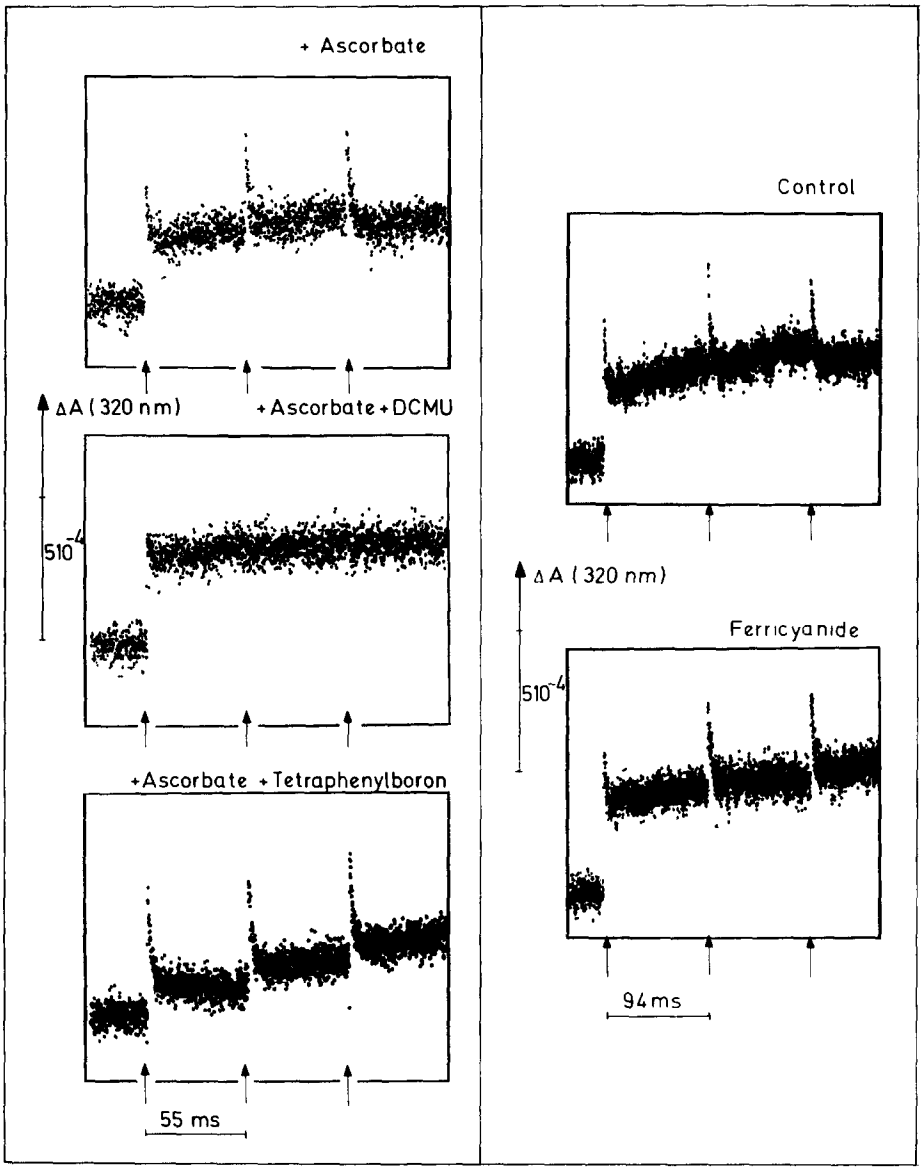

Fig 8. Absorption changes induced at 320 nm by a sequence of three flashes given to suspensions of untreated chloroplasts (chlorophyll concentration $1\,0 \cdot 10^{-5}$ M) Left traces. average of 100 measurements performed upon addition of 0.4 mM ascorbate (top) and also of 5 μM DCMU (middle) or 20 μM tetraphenylboron (bottom) Right traces average of 50 measurements performed with chloroplasts which were osmotically broken (1 min in a 10-fold diluted buffer) and then suspended in the usual buffer with eventual addition of 60 μM ferricyanide Electrical bandwidth 10 kHz.

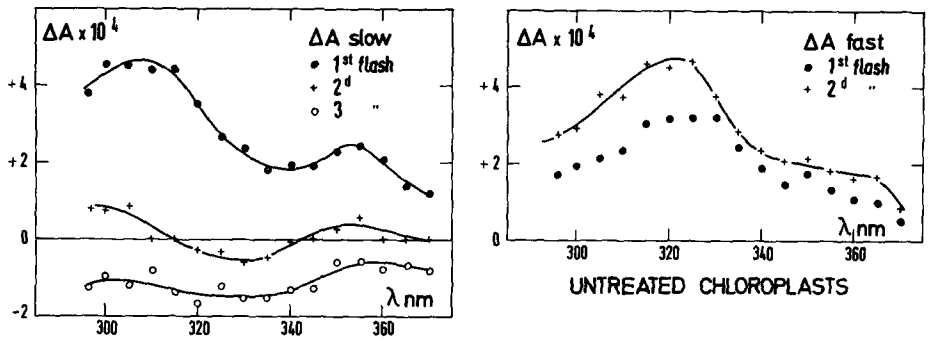

Fig. 9 Spectra of absorption changes induced in suspensions of untreated chloroplasts (chlorophyll concentration 1 65 10^{-5} M) by the 1st, the 2nd and the 3rd flash, spaced by 64 ms. ΔA slow has the same signification as in Fig. 4. ΔA fast is the absorbance variation between just after and 18 ms after the flash.

for the positive ΔA following the 1st, the 5th or the 9th flash.

The decay of ΔA presents a fast phase (Figs. 8 and 10) which is suppressed by DCMU (Fig. 8). Its half-time ($t_{\frac{1}{2}} = 1.2 \pm 0.1$ ms) is independent of the flash number (Fig. 10, right). Its magnitude is nearly the same after the 2nd and the 3rd flash; it is smaller after the 1st flash. The difference spectrum of the fast phase (ΔA decaying between $t = 0$ and 18 ms) is reported in Fig. 9 (ΔA fast). As for ΔA very fast in Tris-treated chloroplasts, this spectrum resembles spectra attributed to the primary acceptor of Photosystem II [11–14] and to the radical anion of plastoquinone [16]. A half-time of 0 6 ms has been reported by Stiehl and Witt [10] for the decay of X-320 at 21 °C. This would fit with our measurement of 1.2 ms at 9 °C, provided that the reaction considered has an activation energy of 9.5 kcal/mol.

In the "series" hypothesis schematized in Fig. 1, the occurrence of a fast phase in the decay after the first flash can be due to some reduction of A_2 in dark-adapted chloroplasts, as for Tris-treated chloroplasts. It can also be due to a dismutation of plastoquinone radical anions formed at two neighbouring reaction centers ("parallel" hypothesis). In the parallel hypothesis the relative magnitude of the fast phase would be largely reduced at non-saturating flash excitation. We found that a 8-fold reduction of the intensity of the excitation flashes, leading to a decrease of the signal on the first flash by 2.6, also decreases (by a factor included between 1.2 and 1.6) the ratio of the amplitudes of the fast and of the stable phases (Fig. 10). The effect is however hardly significant; it is much smaller than the effect predicted by the "parallel" hypothesis [10] and our results argue against this hypothesis. The A_1-A_2 hypothesis of Fig. 1 is strongly supported by our results with Tris-treated chloroplasts. It also receives some support from the results with untreated chloroplasts. Indeed, as predicted by the model, the fast phase is smaller after the 1st than after the 2nd flash (Fig. 10). The difference is increased upon addition of ferricyanide (as predicted by the model since A_2 should be largely oxidized by ferricyanide) (Fig. 8, right), whereas it is decreased by the reductant ascorbate (Fig. 8, left). We found, however, that the fast phases were of nearly equal amplitude after the 2nd and the 3rd flashes. This observation can be reconciled with the two-electron carrier hypothesis only by making additional assumptions, as for instance the contribution of fast decaying ΔA due to the donor side

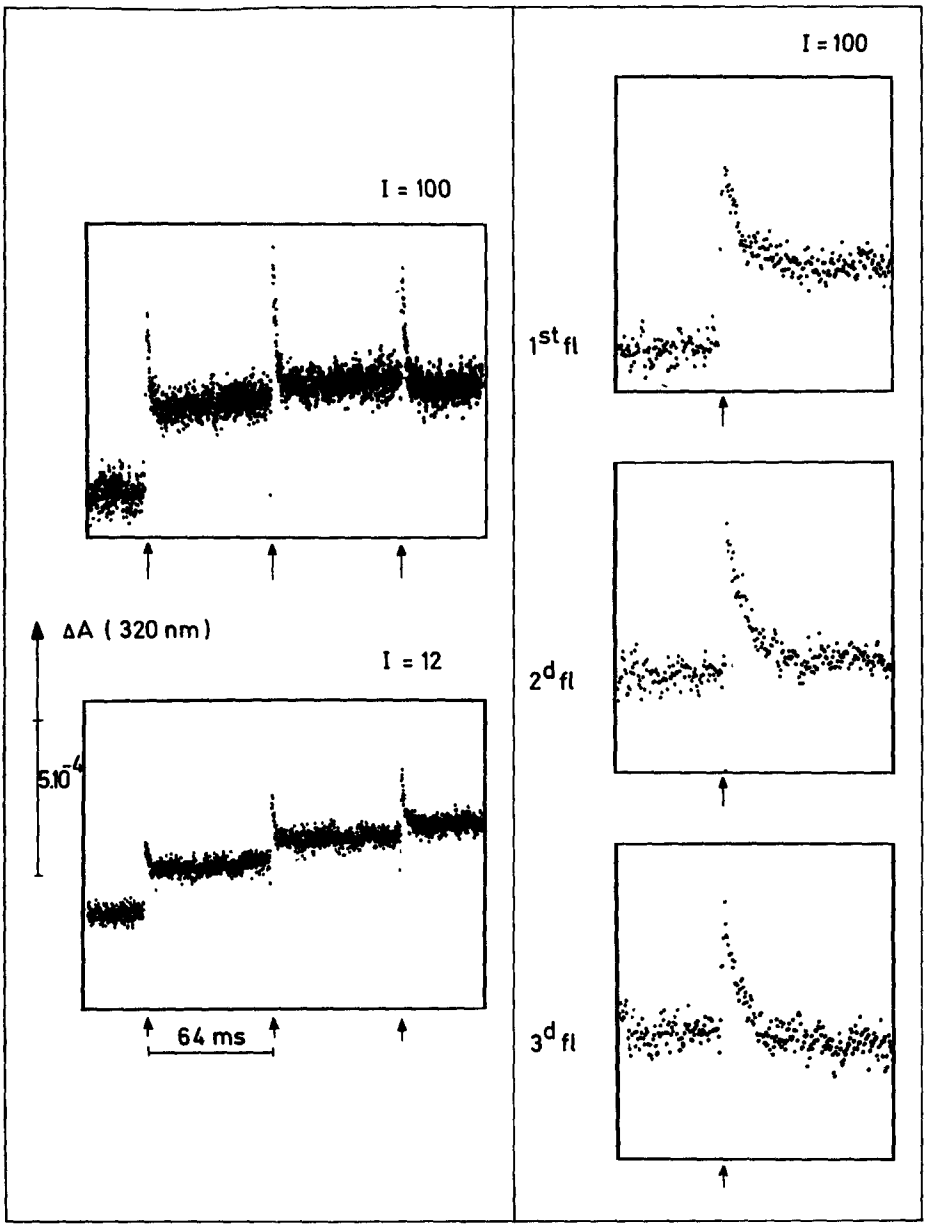

Fig. 10. Absorption changes at 320 nm induced in suspensions of untreated chloroplasts (no addition; chlorophyll concentration: $1.5 \cdot 10^{-5}$ M) excited with flashes at maximum intensity ($I = 100$, average of 50 measurements) or with attenuated flashes ($I = 12$; average of 200 measurements). Right traces: same experiment as with $I = 100$, displayed on a five times enlarged time scale Electrical bandwidth. 10 kHz.

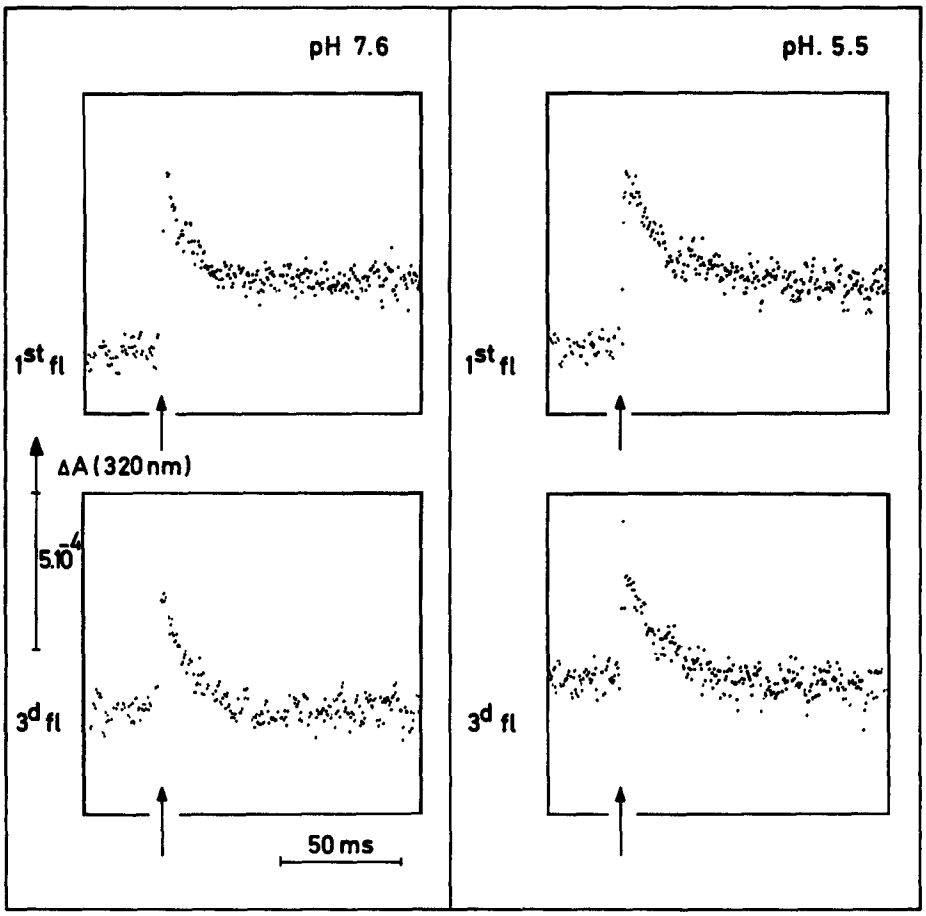

Fig. 11. Absorption changes at 320 nm induced in suspensions of untreated chloroplasts (no addition, chlorophyll concentration: $1.3 \cdot 10^{-5}$ M, average of 100 measurements) in the regular buffer at pH 7.6 or in a 50 mM 2-(*N*-morpholino)-ethane sulfonic acid buffer at pH 5.5 The flashes were spaced by 64 ms. We show the effect of the 1st and 3rd flash, on an enlarged time scale Electrical bandwidth: 10 kHz

of Photosystem II superimposed to the ΔA due to A_1^- or A_2^-.

Diner and Joliot [18] have recently shown that the primary acceptor of Photosystem II is reoxidized more slowly at lower pH. We made a similar observation (Fig. 11) showing that the fast phase of decay at 320 nm is slower at pH 5.5 ($t_{\frac{1}{2}} = 2.3$ ms) than at pH 7.6 ($t_{\frac{1}{2}} = 1.2$ ms), for any flash in a series of three. These decay times are in good agreement with those reported by Diner and Joliot, and also by Haehnel [19], considering the different sample temperature. Since Diner and Joliot probed the reoxidation of A_1^- and since, in our interpretation, the fast phase is due to the simultaneous decay of two plastoquinone radical anions, the kinetic agreement indicates that the electron transfer from A_1^- to A_2 is the limiting step in the disappearance of the radical anions. This disappearance leads to the formation of fully reduced plastoquinone (PQH_2) via the pick-up of two protons. Our spectra for A_1^-

and A_2^- correspond to unprotonated plastoquinone radical anions (compare with ref. 16), both at pH 7.6 and at pH 5 5 (at which we ran only limited spectra, not shown). Our results thus argue against the hypothesis, proposed by Diner and Joliot [18], of a protonation of A_1^-. Haehnel [19] came to the same conclusion as us.

CONCLUSIONS

It appears in our experiments that absorption changes in the studied spectral range (300–360 nm) are very complex, so that all our interpretations must be provisional. Wavelengths under 300 nm are not accessible with our equipment, and wavelengths over 360 nm produce even more complex signals. Concerning the donor side of Photosystem II, we think that the possibility of associating absorption spectra with the S states is a promising approach for studying the oxygen-evolving reactions.

As for the acceptor side of Photosystem II we believe that our results, together with previously published reports [5–8, 11–14], strongly support the hypothesis of two specialized plastoquinone molecules functioning in series, without the direct uptake of H^+. However, the fact that the electron transfer from A_1^- to A_2 is pH sensitive is a further argument for a complex mechanism for this reaction. Several authors recently proposed that a specific protein was involved in that reaction [31–33].

We would like to stress the point that the agreement between the kinetics of reoxidation of the primary acceptor [17, 18] and of disappearance of PQ^- (refs. 10, 14, 19 and this study) implies that the rate of electron transfer is nearly the same for $A_1^- \rightarrow A_2$ or for $A_1^- \rightarrow A_2^-$. The second reaction seems to be rather unfavorable So these results may suggest that A_2 is constituted of two plastoquinone molecules working only between their oxidized and their semi-reduced state, or again that the transfer from A_1^- to A_2 (or to A_2^-) has a complex mechanism.

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