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EFFECTS OF SODIUM AZIDE ON PHOTOSYSTEM II OF *CHLORELLA PYRENOIDOSA*

B. MAISON-PETERI and A. -L. ETIENNE

Laboratoire de Photosynthèse, C.N.R.S., 91190 Gif-sur-Yvette (France)

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SUMMARY

The action of sodium azide on the electron transport chain was investigated by means of oxygen evolution, fluorescence and luminescence measurements.

(1) The damping of the oxygen oscillations is progressively reduced with increasing azide concentration in the range 10^{-5}–10^{-1} M.

(2) The rate of the dark decay of the S_2 and S_3 states is considerably slowed. The degree of slowing is dependent on concentration.

(3) Luminescence is inhibited by azide both in the presence and absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

(4) The fluorescence induction curve in strong light is modified in the presence of azide and its shape depends on azide concentration and on incubation time.

(5) At a given time after a saturating flash, the fluorescence yield in the presence of azide is much higher than that of the control. It seems to be due to a general fluorescence increase rather than to a slower Q^- reduction.

(6) We tentatively propose an accelerated reduction of the primary donor P^+, in state S_2 and S_3, by the intermediate donor Z in the presence of azide. Additionally, we have to assume that in the S_2 and S_3 states, some centers are blocked in an inactive low fluorescent form and that azide decreases their concentration.

INTRODUCTION

Despite the progress of photosynthesis research in recent years (for review, see ref. 1), a number of problems concerning the functioning of System II remain to be solved. Among these are the origin of the "misses" reflected in the damping of the oscillations of oxygen during a flash sequence [2], and the pathways of the deactivation reactions [3].

A number of substances are known to be specific modifiers of System II and are useful tools for the elucidation of the mechanisms involved; e.g. DCMU [4], NH_2OH [5], Adry reagents [6] and anaerobiosis [7].

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Azide, best known as an inhibitor of catalase [8] was also shown to interfere with System II [9]. Experiments on the relation between the azide induced rates of photobleaching of the carotenoids and the decrease of oxygen evolution in spinach chloroplasts, by Yamashita et al. [10], led to the conclusion that azide inhibits the reduction of the intermediate Y^+ by water. Similar conclusions were reached by Katoh [11], also using chloroplasts, who showed the inhibition of the Hill reaction by azide to be progressive, irreversible and specially enhanced by light. On the other hand, Bamberger et al. [12] have described an effect of azide on carbon dioxide fixation and carbonic anhydrase activity in intact lettuce chloroplasts, but these effects were not seen in broken chloroplasts.

Hence, azide could be a useful modifier of System II, and as isolated chloroplasts and whole algae often show different responses to treatments, it seemed worth while to further investigate the action of this substance.

MATERIALS AND METHODS

The experiments were performed with either the alga *Chlorella pyrenoidosa*, grown and harvested daily as previously described [13], or with chloroplasts prepared from greenhouse spinach. Chloroplasts were prepared according to the method of Amesz et al. [14], except that the isolation medium contained 0.1 % bovine serum albumin and was at pH 7.0. Chloroplasts were stored in liquid nitrogen in the isolation buffer containing 5 % (v/v) dimethyl sulfoxide.

The algae were kept aerated in dim light for several hours before use. They were then resuspended in 0.05 M phosphate buffer, pH 6.5, KCl 0.1 M at a concentration of 500 μg chlorophyll $\cdot$ ml^{-1} for oxygen measurements, and in phosphate buffer without KCl at 20 μg chlorophyll $\cdot$ ml^{-1} for fluorescence and luminescence measurements. Chloroplasts were diluted to the same concentrations in 0.05 M Tricine, pH 7.0, KCl 0.1 M $MgCl_2$ 2 mM. 1 μM ferredoxin and $2 \cdot 10^{-4}$ M NADP were added as electron acceptors. Chlorophyll concentrations were estimated by measuring the absorbance at 680 nm (opal glass method), calibrated by the extraction method of Arnon [15].

Oxygen evolution during flashing light was measured using the polarographic method of Joliot and Joliot [16]. After differentiation and amplification, the flash yields of oxygen are displayed on an oscilloscope and recorded photographically. Fluorescence and slow luminescence experiments were carried out as previously described [5]. Rapid luminescence measurements were as previously described [17] except for the use of a pulsed dye laser (Spectra Physics model 375) with Rhodamine G as dye. Pulse duration was 2 μs and frequency of pulsing was 300 Hz. The pulses were non-saturating. The measurements of luminescence intensity at 685 nm were averaged over 10 000 runs with a multichannel analyser. The photomultiplier was turned off during the flash by driving the first dynode negative with respect to the cathode. Dark adaptation of algae and chloroplasts before oxygen sequences, fluorescence and luminescence measurements was at least 10 min.

RESULTS

Oxygen evolution in the presence of azide

The oxygen evolved per flash, by algae and chloroplasts, during a sequence of

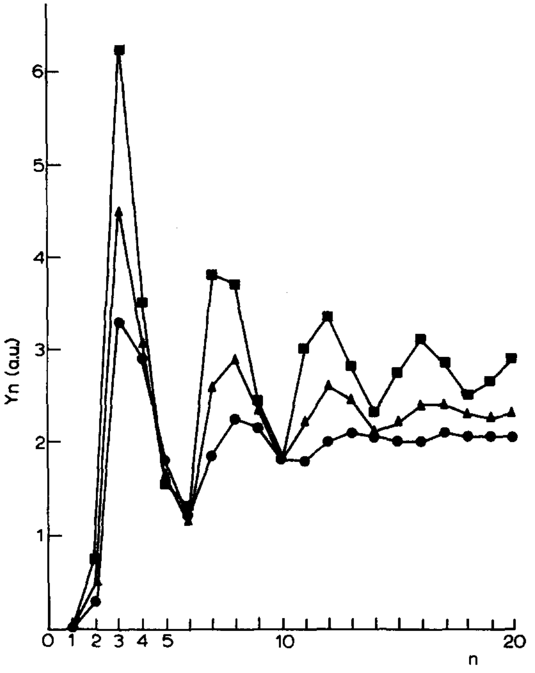

Fig. 1. Oxygen yield for a series of saturating flashes spaced 300 ms apart Y_n represents the flash yield and n the flash number. The azide concentration is as indicated: ●, control; ▲, NaN_3 10^{-3} M; ■, NaN_3 10^{-2} M.

flashes shows characteristic damped oscillations of periodicity four [3]. A number of reagents are able to modify the form of these sequences [18, 19, 4], and we find that sodium azide also has this property.

In whole algae, the presence of azide induces a diminution of the damping of the oscillations as compared with the control (Fig. 1). This decreased damping is dependent on the azide concentration in the range 10^{-5}—10^{-1} M. From Fig. 1, it is also obvious that the mean values of Y_n (oxygen yield on the n'th flash) are greater in the presence of azide than for the control, at least for the first 20 flashes. Depending on the sample, the Y_n increase is more or less pronounced but always present as is the diminution of the damping. We have not undertaken a quantitative correlation of the increase of Y_n versus the decrease in misses. The increase in Y_n is only evident for the first two or three sequences at high azide concentrations since a partially irreversible photoinhibition reduces progressively the Y_n.

The oxygen sequence of chloroplasts was not changed by azide.

Mathematical computations of the oxygen sequences

To evaluate the percentage of misses for a given oxygen sequence, Lavorel [20] has developed a matrix analysis of Kok's model [2]. Table I shows the values for σ_1 (the sum of the misses on the different S states) for various concentrations of azide. There is a decrease in σ_1 with increasing azide concentration, as already suspected from the reduced damping of the oxygen oscillations, with a sharp decrease in going from

TABLE I

VALUES FOR THE SUM OF THE MISSES AND DEACTIVATION HALF TIMES WITH DIFFERENT AZIDE CONCENTRATIONS

NaN_3 (M)	σ_1	$t_{\frac{1}{2}S_3}$ (s)	$t_{\frac{1}{2}S_2}$ (s)
0	0.735	5	30
10^{-5}	–	11	–
10^{-4}	0.685	65	200
10^{-3}	0.660	30	140
10^{-2}	0.400	24	75
10^{-1}	0.325	–	–

10^{-3} M to 10^{-2} M azide. The increase in oxygen yield observed may partly be due to this diminution of the misses.

A general expression for the computed steady state oxygen yield, Y_{ss}, has been derived by Lavorel and Lemasson [21].

$$Y_{ss} = \Sigma S_i/\Sigma\, (1-\alpha_i)^{-1}$$

This equation is a simple function of σ_1 for the two extreme cases: for a single miss $\alpha_i = \sigma_i$, or for a homogeneous distribution of the misses $\alpha_i = \sigma_i/4$.

We computed the ratios $(Y_{ss})N_aN_3/(Y_{ss})$ control for 10^{-2} M and 10^{-3} M azide, for a constant value of ΣS_i. The ratios were higher than those deduced from the above formula, as if there was indeed a change in the concentration of the S states.

The dark decay of the S_2 and S_3 states

It is possible to follow the decay of S_2 and S_3 with dark time by giving 1 or 2 preilluminating flashes and measuring the oxygen evolved on the second or third flash respectively, a time Δt after the last preilluminating flash [3, 22, 23].

We found a considerable slowing of the rate of deactivation of S_2 and S_3 (Table I) at various azide concentrations (10^{-5}–10^{-2} M) in algae. The rate of deactivation is already strongly affected at azide concentrations (10^{-4} M) where the form of the oxygen sequence has hardly changed. At 10^{-3} M azide, the deactivation starts to reaccelerate, although still much slower than for the control. In contrast, the misses continue to decrease from 10^{-5} M to 10^{-1} M azide.

Preliminary results (in collaboration with B. Diner) indicate that the rate of decay of the electric field, measured by the 520 nm absorption change [24] is also slowed down at low azide concentration and reaccelerated when the concentration is increased, in a similar manner to the deactivation. However, the time domain is different, milliseconds rather than seconds.

For spinach chloroplasts the rate of deactivation in the presence of azide remains virtually unchanged compared with the control.

Luminescence decays

Luminescence is considered to be due to the back-reaction $P^+Q^- \rightarrow PQ + h\nu_L$, and in particular, in the microsecond range is indicative of the P^+ concentration [25], since the reduction of P^+ is faster than the reoxidation of Q^-. Bertsch et al. [26]

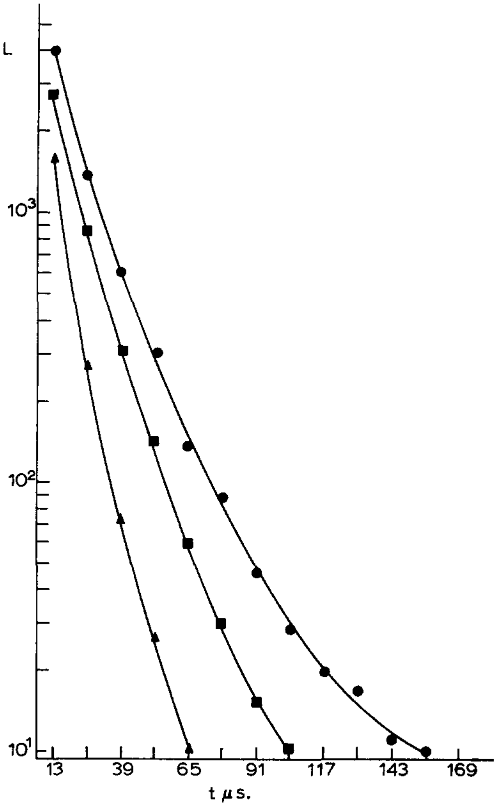

Fig. 2. Fast decay of luminescence intensity (L) as a function of azide concentration. ●, control; ■, NaN_3 10^{-3} M; ▲, NaN_3 10^{-2} M. The points are the average of 10 000 runs; flash duration, 2 μs, frequency, 300 Hz.

have shown in *Chlorella* that the luminescence in the millisecond range is inhibited by azide, but its decay kinetics are not altered.

In the microsecond range, we find an accelerated decay dependent on azide concentration, i.e. a faster decay with 10^{-2} M than with 10^{-3} M azide (Fig. 2). In the second time range, the luminescence intensity during a flash sequence shows oscillations related to the oscillations of the S states [22]. In the presence of 10^{-3} M and 10^{-2} M azide the oscillations remain, but the luminescence intensity is decreased.

In the presence of DCMU, both microsecond and second luminescence intensities are inhibited by 10^{-2} M azide.

Fluorescence induction curves during continuous light

In whole algae, the fluorescence induction curve in strong light shows three distinct phases: a fast rise from the initial fluorescence yield Φ_0 to a plateau Φ_I, followed by a slow rise $\Phi_I \rightarrow \Phi_P$ and a fluorescence decline $\Phi_P \rightarrow \Phi_S$. The fluorescence yield is not only controlled by the oxido-reduction state of Q and A, but also by the electric field [24], the proton gradient [27] and the "state" (i.e. State I-State II) of the algae [28].

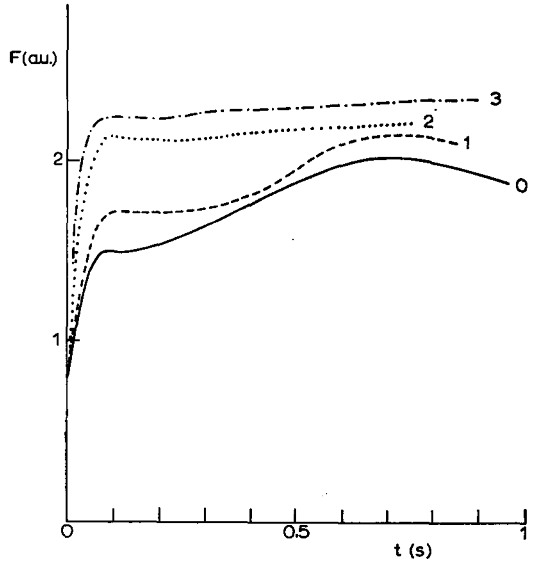

Fig. 3. Fluorescence induction curves as a function of azide concentration. Fluorescence yield in arbitrary units. (0), control; (1), 10^{-4} M NaN_3; (2), 10^{-3} M NaN_3; (3) 10^{-2} M NaN_3.

The effects of azide on the fluorescence induction are found to be complex, they depend on its concentration and in certain experiments on the incubation time. It is difficult to quantify these variations, as such a large number of factors have a role in determining the fluorescence yield, but they may be related to different physiological states of the algae.

Fig. 3 represents the induction curves for various azide concentrations. Azide does not alter the Φ_0 levels, but the Φ_I-Φ_P phases are strongly affected. Φ_I is increased to a level close to Φ_P with increasing azide concentration and the Φ_P-Φ_S decline is suppressed at 10^{-2} M azide. We have occasionally observed a dip between Φ_I andΦ_P. The presence of 10^{-2} M azide does not change the fluorescence induction observed in the presence of $5 \cdot 10^{-5}$ M DCMU.

The slow fluorescence induction also shows modifications dependent on azide concentration which will not be considered in the present publication.

Fluorescence decay after a saturating flash

Since Φ_0 is not modified by azide, Φ/Φ_0 is strictly proportional to the fluorescence yield. The fluorescence yield of the control has declined to a value close to that of the initial level 100 ms after the flash. On the contrary, in the presence of azide, the fluorescence yield is still well above Φ_0 even 1 s after the flash (Fig. 4). This does not necessarily correspond to a slower fluorescence decay, if the maximum fluorescence yield reached during the flash is higher in the presence of azide than in the control. In the presence of DCMU, no modification of the fluorescence decay is observed when 10^{-2} M azide is added.

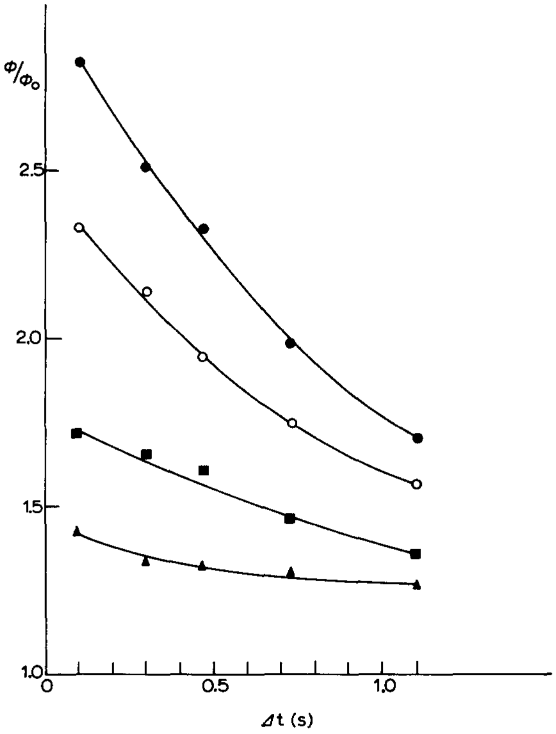

Fig. 4. Dark fluorescence decay after a single flash as a function of azide concentration. The variable fluorescence yield is normalised to Φ_0. ▲, control; ■, 10^{-4} M NaN_3; ○, 10^{-3} M NaN_3; ●, 10^{-2} M NaN_3.

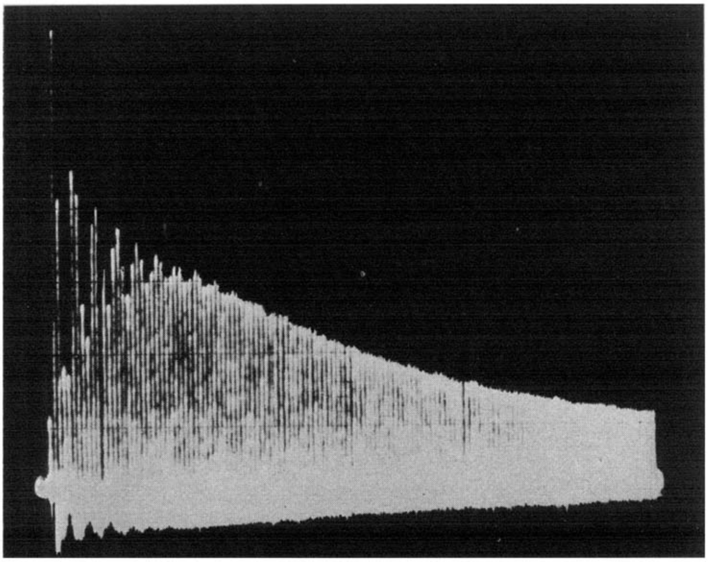

Fig. 5. Oxygen yield for a series of saturating flashes spaced 300 ms apart. Chlorella + 10^{-2} M NaN_3.

Fluorescence oscillations of the low fluorescence yield during a flash sequence

The fluorescence yield measured at least 400 ms after the last flash of a flash sequence shows oscillations correlated to the oscillations of the concentration of (S_2+S_3) [30]. These oscillations are not abolished by various azide concentrations.

Additional effects seen after prolonged illumination

At high azide concentrations ($> 10^{-2}$ M) we observe a gradual photoinhibition of the oxygen yield per flash (Fig. 5). This inhibition manifests itself after 30–40 flashes but never attains 100 %, i.e. zero oxygen yield, even after more than 100 flashes. This reduction of yield is only partially reversible in the dark but completely reversible on washing out the azide.

Possibly related to the above results is the observation that, in the presence of 10^{-2} M azide, after 30 preilluminating flashes, the maximum fluorescence yield attained during a subsequent illumination is lowered as compared with the control.

Lavorel (unpublished results) has observed that after more than 60 flashes the deactivation is accelerated rather than slowed in the presence of azide.

DISCUSSION

Azide, a reagent which is not very specific, is seen to have complex effects on Photosystem II. Fortunately, the results obtained from oxygen measurements provide a good indication that, at least at the beginning of illumination the photosynthetic electron transport chain is still fully functional.

The charge separation produced by a flash can give rise to a luminescence emission if there is a rapid recombination between Q^- and the positive charge. This necessarily leads to misses. Depending on the competition between the stabilisation of the charges and the back reaction, the latter may account to a greater or lesser extent for the misses. Similarly the back reaction may provide a route for the deactivation of the S_2 and S_3 states.

At low azide concentrations, 10^{-5}–10^{-4} M azide, the rates of deactivation of S_2 and S_3 are already slower whereas the misses have only slightly decreased. It is thus unlikely that these phenomena have the same origin.

At higher azide concentrations, the decrease in misses is considerable and the decay of the fast luminescence is accelerated. In the present range, luminescence is indicative of the P^+ concentration since the reduction of P^+ is faster than the reoxidation of Q^-. Thus the decrease in misses and the acceleration of the decay of the fast luminescence component can be explained by an increased rate of P^+ reduction by the normal secondary donor Z.

The reacceleration of the deactivation observed at high azide concentration is probably due to an azide-induced reduction pathway of S_2 and S_3 distinct from the back reaction. The reduction rate is still slow enough to be negligible in the first 300 ms and hence does not increase the misses. The effects seen after prolonged illumination may perhaps result from extensive use of the azide-induced deactivation route (perhaps being due to carotenoid oxidation).

The increase of the oxygen yield at high azide concentrations is larger than can be accounted for by the decrease in misses and the presence of inactive centers in the control must be assumed. These inactive centers do not seem to exist in the S_0 and S_1

states since the fluorescence induction in DCMU treated algae is not modified by azide. Thus the increase in the fluorescence yield observed for the Φ_0 to Φ_I phase or after a saturating flash can be explained if it is assumed that these inactive centers are low fluorescent.

The maximum fluorescence yield cannot be reached at the end of the photochemical rise even in strong light [29] or during a saturating short flash [30]. This was described by Delosme as a non-photochemical quenching R, destroyed after prolonged illumination (when the plastoquinone pool becomes reduced) or by DCMU [29].

At the beginning of a strong illumination or of a flash sequence, the S_2+S_3 concentration reaches a maximum value then decreases to attain its stationary value. We therefore propose that the non-photochemical quenching R corresponds to inactive low fluorescent centers in slow equilibrium with the S_2 and S_3 states and that azide by decreasing their concentration destroys partially the quenching R.

The absence of an azide effect on broken chloroplasts is puzzling. Two explanations seem possible: (i) azide action requires structural integrity of the chloroplast, or (ii) azide has no direct effect on the chloroplast but acts through some modification of another part of the organism.

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