Biochimica et Biophysica Acta, 440 (1976) 176–188

BBA 47103

INHIBITION BY AMMONIUM CHLORIDE OF THE OXYGEN YIELD OF PHOTOSYNTHESIS

M. J. DELRIEU
Laboratoire de Photosynthèse, C.N.R.S., 91190, Gif-sur-Yvette (France)
(Received November 21st, 1975)

SUMMARY

Flash O_2 yield experiments are described in spinach chloroplasts and *Chlorella* after addition of NH_4Cl.

(1) The damping of the sequence is increased by NH_4Cl.

(2) The turnover times are accelerated but the reaction during which O_2 is released ($S_3 \xrightarrow{h\nu} S_4 \rightarrow S_0$) is slowed.

(3) We observed between the end of the turnover kinetic and the beginning of deactivation a latency time t_1 during which the S_2 and S_3 states are perfectly stable. In the presence of NH_4Cl, this latency time for S_2 is shortened, diminishing from 1 s to less than 12 ms; whereas it is lengthened for S_3 (up to 4 s, 5 s).

(4) After this latency time, two phases are clearly distinguished in the S_2 deactivation: the beginning of the deactivation is abrupt, varying like the square root of the time, i.e. as is characteristic of a diffusive process. During the second phase, S_2 is stabilized. Our experiment shows that S_2 deactivation is monitored by the release of some product F after the latency time from one particular locus, so that nearby Photosystem II reaction centers are rapidly deactivated by diffusion of the product, whereas centers far from this locus are very slowly deactivated.

These results are qualitatively complementary to the luminescence experiments of Velthuys (Biochim. Biophys. Acta (1975) 396, 392–401) except for the latency time which is invisible in the Velthuys results. We propose a modified model in which the binding of NH_3 on states S_2 and S_3 occurs during deactivation.

INTRODUCTION

Ammonium chloride and amines are known to uncouple photophosphorylation from electron flow [1, 2]. Hind and Whittingham [2] found that NH_3 or uncharged amine is the active species in amine uncoupling; Crofts [3] proposed a mechanism whereby NH_3 enters the chloroplast membranes and associates with H^+ to form NH_4^+ during illumination; hence, this NH_3 uptake eliminates the H^+ gradient by neutralizing internal H^+.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-dimethylurea.

Inhibition of O_2 evolution by excess amines or NH_4Cl has the same characteristics as uncoupling by these bases [2], i.e. the inhibition increases with the concentration of NH_3. First measurements of the Hill reaction [4] indicate that NH_3 alters electron transfer between the O_2-yielding reactions and the site of hydroxylamine entry into Photosystem II. More recently, Velthuys [5] observed that with NH_4Cl (50 mM) an enhancement of luminescence occurs after the third and following flashes, indicating that ammonia specifically inhibits the transition from S_4 to S_0. Additional experiments by this author [6] indicate that NH_3 is able to bind to the water-splitting system in both states S_2 and S_3.

This paper relates direct measurements of the S_i states with this inhibitor. It gives direct information on their turnover and deactivation kinetics.

MATERIALS AND METHODS

Chlorella pyrenoidosa was grown as previously described [7]. Prior to use, cells were suspended in 0.1 M sodium phosphate (pH 8.0) at the same chlorophyll concentration as used for chloroplasts.

Chloroplasts were prepared from market spinach according to the method of Nelson et al. [8] with the exception that Tricine was used instead of Tris and sorbitol instead of sucrose. The medium was buffered at pH 7.8. The chlorophyll concentration of the chloroplasts was 500 μM/ml during O_2 measurements.

O_2 was detected using the same polarographic method described by Delrieu [9]. Flash illumination was provided by two xenon flash lamps (General Radio Stroboslave) focused by lenses on the sample. All flashes used were saturating.

RESULTS

Experiments with NH_4Cl were performed after an incubation of 30 min with this inhibitor; because the level of inhibition even in the dark decreased with time, we waited until the level of inhibition was stable.

Spinach chloroplasts, after 10 min deactivation in the dark, were exposed to a sequence of short saturating flashes separated by 300 or 400 ms. With 50 mM NH_4Cl, a steady state of 50 % inhibition was usually observed. With this inhibitor, as shown in Fig. 1, the O_2 yield sequence was more damped. If the dark spaces between flashes were slightly longer, the damping increased. Without inhibitor, in chloroplasts, the deactivation of states S_2 and S_3 appears after 1 s of darkness and no change of the sequence is observed when the space between flashes is below 1 s. With 50 mM NH_4Cl, the sequence is significantly changed when the space varies from 300 to 400 ms (Fig. 1).

As we have shown [9, 10] a statistical method like the least squares method applied to the O_2 yield sequences nearly always gives the best fit between experimental and theoretical sequences when only a unique miss is assigned to one state. The misses on the other states are equal to O. In the experiments shown in Fig. 1, the analysis of the six first flash yields gives the values of the misses written on the figure. The double hits do not increase after addition of NH_4Cl; its value is the same, of the order of 0.03. The effect of NH_4Cl on the flash sequence is only an increase of the miss value. To explain this increase we studied the turnover and deactivation of each state.

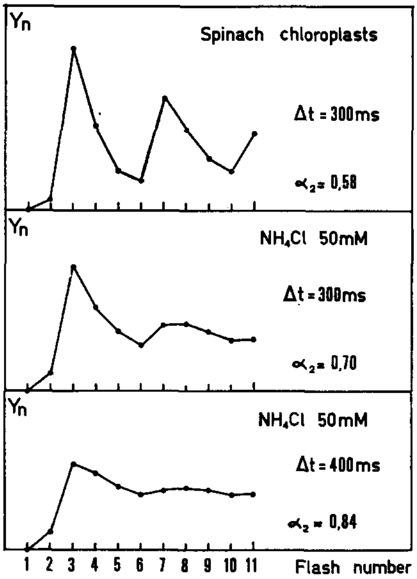


Fig. 1. Flash O_2 yield sequences observed with spinach chloroplasts in the absence (top of the figure) and presence of 50 mM NH_4Cl. Saturating flashes were spaced $\Delta t = 300$ ms or $\Delta t = 400$ ms apart. Chloroplasts were dark adapted for 10 min before flashing. The value of the miss α_2 was obtained from the first six O_2 yields of the sequence by a least squares approximation method [9]. The NH_4Cl curves have been multiplied by a factor of 2.

The measurements of the turnover times were first performed without inhibitor by Kok et al. [11] and by Bouges-Bocquet [12]. The notation $S_n^* \rightarrow S_{n+1}$ used in this paper refers to the dark transition between the intermediate state S_n^* formed by flash excitation of S_n and the following state S_{n+1}. The experiments were performed on dark-adapted chloroplasts by varying the time between the first and second flash ($S_1^* \rightarrow S_2$) or between the second and the third flash ($S_2^* \rightarrow S_3$) of a sequence and detecting O_2 produced at the third flash. For the study of the reaction $S_0^* \rightarrow S_1$ on centers in the S_0 state in the dark, the time between the first and second flash was varied, and O_2 was detected on both the third and the fourth flash. In the case of the turnover $S_3^* \rightarrow S_4 \rightarrow S_0$, we know that after darkness during a sequence of flashes, S_3 is obtained only after the third flash. By varying the time between the third and the fourth flash, O_2 detected on the seventh flash gives information on centers formed at the fourth flash. As demonstrated by Bouges-Bocquet [12], in that case, $Y_7(\Delta t)$ does not give exactly $S_3^* \rightarrow S_0$, but is also a superposition of $S_1^* \rightarrow S_2$ and $S_2^* \rightarrow S_3$.

Turnover times of $S_1^* \rightarrow S_2$ and $S_2^* \rightarrow S_3$ were directly measured in normal and in NH_4Cl-treated chloroplasts (Fig. 2). With NH_4Cl, the reaction rates $S_0^* \rightarrow S_1$, $S_1^* \rightarrow S_2$ and $S_2^* \rightarrow S_3$ show an acceleration. The sigmoidal curve for $S_2^* \rightarrow S_3$ with two phases is transformed in a one phase accelerated curve (NH_4Cl). On the other hand, the $S_3^* \rightarrow S_0$ kinetic is slowed down (Fig. 3). Thus, we observe that NH_4Cl directly affects the water-splitting reaction as predicted from delayed light measurements [5]. In Fig. 3 we have not shown the time, 200 ms, taken by the conversion $S_3^* \rightarrow S_0$. For this reason, in the presence of 50 mM NH_4Cl and with a flash spacing

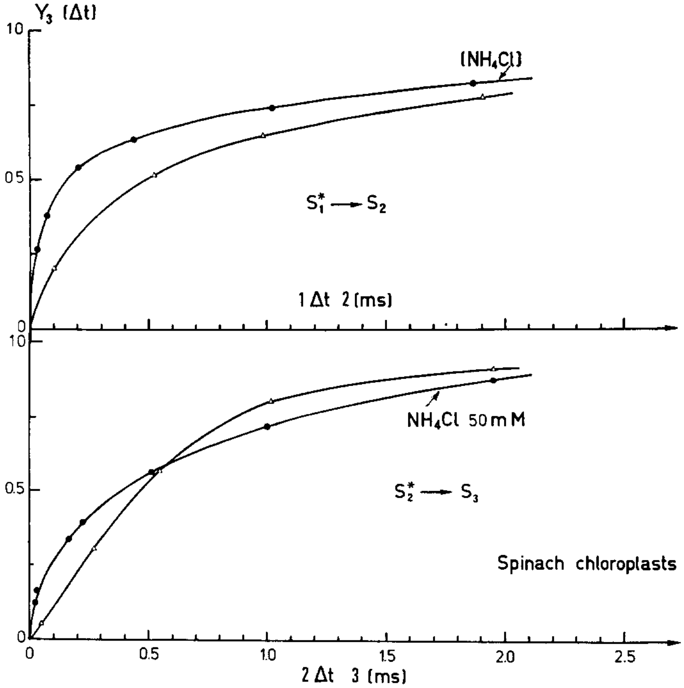


Fig. 2. Turnover times measured by O_2 detection. For $S_1^* \rightarrow S_2$, the first flash of the sequence is followed a variable time later (1 Δt 2) by a second flash. O_2 was detected 400 ms later by the third saturating flash. For $S_2^* \rightarrow S_3$, after a first flash followed 400 ms later by a second flash, O_2 was detected a variable time later (2 Δt 3) by the third flash. Chloroplasts were dark adapted for 10 min before flashing. (△) Chloroplasts without NH_4Cl; (●) chloroplasts in the presence of 50 mM NH_4Cl.

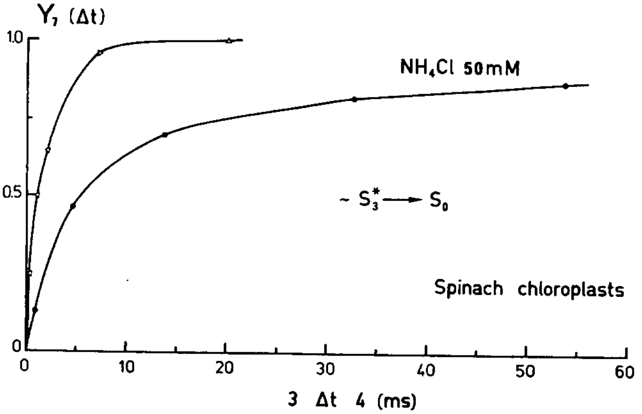


Fig. 3. Y_7, oxygen yield of the seventh flash of a sequence as a function of the time between the third and the fourth flash (3 Δt 4). The time between the remaining flashes of the sequence was 300 ms. Chloroplasts were dark adapted for 10 min before flashing. (△) Chloroplasts without NH_4Cl; (●) chloroplasts in the presence of 50 mM NH_4Cl.

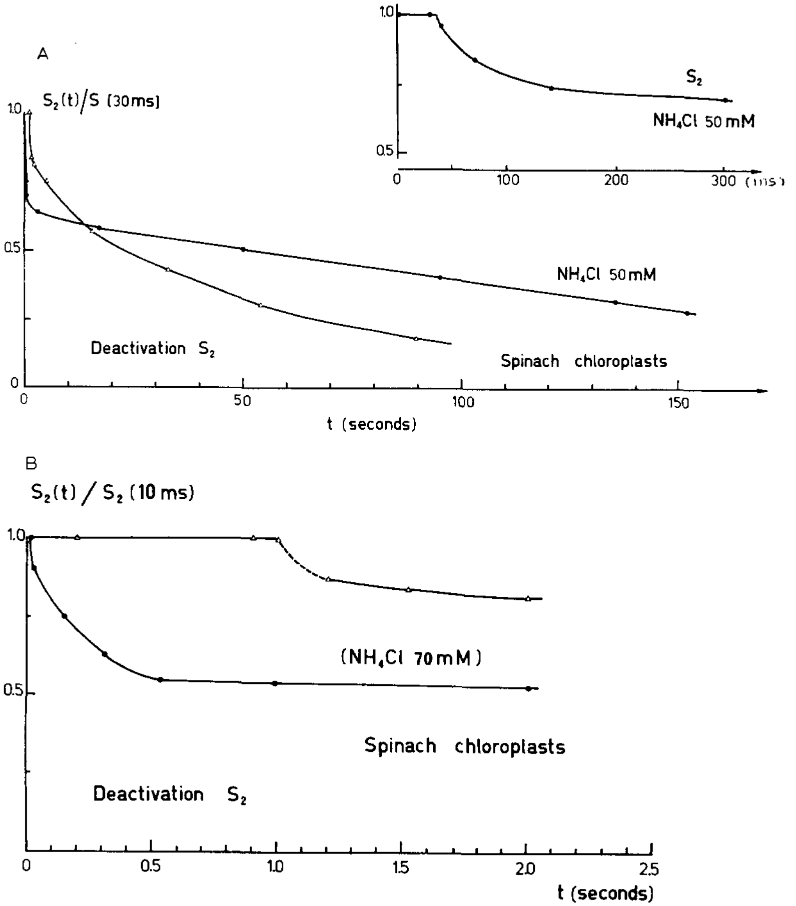


Fig. 4. Deactivation of S_2 in the presence (●) and absence (△) of NH_4Cl with chloroplasts. S_2 was measured by the O_2 yield following the third flash of a sequence as a function of the time between the first and the second flash. The time between the second and the third flash was 300 ms. Chloroplasts were dark adapted for 10 min before flashing. S_2 is formed in 5 ms, then S_2 keeps it at maximum value before deactivation. With untreated chloroplasts the deactivation begins at Δt around 1 s. With NH_4Cl-treated chloroplasts the deactivation begins earlier: in the two examples shown, at Δt = 40 ms for 50 mM NH_4Cl and at Δt = 12 ms for 70 mM NH_4Cl.

of less than 200 ms, we observed a decrease of the stationary O_2 yields in the flash sequence.

The deactivation of state S_2 or S_3 is observed as a function of the time between the first and second flashes, respectively, or between the second and third. In NH_4Cl-treated chloroplasts, the time of stability of state S_2 (i.e. the latency time before the beginning of a measurable deactivation) is shortened. In Fig. 4A, S_2 deactivates abruptly after 40 ms of darkness (50 mM/NH_4Cl). With a slightly larger concentration of NH_4Cl (70 mM) the deactivation begins earlier (12 ms of darkness) (Fig. 4B). Two distinct phases in the deactivation curve of S_2 are clearly observed: a rapid one which decreases S_2 at about half of its maximum value in 0.5 s and, after

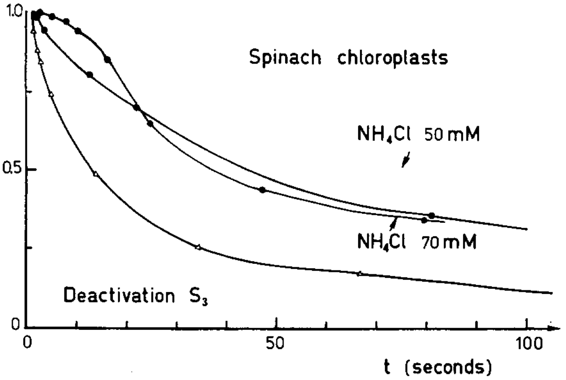


Fig. 5. Deactivation of S_3 in the presence (●) and absence (△) of NH_4Cl with chloroplasts. S_3 is measured by the O_2 yield on the third flash Y_3 of a sequence as a function of the time between the second and the third flash; same conditions as Fig. 4.

that, a very slow one. On the other hand, S_3 is stabilized by NH_4Cl (Fig. 5). For this reason, the beginning of the S_2 deactivation is observed without interference from S_3, i.e. without a superimposed conversion of S_3 into state S_2. In untreated chloroplasts, the true beginning of the S_2 deactivation is never easily seen because S_3 deactivates faster than S_2. Fig. 4B shows the S_2 deactivation in normal chloroplasts. The beginning of the S_3 deactivation is never as abrupt as that of S_2 (with or without inhibitor) (Fig. 5). In the presence of NH_4Cl, the deactivation of S_3 is slowed (Fig. 5) and the dark latency time of S_3 is lengthened (4s, 5s).

In *Chlorella*, under the same experimental conditions, a larger concentration of NH_4Cl is necessary to obtain the same effect as with chloroplasts. An accelerated turnover of $S_0{}^* \rightarrow S_1$, $S_1{}^* \rightarrow S_2$, $S_2{}^* \rightarrow S_3$ also occurs with NH_4Cl. As shown before, for the reaction $S_2{}^* \rightarrow S_3$, with NH_4Cl, two modifications of the kinetics are observed: an acceleration of the reaction rates and also a conversion of the two kinetic phases

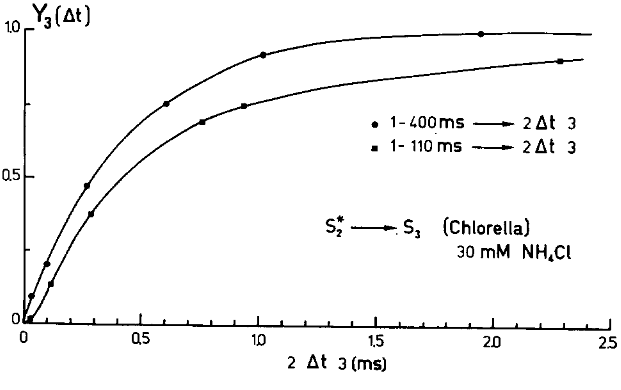


Fig. 6. Turnover time of the reaction $S_2{}^* \rightarrow S_3$ in the presence of 30 mM NH_4Cl with *Chlorella*. The turnover time after the second flash following the first flash at either 110 ms or 400 ms was measured by varying the time on abscissa between the second and the third flash and detecting the O_2 yield, Y_3, on the third flash.

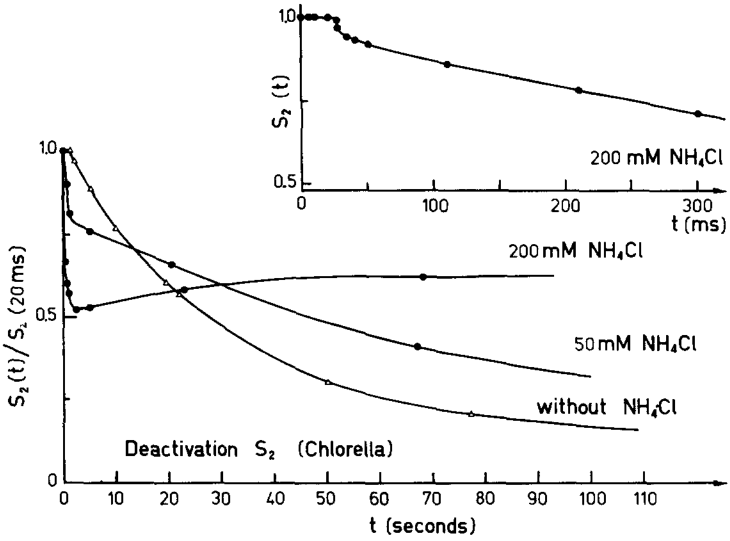


Fig. 7. Deactivation of S_2 in the presence (●) and absence (△) of NH_4Cl with *Chlorella*. S_2 was measured as described in Fig. 4. *Chlorella* was dark adapted for 6 min before flashing. S_2 begins to deactivate at $\Delta t = 500$ ms with untreated chloroplasts, at $\Delta t = 250$ ms with 50 mM NH_4Cl and at 26 ms with 200 mM NH_4Cl in these experiments.

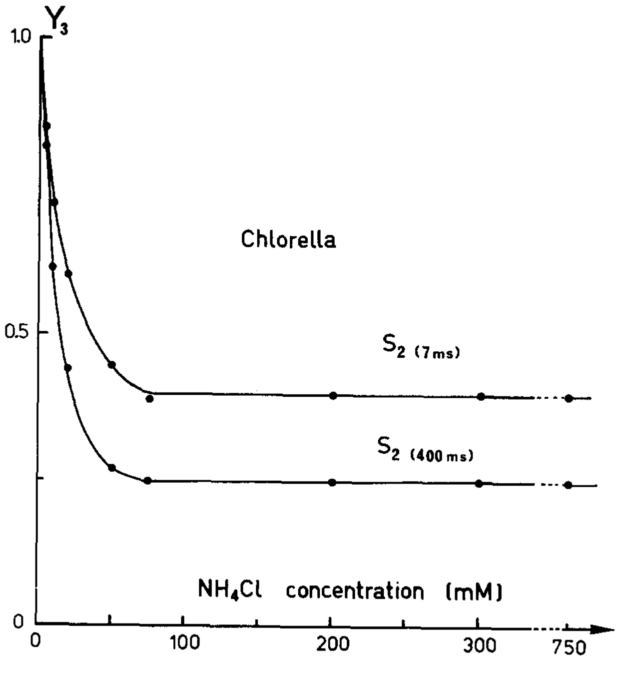


Fig. 8. Inhibition of S_2 at $\Delta t = 7$ ms and at $\Delta t = 400$ ms as a function of NH_4Cl concentration with *Chlorella*. S_2 is measured as in Fig. 7, at its maximum and constant value $\Delta t = 7$ ms, and at the end of the very fast phase of deactivation, $\Delta t = 400$ ms.

in a single kinetic phase. The modifications of turnover are related to the modifications in deactivation. As shown in Fig. 6, if turnover measurements of $S_2^* \rightarrow S_3$ are performed when S_2 has not yet deactivated (for example just after 110 ms of darkness with 30 mM NH_4Cl) we observe accelerated sigmoidal kinetics. On the other hand, at the end of the rapid deactivation phase, single phase kinetics are observed for $S_2^* \rightarrow S_3$ similar to those of $S_1^* \rightarrow S_2$.

In *Chlorella*, as in chloroplasts, there are two phases in the S_2 deactivation, a rapid one (inset of Fig. 7) and a very slow one. As the concentration of NH_4Cl increases, the level at the end of the first phase decreases to nearly half of the maximum value of S_2. The second phase is very slow; it is curious to observe during this second phase (for 200 mM NH_4Cl) an increase of S_2. It is evident that the faster deactivation of S_3 gives S_2. Velthuys [6] has observed a decrease of the slow luminescence phase in NH_3-treated chloroplasts preilluminated by one or two flashes; in the interpretation of this experiment, he assumed a stabilization of the positive charge in states S_2 and S_3 by NH_3. This result is comparable to ours: half of S_2 never deactivates and nearly half of S_3 deactivates slowly (Figs. 4, 7 and 5).

With *Chlorella*, the inhibition of the O_2 evolution is far from complete even with very high concentration (500 mM, 1 M). The inhibition is strong during the

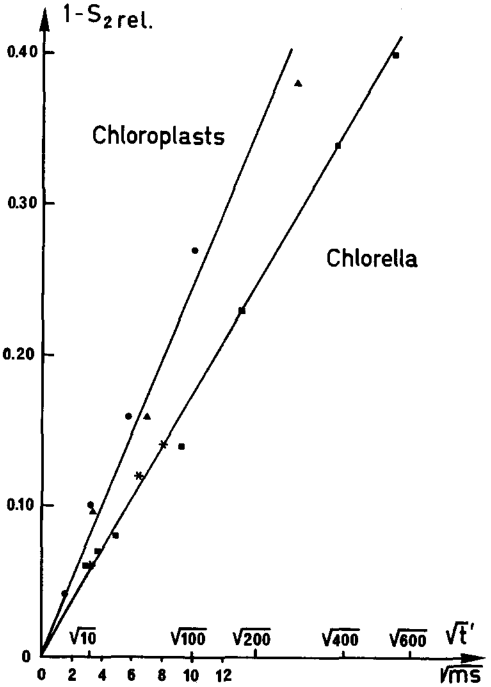


Fig. 9. Study of the fast beginning of the deactivation kinetic of S_2 in the presence of different concentrations of NH_4Cl as a function of the square root of the time from the beginning t_1 of the deactivation (at the end of the constant value of S_2 as shown in Figs. 4 and 7). Only the fast beginning of the S_2 deactivation as a function of $\sqrt{t'}$ is plotted, with an infinite slope at $t' = 0$ ($t_1 = 26$ ms and $t' = \Delta t - 26$ ms for example in Fig. 7). The slope of straight lines obtained is independent of the NH_4Cl concentration and slightly different in *Chlorella* and in chloroplasts.

first seconds but then an adaptation to this inhibitor NH_3 takes place for *Chlorella*, and the level of inhibition decreases until a stationary level. Fig. 8 shows that with *Chlorella* at pH 8.0 this stationary level represents an inhibition of 75 % of the O_2 yields. This adaptation of algae to NH_3 is perhaps connected to their buffering capacity [16].

We have studied the fast phase of S_2 deactivation. Its shape, with a rapid decline at the end of the stable latency time t_l and a progressive decrease, is very characteristic, and decrease of this rate with time is kinetically rare. We propose an explanation based on diffusion kinetics of a chemical product which triggers the deactivation. For a quantitative study, we plotted the decrease of S_2, i.e. $(S_{2\max}-S_2)$ as a function of the square root of the time $t-t_l$ (ms) during which S_2 effectively deactivates. These experimental points produce straight lines (Fig. 9). The slopes of these lines which go through the origin are slightly different for chloroplasts and *Chlorella*, but they are always the same in different experiments even at different temperature. The slope for chloroplasts is greater. This first phase is short (maximum time obtained 600 ms) but longer than the latency time, and is followed by a slower and quite different phase. Preliminary results on the effect of methylamine of *Chlorella* confirm the general effect of this kind of inhibitor. As with NH_4Cl, with methylamine, the S_2 deactivation is strongly accelerated, but, the S_3 deactivation is delayed (Fig. 10). When a large concentration of methylamine is added (500 mM), giving a 90 % inhibition of the O_2 yield, only the rapid phase of the S_2 deactivation exists and as long as S_3 does not deactivate (10 s), the decrease of S_2 as a function of the square root of the time of deactivation gives a straight line (Fig. 10). The slope of the line obtained with methylamine $(S_{2\max}-S_2)$ as a function of $\sqrt{t' = t-t_l}$ is smaller (by a factor of 1/6) than with NH_4Cl.

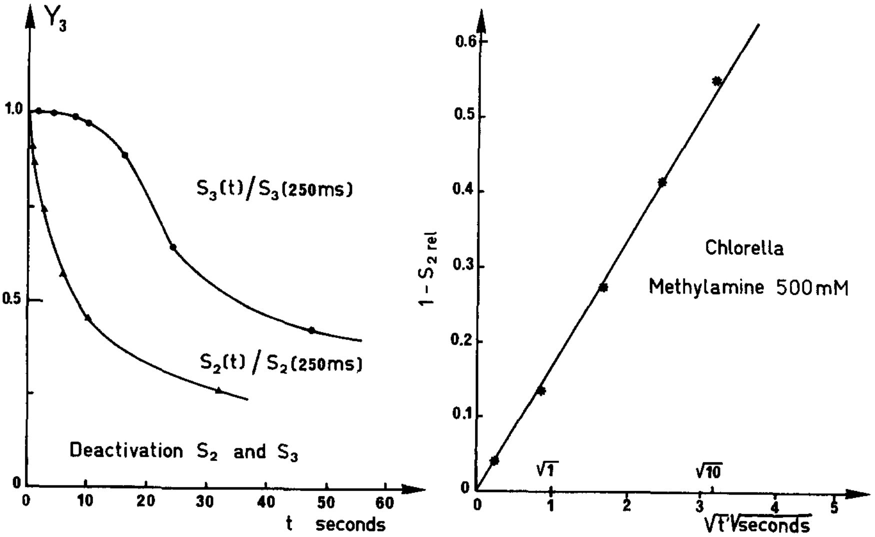


Fig. 10. Deactivation of S_2 and S_3 in the presence of 500 mM methylamine with *Chlorella* measured as in Figs. 7 and 5, and at the right, fast phase of S_2 deactivation plotted as $\sqrt{t'}$ as in Fig. 9. The straight line shown has a slope equal for methylamine to a sixth of that obtained with NH_4Cl (Fig. 9).

INTERPRETATION AND DISCUSSION

(1) As shown in Fig. 4, the dark time of stability of S_2 is shortened by NH_4Cl. This effect explains the increase of the damping of the O_2 yields in a sequence of flashes in the presence of NH_4Cl. During the dark spaces between flashes (300 or 400 ms in Fig. 1) S_2 partially deactivates. This decrease of S_2 between flashes is equivalent to a miss attributed to the state S_2. There is no miss on another state because S_3 is stabilized by NH_4Cl, and the reaction $S_3 \rightarrow S_0$ is finished in 300 ms. This result gives another proof of our preceding result [9, 10] that the only miss found in normal chloroplasts is on state S_2. If this was not the case, we should find two misses in the presence of NH_4Cl, because our analysis is sufficiently precise to distinguish two misses on two different states.

(2) The action of NH_4Cl on turnover time experiments and deactivation has some similarity with that of 3-(3,4-dichlorophenyl)-dimethylurea (DCMU) at high concentration observed by Diner [13], although with DCMU the effects are less important. He showed, only with chloroplasts, that with DCMU some cooperation occurs between Photosystem II centers. Our experiments give nearly identical results in *Chlorella* and chloroplasts, so that if cooperation between centers is a necessary condition, it is the same for both. The principle of the interpetation of Diner [13] is a pairing of the centers on the donor side or on the acceptor side. Unfortunately, this hypothesis cannot explain why, contrary to S_2, the deactivation of S_3 is delayed.

The turnover kinetic with NH_4Cl, for all states S_i, is very accelerated at the beginning, and slowed at the end giving a very nonexponential kinetic. Bouges-Bocquet [12] fixing membranes with glutaraldehyde obtained first order kinetics. We can suppose that NH_4Cl increases conformational changes of membranes. We fixed *Chlorella* cells with 2 % glutaraldehyde [14] before treatment with 200 mM NH_4Cl. In this condition, the turnover kinetic of $S_1{}^* \rightarrow S_2$ is not far from exponential, as for untreated *Chlorella*. Therefore, the shape of the turnover kinetics in the presence of NH_4Cl is a property of centers.

(3) There is a latency time t_l before the beginning of the deactivation of states S_2 and S_3, which is a function of the concentration of NH_4Cl. This latency time t_l decreases for S_2 and increases for S_3. For S_2, the analysis shows that the beginning of the deactivation after the latency time t_l is abrupt, varying like $\sqrt{t-t_l}$. Generally, the kinetic laws as the square root of the time occur in diffusive processes [15]. Therefore, we can speculate that a chemical product F which deactivates centers, is released after this time t_l from one particular locus A and diffuses to the centers (one possibility is that the membrane becomes suddenly permeable to the product F); the centers near A are very rapidly deactivated by diffusion of the product F and the centers far from A are very slowly deactivated, explaining the $\sqrt{t}$ kinetics observed at the beginning of the deactivation of S_2. If we suppose that there is a constant concentration of F at the point A, the number of deactivated centers ramdomly dispersed in the medium is proportional to the total quantity of product F released from A by diffusion (i.e. the integral on all spaces of the concentration of F). One shows easily that the kinetic begins as $\sqrt{t}$ for all geometric configurations, because the distance x of diffusion from A increases linearly with $\sqrt{t} : x = \alpha\sqrt{Dt}$ (where $\alpha \simeq 1$ is a number function of the geometry of medium and D is the diffusion

constant). One proof of this model is that we find the constant $\alpha\sqrt{D}$ in front of $\sqrt{t}$ experimentally independent of temperature, nearly identical on *Chlorella* and chloroplasts and of the same order for NH_4Cl and methylamine (1/6 of that obtained with NH_4Cl). On the other hand, the latency time t_l is very sensitive to the temperature; for example (unpublished results) in *Chlorella* at 31 °C the two states S_2 and S_3 are destabilized after 100 ms of darkness. The latency time depends on chemical processes with a large activation energy and not on physical processes like the diffusion. Our experiments show that the deactivation does not occur only by charge recombination, but is monitored by a product F released after the latency time. The latency time of S_3 increasing with NH_3, is not monitored by the same product. These products F_2 (for S_2) and F_3 (for S_3) cannot be identical to NH_3; in particular, they cannot diffuse freely through the membrane.

(4) Our results agree in qualitative terms with those of Velthuys [6]: with NH_4Cl, only the reaction $S_4 \rightarrow S_0$ is slowed, no inhibition is observed for the other states which are able to accumulate positive charges. Velthuys [6] found that in state S_2 the binding of ammonia takes place with a half-time of about 0.5 s after the flash and that in state S_3, the binding is slower (half-time of about 10 s). These times correspond in our experiments to the first phase of deactivation of states S_2 and S_3, the second phase being the stabilization of these states. With NH_3, the luminescence increases for S_2 in times smaller than 0.1 s and decrease after in agreement with our result. As in our experiments, the stabilization of S_2 is better than that of S_3. Velthuys found that the binding of NH_3 on S_2 is rapid and easily reversible [6]; we observe that in the presence of methylamine at high concentration (500 mM) in *Chlorella*, S_2 is no longer stabilized (Fig.10) (with a lower concentration S_2 is stabilized as with NH_3); but S_3 is similarly stabilized by methylamine over a wide range of concentration. Nevertheless, there is no exact correspondence between luminescence and O_2 evolution. In particular, for states S_2 and S_3, no deactivation occurs during the latency time just after the flash (there is no equivalent result for luminescence). During this latency time the luminescence is large, so it seems difficult to interpret the beginning of deactivation with only a simple model of charge recombination.

According to our experiments, the states S_2 and S_3 complex with NH_3 only during deactivation. We suppose that there are two quite different products F_2 and F_3 able to deactivate S_2 and S_3; they bind freely to NH_3 (F_2NH_3, F_3NH_3). After the flash, at the end of the latency time, the diffusion through the membrane for F_2NH_3 and F_3NH_3 becomes abruptly allowed; the two products can bind to the centers. When the binding occurs, there are two possibilities as a function of the nature of these centers: (a) a deactivation for some unstable part of S_2 (or S_3), and (b) a stabilization for another part of S_2 (or S_3). As shown in this paper (Fig. 6), the unstable part of S_2 is characterized by a biphasic turnover $S_2{}^* \rightarrow S_3$ (the kinetic of $S_2{}^* \rightarrow S_3$ remains biphasic if S_2 has not yet begun to deactivate; it is monophasic if S_2 is taken after the fast phase of the S_2 deactivation). As interpreted by Diner [17], this biphasic kinetic is related to Q^- associated with the state S_2. Velthuys and Amesz [18] and Bouges-Bocquet [19] demonstrated that the first electron transferred from Photosystem II to Photosystem I is stored on a secondary acceptor B; upon transfer of a second electron, the accumulated pair is transferred from B to the pool. The unstable state S_2 has not yet transferred its electron to B, as shown in Scheme 1.

During the flash, for the state S_2Q^-B, the turnover kinetic is biphasic because Q^- must be oxidized before S_2 becomes active.

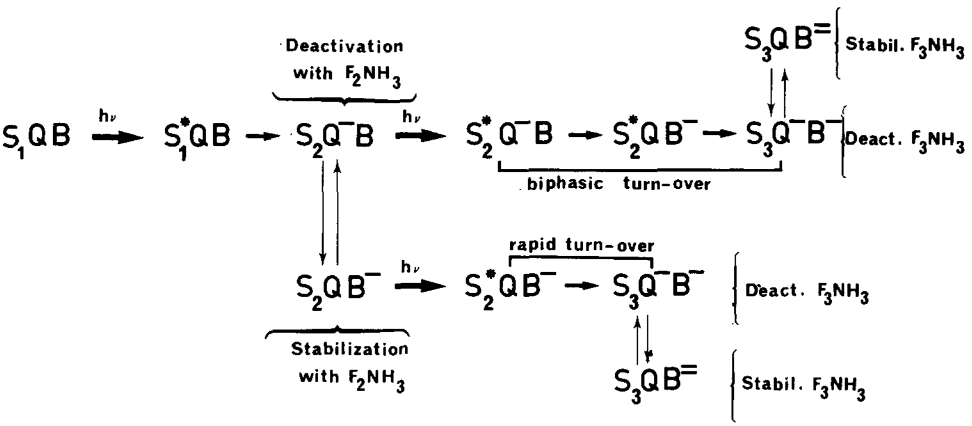
We suppose that the fixation of F_2NH_3 on the state S_2Q^-B leads to a deactivation without fixation:

$$S_2Q^-B+F_2NH_3 \rightarrow S_1QB+F_2NH_3$$

and on the state S_2QB^-, to a stabilization:

$$S_2QB^- + F_2NH_3 \rightarrow \underset{\substack{|\\ NH_3+F_2}}{S_2QB^-}$$

In that case, NH_3 hinders the reverse transfer of the electron to Q. This action of NH_3 is qualitatively in accord with its nucleophilic properties, decreasing the probability of an electron being on Q near S_2. The same interpreation must be true for the state S_3, only its latency time is longer.



Scheme I

This kind of interpretation does not explain all the details of deactivation kinetics. In particular, with anaerobic conditions [17], DCMU treatment [13] and with NH_4Cl treatment, an acceleration of the S_2 deactivation is always associated with a slowing of that of S_3. These opposite modifications for S_2 and S_3 also exist in the difference between chloroplasts and *Chlorella* deactivation. The experimental simple law for opposite stabilization-destabilization of S_2 and S_3 must have a general explanation which is unknown.

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