Biochimica et Biophysica Acta, 396 (1975) 392–401

BBA 46974

BINDING OF THE INHIBITOR NH_3 TO THE OXYGEN-EVOLVING APPARATUS OF SPINACH CHLOROPLASTS

B. R. VELTHUYS

Department of Biophysics, Huygens Laboratory of the State University, Wassenaarseweg 78, Leiden (The Netherlands)

(Received April 14th, 1975)

SUMMARY

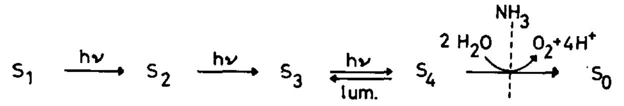
Experiments are described on flash-induced luminescence of isolated spinach chloroplasts after addition of NH_4Cl. The results indicate a binding of NH_3, presumably in competition with water, in the oxidation states S_2 and S_3, i.e. the states reached upon illumination of dark-adapted material with one and two flashes, respectively. In the initial state S_1, no binding of NH_3 occurs. In state S_2 the binding of ammonia is rapid (half-time about 0.5 s) and rapidly reversible; in state S_3 the binding is slower (half-time about 10 s) and slowly reversible. NH_3 bound to S_4 prevents the oxidation of water. NH_3 bound to S_2 decreases the rate of the back reaction of reduced primary acceptor (Q^-), indicating a charge stabilization, i.e. a decrease in the redox potential of S_2 due to interaction with ammonia. In Tris-washed chloroplasts, the stability of the positive charge generated in a flash is much smaller than in normal chloroplasts and not increased by NH_3. On the basis of these observations it is postulated that, in the absence of NH_3, states S_2 and S_3 are stabilized by manganese-coordinated, bound water.

INTRODUCTION

Evidence both from measurements of the Hill reaction [1, 2] and luminescence [3–5] of isolated chloroplasts indicates that the oxidizing side of Photosystem II is affected by NH_4Cl, or, rather, by the unprotonated base NH_3 (ammonia) [1, 2, 5]. It was shown by Izawa et al. [2] that in the presence of this substance, Photosystem II is no longer able to use water as the electron donor; an artificial electron donor, hydroxylamine, however, can still be oxidized. Measurements by Zankel [3] and by this laboratory [4, 5] have shown that in the presence of ammonia the intensity of luminescence induced by short saturating flashes is strongly dependent on the number of flashes given, also in a time region (around 40 ms) where in the absence of NH_3 very little flash-number dependence is observed with isolated chloroplasts [4, 5]. The most conspicuous effect of NH_3 was an enhancement of luminescence after the third and following flashes [4, 5].

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

In terms of the model of Kok et al. [6] for water oxidation these results might seem to indicate that ammonia specifically inhibits the transition from S_4 to S_0, according to the following scheme:



(1)

Scheme (1)

Additional evidence [4], however, indicated that the action of ammonia is more complex than suggested in this scheme. In particular, no satisfactory explanation is given for the observation that a strongly enhanced luminescence after the third flash was obtained only when ammonia was added before or after the first flash. When it was added after the second one, i.e. in state S_3, most of the stimulation of luminescence was delayed till after the seventh flash [4], demonstrating that in this case the inhibition of the S_4 to S_0 transition, i.e. water oxidation, had been ineffective. Apparently, already earlier states than the S_4 state are affected by NH_3.

This paper describes further experiments about flash-induced luminescence in the presence of NH_3. The results indicate that both in S_2 and S_3, but not in S_1, ammonia is able to bind to the water-splitting system. In S_2 this binding is rapid and rapidly reversible, and leads to a slowing down of the back reaction of the stored positive charge with reduced products of Photosystem II. In S_3, the binding of NH_3 is less rapid and only slowly reversible. The results are tentatively explained in terms of a competition between water and ammonia for binding site(s) at the oxidizing side of Photosystem II.

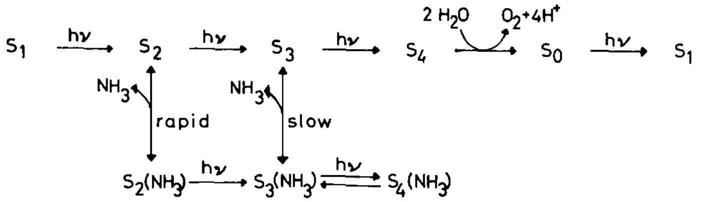
MATERIALS AND METHODS

Chloroplasts were prepared from spinach leaves as described in ref. 7. Tris-washed chloroplasts (Fig. 8) were obtained by adding to a concentrated chloroplast suspension (5 mM chlorophyll) an equal volume of 0.2 M Tris (final pH 10.0). After 15 min incubation the final suspension was prepared by 100-fold dilution with the medium used in the experiments: a solution of pH 7.8 containing 0.2 M sucrose, 0.05 M KCl, 0.04 M NaCl and 0.01 M sodium morpholinopropane sulfonate (MOPS) buffer. Other conditions and procedures were as described in ref. 8.

RESULTS AND INTERPRETATION

NH_3-induced transformation of S_2 and S_3

In the presence of ammonia, luminescence after the third one of a series of flashes is strongly enhanced [4, 5]. In one of the previous papers [4] an experiment was shown which demonstrated that the degree of enhancement of luminescence was dependent on the time of addition of the inhibitor: when NH_4Cl was added just before the third flash, i.e. in state S_3, the effect was much smaller than when it was already added in state S_1 or S_2. Further experimentation, to be described below, yielded evidence suggesting the following scheme to explain the effects of NH_3 on Photosystem II:



(2)

Scheme (2)

In this scheme the symbol $S_n(NH_3)$ ($n = 2, 3, 4$) represents a complex of NH_3 with an oxidized intermediate in state S_n. The back reaction $S_4(NH_3) \rightarrow S_3(NH_3)$ is postulated to be responsible for strong luminescence (around 40 ms).

The main evidence for Scheme 2 comes from the experiments described in Figs 1 and 2. Isolated spinach chloroplasts were subjected to a series of three short saturating flashes. Luminescence was recorded 40 ms after the third flash. For Fig. 1A, NH_4Cl (50 mM) was added 10 s after the first or second flash of the series. The additional flash(es) were given after a variable incubation time. It can be seen that in contrast to what was concluded before [4], a strongly enhanced luminescence after the third flash can also be obtained when ammonia was added after the second flash.

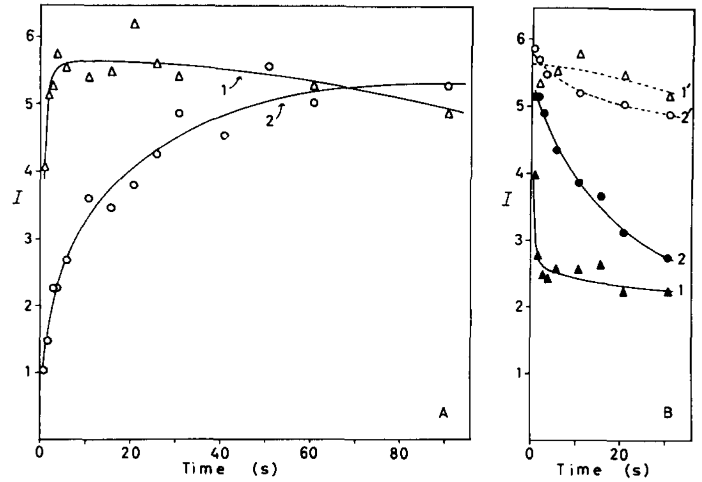


Fig. 1. Intensity of luminescence (in arbitrary units) (I) after the third flash, as function of time after a change in the concentration of NH_3 made after the first or second flash. (A) Effect of an increase in NH_3 concentration. Curves 1 and 2, resp., chloroplasts were preilluminated by one or two flashes, resp. (flash-interval time: 1 s). 10 s later 50 mM NH_4Cl was added. After a variable incubation time (indicated at the ordinate) two or one additional flashes were given (flash-interval time, 1s) so that the total number of flashes was three in all experiments. Luminescence was recorded 40 ms after the last flash. Without addition of NH_4Cl the luminescence intensity 40 ms after the third flash was about 1. Chlorophyll concentration: 12.5 μM. Cuvette thickness, 2 mm; pH 7.8 temperature, 16 °C. (B) Effect of a decrease in NH_3 concentration. The data of curves 1 and 2 were obtained in a similar way as the corresponding curves of A, except that 50 mM NH_4Cl was now added 30 min before the first flash and instead of adding NH_4Cl after the first or second flash, the concentration of NH_3 was lowered 20-fold by adding an equal volume of acidified buffer, decreasing the pH to 6.8. Curves 1′ and 2′, control experiments in which, instead of acid, buffer of pH 7.8 was added (containing 50 mM NH_4Cl).

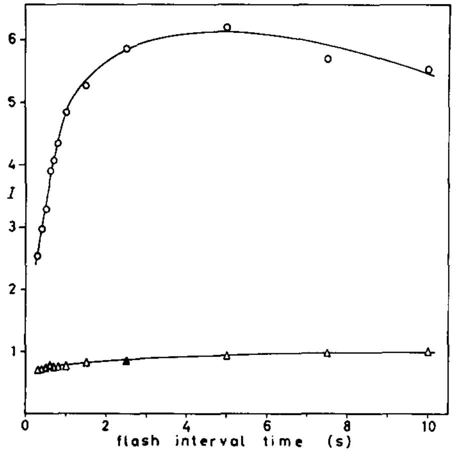


Fig. 2. Intensity of luminescence (I) 40 ms after the second (△ — △) and third (○ — ○) flash of a series given 10 s after the addition of 50 mM NH_4Cl to dark-adapted chloroplasts, as function of the flash-interval time. In the absence of NH_4Cl the luminescence intensity 40 ms after the third flash was about 0.7.

In this case, however, a much longer incubation time was needed than when ammonia was added after the first flash. Evidently, the dark transformation of S_3 into $S_3(NH_3)$ is possible, but it occurs slowly. The result obtained when NH_3 was added after the first flash can be explained (see Scheme 2) by assuming that NH_3 bind rapidly to S_2, giving $S_2(NH_3)$, which is transformed to $S_3(NH_3)$ by the second flash. An alternative explanation could be that from S'_2, the intermediate state formed by flash excitation of a center in state S_2 [9], both a transition to S_3 and to $S_3(NH_3)$ is possible, depending on the absence or presence of NH_3. However, as will be shown below (Figs 2–7), there is additional evidence that already S_2 complexes with ammonia.

Fig. 1B indicates that the transformations of S_2 and S_3 by ammonia are reversible, which is easily explained as a dissociation of the complex. After one or two flashes in the presence of 50 mM NH_4Cl the concentration of NH_3 was decreased by adding an equal volume of a more acid buffer, lowering the pH from 7.8 to 6.8 and the concentration of NH_4Cl to 25 mM. Due to both the dilution and the pH lowering, the concentration of NH_3 decreases almost 20-fold. This resulted in a much less enhanced luminescence after the third flash. Again, the incubation time needed was much longer with two than with one flash preillumination. Apparently, we may conclude that state $S_3(NH_3)$ is not only formed more slowly, but that the complex also dissociates more slowly than the complex between S_2 and NH_3.

Fig, 2 shows the luminescence intensity after the third flash as function of the dark-time between the flashes. 50 mM NH_4Cl was added 10 s before the first flash. The stimulation of luminescence increased with increasing dark-time between flashes. A half-maximal stimulation of luminescence after the third flash was obtained at a flash interval time of about 0.5 s, a time constant that is of the same order as that which appeared in Fig. 1A for the formation of $S_2(NH_3)$. This result indicates that state S_2 is the first state which is transformed by NH_3, and that no binding to state S_1 occurs.

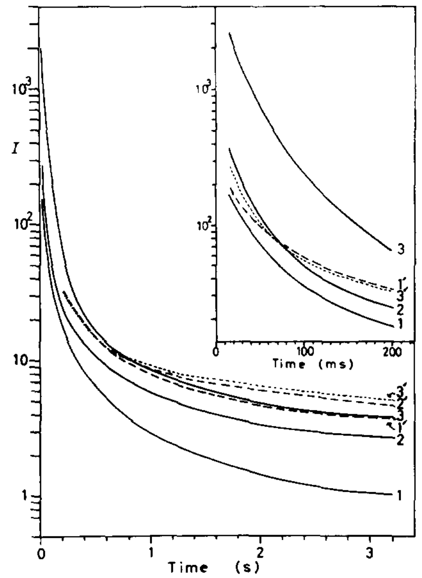


Fig. 3. Kinetics of luminescence (I) of chloroplasts after the first (curve 1), second (curve 2) and third flash (curve 3) in the presence of 50 mM NH_4Cl. Curves 1′–3′, the corresponding luminescence kinetics in the absence of NH_4Cl. The insert shows the decay in the region 15–200 ms at an extended time scale; curve 2′, almost coinciding with 1′, has been omitted here. Flash-interval time, 5 s.

Charge stabilization by ammonia in state S_2

The evidence discussed above concerning the action of NH_3 was all based on the increase of luminescence after the third flash, in the presence of this substance. This effect, though, is not the only effect of NH_3 on luminescence.

Fig. 3 shows the kinetics of luminescence from 0.02 to 3 s after one to three flashes in the absence and presence of ammonia. It can be seen that, apart from the large stimulation after the third flash, which lasts for about 0.5 s, luminescence in the seconds region is also affected. In this time region luminescence is diminished. This effect is the largest for the first flash, i.e. for luminescence from centers in S_2. This decrease of slow luminescence by NH_3 can also be observed when the inhibitor is added after the illumination. This is shown in Fig. 4 where 50 mM NH_4Cl was added

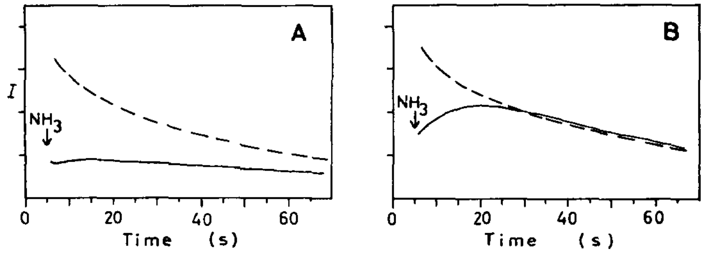


Fig. 4. Luminescence intensity (I) of chloroplasts, preilluminated by one (Fig. 4A) or two (Fig. 4B) flashes, after the addition of 50 mM NH_4Cl (5 s after the preillumination). Broken lines, no NH_4Cl added (buffer added instead).

5 s after one or two flashes preillumination. Again, the decrease is much larger after one than after two flashes. One way to interpret this decrease of slow luminescence is to assume that the rate of the back reaction responsible for luminescence is lowered, which would indicate a stabilization of the positive charge in S_2 by NH_3. An alternative explanation could be that the 'exciton yield' [10] of the back reaction is affected by NH_3.

In order to be able to decide between these assumptions, we have studied luminescence in the presence of DCMU. In this case the rate of back reaction of the stored positive charge is enhanced, due to the presence of reduced primary acceptor. This substance, Q^-, is presumably the sole source of electrons for the back reaction now [11], which means that the rate of back reaction can be independently followed by measuring the redox level of Q as function of time. This can be easily done by measuring the area above the fluorescence induction curve obtained in strong light, [11].

Fig. 5 shows that, as was the case after the first flash in the absence of DCMU, luminescence is diminished by NH_4Cl. In contrast to NH_4Cl, valinomycin and nigericin only little affected the intensity of luminescence, indicating that the well-known uncoupling activity of NH_4Cl is not responsible for the change in luminescence emission. A change in the decay rate of luminescence can also be observed in Fig. 5. This is more clearly seen in Fig. 6A, where instead of luminescence intensity the luminescence intensity integrated from 0.2 s after the flash is plotted as function of time. It is obvious from this figure that in the presence of NH_4Cl the luminescence is only emitted more slowly; the total amount of luminescence is the same or almost the same. It may be remarked that this result not only shows that the 'exciton yield' of the back reaction between positive charge and Q^- is not affected by NH_3, but also

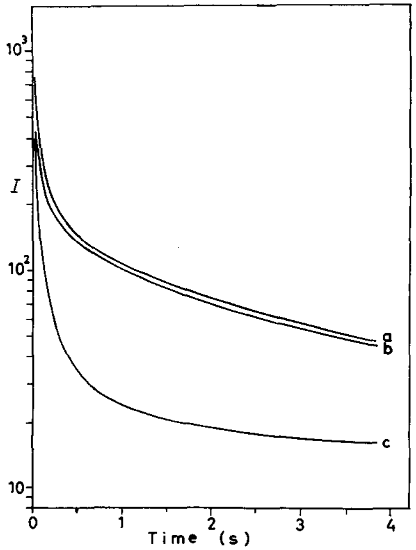


Fig. 5. Kinetics of luminescence (I) of chloroplasts after a flash given in the presence of 10 μM DCMU (curve a), 10 μM DCMU, 1 μM nigericin and 1 μM valinomycin (curve b) or 10 μM DCMU and 50 mM NH_4Cl (curve c).

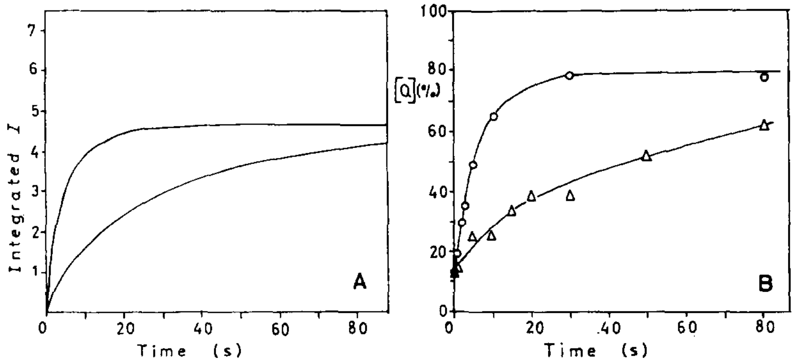


Fig. 6. (A) Luminescence (I) integrated from 0.2 s after a flash given in the presence of 10 μM DCMU (curve A) or 10 μM DCMU plus 50 mM NH_4Cl (curve B). (B) Reoxidation of Q^- after a flash given in the presence of 10 μM DCMU (curve A) or 10 μM DCMU plus 50 mM NH_4Cl (curve B). The amount of oxidized Q ([Q]) was measured as the area above the fluorescence induction curve obtained in strong blue light (0.4 mW/cm^2), and plotted as percentage of the area obtained before the flash.

that this back reaction, in the presence of DCMU, occurs only or mainly via the exciton producing pathway (see also refs 11–13). In Fig. 6B the decrease in the concentration of Q^- (the area above the fluorescence induction curve) is shown, measured under the same circumstances. The rate of oxidation of Q^- is diminished by NH_4Cl, in agreement with Fig. 6A.

Fig. 7 shows that the decrease of luminescence in the presence of DCMU and

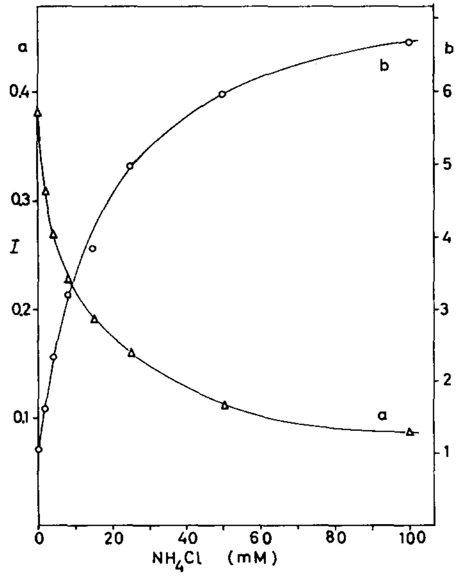


Fig. 7. Decrease of luminescence (I) 1 s after the first flash (in the presence of 10 μM DCMU) and increase of luminescence 40 ms after the third flash (flash-interval time, 5 s; no DCMU present), as function of the concentration of NH_4Cl (pH 7.8). The additions were made 10 s before the illumination.

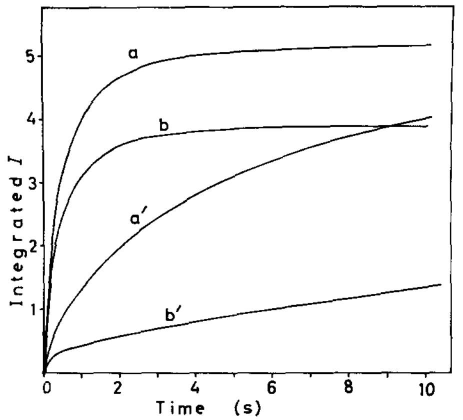


Fig. 8. Luminescence (*I*) integrated from 0.04 s after the first flash given to Tris-washed chloroplasts in the presence of 10 μM DCMU and in the absence (curve a) and presence (curve b) of 50 mM NH_4Cl (pH 7.8). Curves a′ and b′, untreated chloroplasts instead of Tris-washed chloroplasts were used.

the increase of luminescence after the third flash (in the absence of DCMU) are similarly dependent on the concentration of ammonia, supporting the assumption that both effects of ammonia have an identical cause (i.e. the formation of the complex $S_2(NH_3)$).

Luminescence of Tris-washed chloroplasts

After incubation at a high concentration of un-ionized Tris electron donation by water is irreversibly inhibited [14–17]. A single flash still induces electron transfer by Photosystem II, but subsequent flashes do not induce additional electron transfer [17]; evidently, only one positive charge can be stored at the oxidizing side of Photosystem II. This charge is much less stable than in untreated chloroplasts: Fig. 8 shows that in the presence of DCMU luminescence is much more rapidly emitted than in normal chloroplasts. NH_4Cl, in contrast to what was observed with untreated chloroplasts, did not substantially decrease the rate of emission. This last observation does not seem to be merely a consequence of the rapidity of the back reaction. Even at, e.g. 1 s, a time longer than required for NH_3 to bind in untreated chloroplasts, the rate of emission of luminescence is much less diminished by NH_4Cl in Tris-washed than in untreated chloroplasts. This might indicate that Tris-washed chloroplasts are unable to bind NH_3.

DISCUSSION

The results presented in this and previous papers [4, 5] demonstrate that at high NH_3 concentration both states S_2 and S_3 are transformed to states which we called $S_2(NH_3)$ and $S_3(NH_3)$, respectively. The transformations are reversible and are presumably due to binding of the inhibitor. The reactions between the various states were summarized in Scheme 2.

The presence of $S_3(NH_3)$ is demonstrated by the effect of another flash. Luminescence after such a flash is strongly enhanced (in the time region around 40 ms).

This is also true for subsequent flashes [4], indicating that for the centers responsible for the intense luminescence the transition to S_0 cannot occur. This explains the inhibition of oxygen evolution observed earlier [2]. State $S_2(NH_3)$ is characterized by a low rate of deactivation via the back reaction which is accompanied by luminescence, and by the efficient formation of $S_3(NH_3)$ in a flash.

Binding and release of inhibitor were slower in state S_3 than in state S_2. This might be explained in terms of differences in conformational state. One could e.g. assume, that in state S_3 the immediate environment of the binding site of ammonia equilibrates less easily with the medium than in state S_2. The fact that binding and release of NH_3 takes about the same time (cf. Figs 1A and 1B) agrees with this assumption. Another kind of explanation, which we prefer, is based on the assumption that ammonia competes with another molecule for the binding site. If the binding of this other molecule and of NH_3 is stronger in S_3 than in S_2, differences in activation energy needed for displacement of one molecule by another then would explain the differences in rates of binding and release of NH_3.

It is attractive to assume that the molecule competing with ammonia for binding at the oxidizing side of Photosystem II is water itself, the substrate of the oxygen-evolving reaction. If the S_4 to S_0 transition is dependent on a pair of bound water molecules, a replacement of one, or both, of these molecules by NH_3 would make this reaction no longer possible, which explains the inhibitory action of ammonia. If this hypothesis is correct our results strongly suggest that water is already involved in the stabilization of the positive charge in state S_2, since also binding of ammonia seems to increase the stability of the charge.

It may be speculated (see also ref. 18) that bound water is present in a manganese-coordinated form. To explain the function of manganese in Photosystem II it has been proposed that higher oxidation states of this metal are used for the accumulation of oxidation equivalents [19, 16]. It may be remarked, however, that a merely structural function of manganese in providing part of the binding site for water would already explain why it is indispensable for the oxygen-evolving apparatus. Such an assumption would also be sufficient to explain the high rate of luminescence emission in chloroplasts washed with Tris, a treatment which results in the loss of a large part of the bound manganese [16]: the lower stability of the photo-generated positive charge in these chloroplasts, compared with normal chloroplasts, might not be due to a difference in the identity of the oxidized electron donor, but to the absence of bound water interacting with the oxidized electron donor. Similarly, ammonia can no longer be bound, and therefore does not decrease the lability of the positive charge(Fig. 8).

A competition with water for binding sites in Photosystem II has earlier been proposed for another amine, hydroxylamine, of which two molecules can be irreversibly bound [20, 9]. This binding does not result in a stabilization of photo-generated positive charge, as in the case of NH_3, but in a rapid decay of the charge, due to oxidation of the bound hydroxylamine. Another difference with ammonia is that the binding occurs already in dark-adapted material. This does not exclude, of course, that the binding site of hydroxylamine and ammonia may be the same. An observation supporting such an assumption is that in Tris-washed chloroplasts, which presumably do not bind NH_3, binding of hydroxylamine in the dark does not seem to occur either (Velthuys, B. R., unpublished observations).

ADDENDUM

Preliminary results obtained after submission of the manuscript indicate that the inhibition of the S_4 to S_0 transition, and enhanced 40 ms-luminescence, is dependent on the binding of two molecules of NH_3 in state S_3; only one of these molecules can be bound in state S_2. This suggests a different interpretation of Fig. 1B, curve 1: lowering of the concentration of NH_3 after the first flash results in a relatively low intensity of luminescence not because of a rapid release of the first NH_3-molecule but because the second one is not bound.

ACKNOWLEDGEMENTS

The investigation was supported by the Netherlands Foundation for Chemical Research (SON), financed by the Netherlands Organization for the Advancement of Pure Research (ZWO). I wish to thank Dr J. Amesz for his continuous interest and for a critical reading of the manuscript.

REFERENCES

1 Hind, G. and Whittingham, G. P. (1963) Biochim. Biophys. Acta 75, 194–202
2 Izawa, S., Heath, R. L. and Hind, G. (1969) Biochim. Biophys. Acta 180, 388–398
3 Zankel, K. L. (1971) Biochim. Biophys. Acta 245, 373–385
4 Velthuys, B. R. and Amesz, J. (1975) Biochim. Biophys. Acta 376, 162–168
5 Velthuys, B. R. (1974) in Proceedings of the Third International Congress on Photosynthesis, Rehovot, (Avron, M., ed.), pp. 93–100, Elsevier, Amsterdam
6 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475
7 Amesz, J., Pulles, M. P. J. and Velthuys, B. R. (1973) Biochim. Biophys. Acta 325, 472–482
8 Velthuys, B. R. and Amesz, J. (1973) Biochim. Biophys. Acta 325, 126–137
9 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785
10 Van Gorkom, H. J. and Donze, M. (1973) Photochem. Photobiol. 17, 333–342
11 Bennoun, P. (1970) Biochim. Biophys. Acta 216, 357–363
12 Etienne, A. L. (1973) Biochim. Biophys. Acta 333, 320–330
13 Joliot, A. (1974) Biochim. Biophys. Acta 357, 439–448
14 Yamashita, T. and Butler, W. L. (1968) Plant Physiol. 43, 1978–1986.
15 Yamashita, T. and Butler, W. L. (1969) Plant Physiol. 44, 435–438
16 Cheniae, G. M. and Martin, I. F. (1970) Biochim. Biophys. Acta 197, 219–239
17 Velthuys, B. R. and Amesz, J. (1974) Biochim. Biophys. Acta 333, 85–94
18 Renger, G. (1973) Eur. J. Biochem. 27, 259–269
19 Fowler, C. F. and Kok, B. (1974) Biochim. Biophys. Acta 357, 299–307
20 Bennoun, P. and Bouges, B. (1971) in Proceeding of the Second International Congress on Photosynthesis, Stresa (Forti, G., Avron, M. and Melandri, M., eds), pp. 569–576, Junk, The Hague