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NH_4Cl ACTIVATION OF THE FLUORESCENCE YIELD IN CO_2-STARVED *CHLORELLA PYRENOIDOSA*

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SUMMARY

The addition of NH_4Cl to *Chlorella* deprived of CO_2, raises the fluorescence yield about 2.5-fold. This effect does not appear to be related to nitrate reduction, and is specific for $NH_4{}^+$. Concentration curves, at varying pH, indicate that the unprotonated ammonia is responsible for this effect.

Large increases in the yield of chlorophyll fluorescence, accompanied by parallel increases in the rate of net photosynthetic electron transport, have been previously demonstrated (i) during induction in *Chlamydomonas reinhardti*[1], (ii) when Mg^{2+} is added to chloropasts[2,3] and (iii) when the CO_2 supply is restored to *Chlorella pyrenoidosa*[4]. These instances of parallelism are not consistent with a simple photochemical system, and hypotheses of α-changes[5] or of activation of Photosystem II units[1] have been offered in explanation. In this note, we show that 0.4 mM NH_4Cl increases by about 2.5-fold the steady-state fluorescence yield of *Chlorella* deprived of CO_2. This effect provides further evidence for α-changes or for a modification of the deexcitation processes within Photosystem II units. The NH_4Cl effect on fluorescence does not appear to be associated with nitrate reduction and seems specific for $NH_4{}^+$.

C. pyrenoidosa was grown synchronously as previously described[4]. Two growth media were employed: (i) a "Nitrate medium" containing 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4 \cdot 7H_2O$, 0.04 mM K_2HPO_4, 0.5 $ml \cdot l^{-1}$ of Hutner's trace element solution and 2.0 mM KNO_3, and (ii) a "NH_4Cl medium" containing 0.2 mM $CaCl_2$, 1.0 mM $MgSO_4 \cdot 7H_2O$, 0.82 mM K_2HPO_4, 0.53 mM KH_2PO_4, 0.5 $ml \cdot l^{-1}$ of Hutner's trace element solution and 4.0 mM NH_4Cl.

Cells were harvested 3 h after the onset of the light period, washed several times, resuspended at 7 μl packed cellvolume per ml, and mounted on the platinum electrode. The electrolyte solution used for washing the cells and also in the electrode vessel, was identical to the "Nitrate medium" except that nitrate was omitted and 0.05 M KCl and 0.05 M NaCl were added. During the experiments, either 2.0 mM KNO_3 or NH_4Cl were added to the electrolyte. The rate of O_2 evolution and the chlorophyll fluorescence (680 nm) were measured simultaneously as previously described[4]. A saturating actinic illumination (6.5 $mW \cdot cm^{-2}$ of "broad band blue", 440–660 nm) was applied in repeated cycles of 5 min light–3 min dark. "Broad band blue" illumination was obtained with two Corning 4303 blue green filters, a KG-1 infrared filter and a 1000-W tungsten lamp.

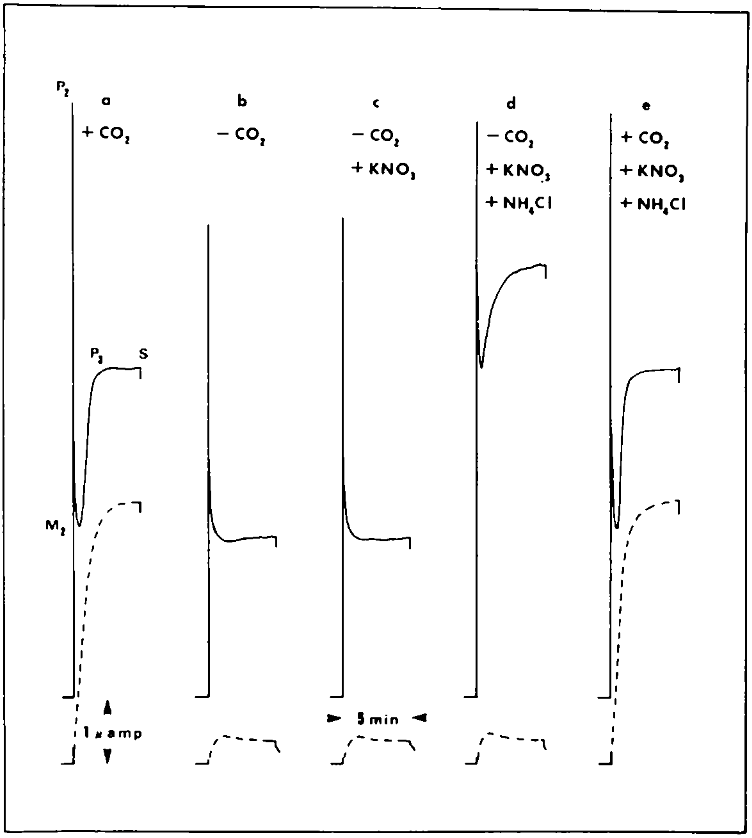

Fig. 1. Effect of NH_4Cl on chlorophyll fluorescence (——) and rate of O_2 evolution (- - - - -) in nitrate-grown *Chlorella* deprived of CO_2. Selected curves from an experiment in which repeated cycles of 5 min light – 3 min dark were given. Conditions: 26 °C, pH 5.3, with 6.5 $mW \cdot cm^{-2}$ "broad band blue" actinic light. The electrolyte was equilibrated with (and stirred by) either 5.0% CO_2 ($+CO_2$) and 6.0% O_2 in N_2 or 0% CO_2 ($-CO_2$) and 6.0% O_2 in N_2. Curves a, normal induction showing typical P_2, M_2, P_3 and S levels of fluorescence; Curves b, 0.5 h after CO_2 deprivation; Curves c, after adding 2.0 mM KNO_3; Curves d, after subsequent addition of 2.0 mM NH_4Cl; Curves e, after restoring the CO_2 supply.

When nitrate-grown cells were mounted in the electrolyte containing no nitrogen source and illuminated, the O_2 evolution rate and chlorophyll fluorescence exhibited the well-known features of induction (Fig. 1, Curves a). The P_2, M_2, P_3 and S levels (see refs 1 and 4) of fluorescence induction are clearly manifested. When CO_2 was removed (Curves b), O_2 evolution was suppressed and, as Slovacek and Bannister[4] recently showed, the M_2–P_3 fluorescence transient was abolished. When 2.0 mM KNO_3 was added (Curves c), there was no effect at all on either O_2 evolution or fluorescence. In contrast, the addition of 2.0 mM NH_4Cl had a marked effect (Curves d); the P_2, M_2 and P_3 levels were all raised up to, or above, the original levels seen in Curves a. O_2 evolution remained suppressed for lack of CO_2. Finally, when the CO_2 supply was restored, both O_2 evolution and fluorescence induction returned to the original states. We previously emphasized that the O_2 evolution and fluorescence declined in parallel when CO_2 is removed. Fig. 1 shows that, in the presence of NH_4Cl, the addition of CO_2 brought about complimentary changes —O_2 evolution increased while fluorescence levels declined. Complimentary responses to the removal of CO_2 have also been reported for *C. reinhardti*[4], an organism which requires NH_4^+ as a nitrogen source for growth.

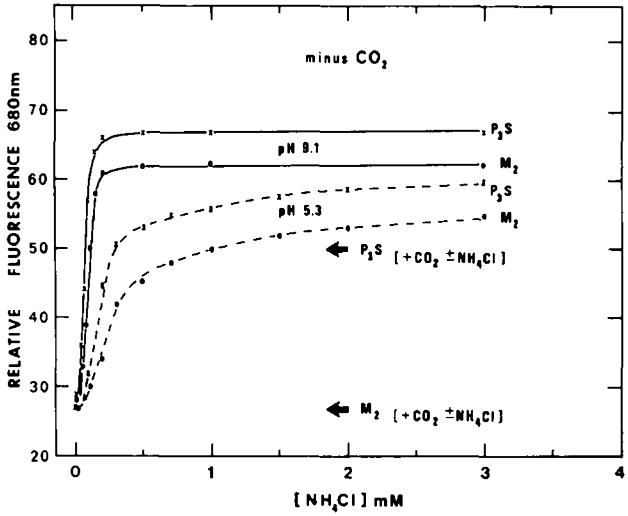

Fig. 2. Dependence of the M_2 and P_3–S levels of fluorescence (in the absence of CO_2) on NH_4Cl concentration. Conditions: same as for Fig. 1 except pH either 9.1 (——) or pH 5.3 (- - -). Arrows indicate the M_2 and P_3–S levels of fluorescence in the presence of CO_2 with or without NH_4Cl.

Fig. 2 shows how the M_2 and P_3–S levels of fluorescence, in the absence of CO_2, depend on the concentration of added NH_4Cl. The dependence was investigated at pH 5.3 and 9.1. At pH 5.3 400 μM NH_4Cl almost saturates the fluorescence increase dependence. At pH 9.1, the effects are slightly more pronounced and saturation occurs at about 150 μM NH_4Cl. NH_4Cl was markedly less effective at pH 4.5; even 2.0 mM NH_4Cl did not appreciably raise the fluorescence. These results suggest that the unprotonated form NH_3 (ammonia) may be responsible for the effect. The arrows in Fig. 2 indicate the M_2 and P_3–S levels which were measured early in the experiment in the presence of CO_2 and later when CO_2 was added back after the addition of NH_4Cl.

It is known that the synthesis of nitrate reductase is repressed by NH_4Cl (ref. 6) and there is also evidence that when nitrate reductase is present, NH_4Cl incorporation quickly displaces nitrate reduction[7]. It therefore seemed plausible that the effect of NH_4Cl on fluorescence induction, might be related to the presence of nitrate reductase. To investigate this possibility, *Chlorella* was grown on a medium containing only NH_4Cl as a nitrogen source; such cells, we presume, contain no nitrate reductase. In Fig. 3A, O_2 evolution and fluorescence induction is recorded

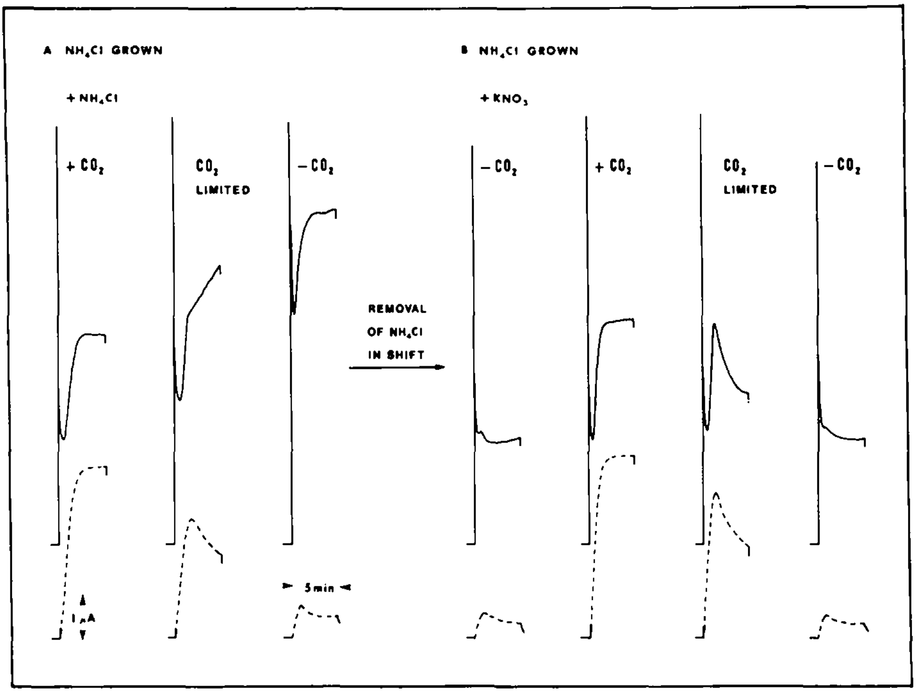

Fig. 3. Fluorescence (——) and O_2 evolution (- - -) induction in NH_4Cl-grown *Chlorella*. Conditions: light intensity, temperature, pH and gases as in Fig. 1. Curves A, cells measured in 2.0 mM NH_4Cl electrolyte; Curves B, after replacing the NH_4Cl with KNO_3 electrolyte.

for cells grown and measured in the presence of NH_4Cl. When CO_2 was removed, a rise in the M_2, P_3 and S fluorescence levels and a complementary decline of O_2 evolution occurred as seen in the second and third traces. This result is identical to that obtained with nitrate-grown cells measured in the presence of NH_4Cl (see Fig. 1). In the space of one light-dark cycle, the NH_4Cl electrolyte was withdrawn and replaced with nitrate electrolyte at the same temperature, pH and gas composition. The first curves in Fig. 3B, after the shift, show that the removal of NH_4Cl causes the fluorescence to fall to the same low level found in nitrate-grown cells in the absence of CO_2. Thus, the NH_4Cl effect does not appear to have anything to do with the presence or absence of nitrate reductase. When the CO_2 was added back, O_2 evolution was restored and a parallel increase in the fluorescence was observed, in the same manner as with nitrate-grown cells. Neither arginine nor glutamine (which also suppress nitrate reductase activity[8]) at twice the concentration of NH_4Cl used, had any effects on the fluorescence.

Since $MgCl_2$ is known to cause a 2.5-fold rise in the fluorescence yield when added to chloroplasts[2,3], an attempt was made to see if $MgCl_2$ would raise the fluorescence yield of CO_2 deprived *Chlorella*. It was found that even 100 mM $MgCl_2$ had no effect at all.

It is evident that, with cells unable to carry out significant electron transport, the yield of steady state chlorophyll fluorescence can vary 2.5-fold depending on whether NH_4^+ is present or not. Such a large change in the fluorescence yield unaccompanied by any detectable change in the rate of O_2 evolution, must be ascribed to an important modification of, at least, the relative rates of the two deexcitation processes—fluorescence and internal conversion.

Smaller parallel changes of electron transport and fluorescence yield have been attributed to "α-changes", *i.e.* changes in the partitioning of excitation between Photosystems I and II[5]. According to this hypothesis, in the absence of NH_4Cl α(the fraction of the excitation acting in Photosystem II) would be large in the steady state in the presence of CO_2, and must fall to a low value in the absence of CO_2. In contradiction to Bonaventura and Myers[5], we are obliged to suppose that α has a maximum value in Photosystem II light ("broad band blue") in the presence of CO_2. Since the steady-state fluorescence yield falls 2-fold when CO_2 is removed, the α-value must decline at least 2-fold and perhaps more since the inhibition of electron transport would be expected to lead to a more completely reduced condition of the Photosystem II acceptor Q. The maximum estimated values of α being about 0.6 (ref. 9), it must be concluded that CO_2 deprivation causes the value of α to fall to less than 0.3; that is, that Photosystem II units are converted into Photosystem I units! Similarly, we would have to conclude that NH_4Cl restored α to about 0.6. Both the qualitative contradiction requiring α to be a maximum in Photosystem II light, and the extremely large α-changes required to explain the effects of CO_2 and NH_4Cl on fluorescence, lead us to disfavor the Bonaventura and Myers[5] hypothesis.

In terms of the unit activation hypothesis of Bannister and Rice[1], we would say that in the absence of NH_4Cl, the removal of CO_2 leads to a conversion of roughly half the active Photosystem II units into the inactive form, incapable of sensitizing electron transport and characterized by a low fluorescence yield. NH_4Cl would then be supposed to reconvert Photosystem II units to the active state. As a result, when CO_2 is added back, only complementary changes in the steady-state fluorescence (dependent on the redox level of Q) would be observed. The fact that NH_4Cl has a large effect on the fluorescence yield, when net electron transport is almost entirely inhibited by the absence of CO_2, is strong evidence for two distinct physical states of Photosystem II units.

It is possible that the mechanism by which NH_4Cl modifies deexcitation processes is related to the ability of NH_4^+ to diminish the H^+ gradient formed in the light in chloroplasts[10]. Murata and Sugahara[11] and Wraight and Crofts[12] have shown the high fluorescence yield of 3-(3,4-dichlorophenyl)-1,1-dimethylurea-poisoned and unpoisoned chloroplasts falls when phenazine methosulfate or cofactors of cyclic electron transport are added. The quenching can be reversed by the addition of an uncoupler such as NH_4Cl. From these observations it was hypothesized that steep ion gradients can cause fluorescence quenching. Some of our observations can be interpreted in terms of the combined effects of a proton gradient and the reduction level of Q[13]. The low fluorescence yield of CO_2-deprived cells can be ascribed to a proton gradient supported by an endogenous cyclic electron transport. (It is necessary to assume that the quenching effect of the gradient predominates over the elevating tendency of QH.) The high fluorescence resulting from the addition of NH_4Cl to CO_2-free cells, can then be attributed to the dissipation of the H^+ gradient and to fully reduced Q. In the presence of both CO_2 and NH_4Cl, the intermediate fluorescence level would be the result of a combination of no proton gradient and a partially oxidized level of Q. Since in the presence of CO_2, the same fluorescence level occurs both with and without NH_4Cl, it must be supposed there is no appreciable gradient in either case. Thus quenching of the steady state fluorescence by an H^+ gradient would only occur in the absence of both CO_2 and NH_4Cl.

However, some of our observations are difficult to reconcile with H^+ gradient quenching. For example, the low M_2 level seen during induction when CO_2 is present also occurs when CO_2 is absent and NH_4Cl is present. In the latter case, Q is presumed to be fully reduced within the first second of illumination (P_2) and should remain so thereafter; the low M_2 level would have to be ascribed to a transitory H^+ gradient existing in the presence of uncoupler. A second troublesome point is that *Chlorella* grows well in cultures containing up to 10 mM NH_4Cl, a concentration 50 times that required to fully stimulate the fluorescence of CO_2-free cells at pH 5.3. If NH_4Cl is to be regarded as an uncoupler in whole cells, one would have to conclude that an H^+ gradient is not present during normal steady-state photosynthesis.

For the present these difficulties restrain us from fully embracing the hypothesis of H^+ gradient quenching in intact cells. We are, however, investigating the role of Photosystem I, which presumably generates ion gradients, in fluorescence induction of whole cells.

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