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ACTION OF HYDROXYLAMINE IN THE RED ALGA *PORPHYRIDIUM CRUENTUM*

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## SUMMARY

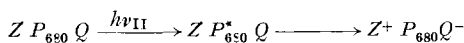
1. Oxygen exchange and fluorescence transient studies, made with the intact cells of red alga *Porphyridium cruentum*, suggest that hydroxylamine (NH<sub>2</sub>OH) inactivates the oxygen evolving capacity; at high enough concentrations, it also feeds electrons to the reductant side of Photosystem II. This result is in conformity with the studies made earlier with spinach chloroplasts and green alga *Chlorella* by other workers. Fluorescence transient data further indicate that the maximal rate of feeding of electrons by this reductant to Photosystem II is not as high as by water.

2. In Porphyridium, the early phase of the fluorescence transient is not markedly changed by NH<sub>2</sub>OH, but characteristic slow complex decay part of the fluorescence transient is completely abolished. However, the treated cells, like the normal cells, do need a dark exposure before subsequent illumination to fully restore the transient. NH<sub>2</sub>OH, at substrate concentrations, eliminates the recovery of the fluorescence rise curve (OI) in 3-(3',4'-dichlorophenyl)-1,1 dimethyl urea (DCMU)-treated cells. Also NH<sub>2</sub>OH together with DCMU abolishes all delayed light emission measured after a msec of illumination. These observations indicate that NH<sub>2</sub>OH inhibits the chemical back recombination reaction between Z<sup>+</sup> (the primary oxidant produced by light reaction II) and Q<sup>-</sup> (the primary reductant produced by light reaction II). These results, obtained with Porphyridium were obtained independently of BENNOUN (1970) who reached similar conclusions from his studies with green alga *Chlorella*.

3. Furthermore, the feeding of electrons by NH<sub>2</sub>OH appears to be very close to the reaction center of Photosystem II, as the photooxidation of NH<sub>2</sub>OH quenches the F696 band which has been suggested to be linked with the reaction center II.

## INTRODUCTION

It is currently believed that the excitation of the reaction center of pigment System II results in the photoreduction of an unknown acceptor Q and the photooxidation of another unknown donor Z. The reaction is represented as follows:



Abbreviation: DCMU, 3-(3',4'-dichlorophenyl)-1,1 dimethylurea.

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where  $P_{680}$  refers to the trap of pigment System II<sup>1</sup>. More than one oxidized donor equivalents per trap are postulated to react, in an unknown fashion, with water to evolve oxygen<sup>2-4</sup>. Hydroxylamine and 3-(3',4'-dichlorophenyl)-1,1 dimethylurea (DCMU) inhibit oxygen evolution<sup>5,6</sup>. DCMU has been known to block the flow of electrons from  $Q^-$  to the next intermediate in the electron transport chain between the two photosystems<sup>7</sup>. However, the inhibitory effect of hydroxylamine has been thought to be due to a block in the re-reduction of  $Z^+$  by water<sup>8</sup>. Also, it has recently been shown<sup>8-14</sup> that hydroxylamine, at high enough concentrations, feeds electrons on the reductant side of the pigment System II in isolated spinach chloroplasts and in green alga *Chlorella*. In this report, attempts have been made to evaluate the mode of action of hydroxylamine in a different test system -- the red alga *Porphyridium cruentum* which is a prototype of photosynthesis research<sup>15-17</sup>. Also, we have explored the functional site of action of this potent inhibitor of photosynthesis.

We report here the effects of hydroxylamine, in the presence and the absence of DCMU, on the fluorescence yield changes, fluorescence emission at 77°K, the delayed light emission, and on the rate of electron flow as measured by a viologen dye photo-reduction in *Porphyridium*. Our studies in *Porphyridium* have shown that (1) the measured luminescence changes are consistent with the dual biochemical action (blocking of electron flow from  $H_2O$  and feeding of electrons to System II) of hydroxylamine and (2) hydroxylamine feeds electrons to  $Z^+$ , very near the reaction center of System II. Independently, BENNOUN<sup>18</sup> came to similar conclusions in the green alga *Chlorella* and in spinach chloroplasts. His paper came to our attention during the preparation of this manuscript.

#### MATERIALS AND METHODS

*Porphyridium cruentum* was grown as previously described<sup>19</sup>. Cells grown in low light, that had smaller mucilaginous envelopes, were collected and used; they were suspended in a buffer consisting of 0.1 M  $NaHCO_3$  and  $K_2CO_3$  with 15.2 g of NaCl per l of buffer (pH 8.5).

Oxygen evolution was measured with a rate electrode<sup>20</sup>. The cells were deposited on a flat platinum cathode that was polarized -0.6 V with reference to a large Ag/AgCl electrode. The electrodes were immersed in an electrolyte (consisting of 0.05 M phosphate buffered at pH 7.8, 0.1 M KCl and hydroxylamine when used). 2%  $CO_2$  in argon was bubbled. The sample was illuminated with white light of saturating intensity.

For the determination of the  $O_2$  uptake by a reduced low-potential dye, methyl viologen, reduced by reactions of photosynthesis, a Clark type concentration electrode was used<sup>21</sup>. The electrode was separated from the magnetically stirred medium (consisting of phosphate buffer, KCN or  $NaN_3$  and hydroxylamine if used.) The temperature was 20°. The cells were illuminated from both sides with two 300-W incandescent lamps; the intensity of this illumination was high enough to saturate photosynthesis.

Fluorescence measurements were made with a spectrofluorometer described earlier<sup>22</sup>. The protocol for measuring fluorescence transients was the same as that of MUNDAY AND GOVINDJEE<sup>23</sup>. The excitation was by a broadband green light peaking at 540 nm with an intensity of approximately  $4.4 \cdot 10^4$  ergs  $\cdot$  cm<sup>-2</sup>  $\cdot$  sec<sup>-1</sup>. Fluorescence was measured at 685 nm with 6.6-nm band width. A Corning 2-63 glass filter was used

before the analyzing monochromator to cut off the exciting light. The procedure of measuring emission spectra at 77°K was the same as that of CHO AND GOVINDJEE<sup>24</sup>. The spectra were corrected for the spectral efficiency of the monochromator and photomultiplier (EMI 9558 B).

Delayed light emission was measured with an apparatus as outlined by STACY *et al.*<sup>14</sup>. Algal suspension was excited by a rectangular pulse of He-Ne laser light ( $\lambda$ , 633 nm) with an intensity of  $5.6 \cdot 10^3$  ergs·cm<sup>-2</sup>·sec<sup>-1</sup>, 4.5 msec in width and repeated 25 times/sec.

## RESULTS AND DISCUSSION

### Oxygen exchange

First, we had to establish that in *Porphyridium* hydroxylamine indeed inhibits oxygen evolution as it does in green plant system. For this purpose O<sub>2</sub> exchange measurements were made. When *Porphyridium* cells were placed on a rate electrode, replacement of buffer with buffer containing 1 mM hydroxylamine led to a substantial inhibition of oxygen after 10 min of incubation (Fig. 1). Longer incubation decreased the rate further. It took about an hour for the complete inhibition of O<sub>2</sub> evolution. At higher concentrations no O<sub>2</sub> evolution could be detected with our system.

Feeding of electrons by NH<sub>2</sub>OH, at higher concentrations, was assayed by following O<sub>2</sub> uptake when low-potential dye methyl viologen was used. It has been shown that viologen dyes accept electrons from the terminal Photosystem I reduced acceptor X<sup>25</sup>. As reduced viologens are highly auto-oxidizable they react with molecular oxygen yielding H<sub>2</sub>O<sub>2</sub>. If the catalytic splitting of H<sub>2</sub>O<sub>2</sub> by endogenous catalase is prevented (by cyanide or azide), it is expected that there will be twice as much O<sub>2</sub> consumption in cells when hydroxylamine is a donor than when H<sub>2</sub>O is a donor to Photosystem II<sup>26</sup>. Our results (Fig. 2) indeed show that *Porphyridium* cells with 10 mM NH<sub>2</sub>OH have 2-fold (0.039  $\mu$ mole O<sub>2</sub> per ml) rate of O<sub>2</sub> uptake than those without it (0.021  $\mu$ mole O<sub>2</sub> per ml). Thus, the higher rate of O<sub>2</sub> uptake in the NH<sub>2</sub>OH-treated than in the normal *Porphyridium* cells confirm that hydroxylamine feeds electrons to the pigment System II replacing water, as it does in isolated chloroplasts<sup>8</sup>.

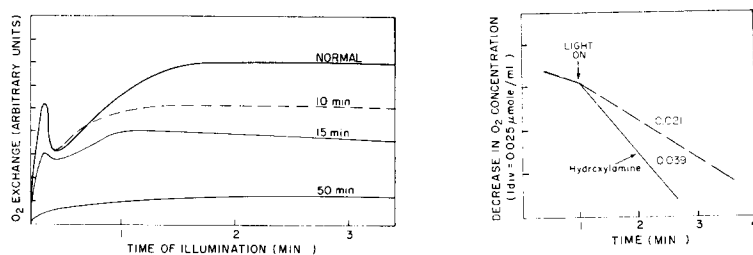


Fig. 1. Time course of the rate of oxygen evolution in the red alga *P. cruentum* before (normal) and after the addition of hydroxylamine. The numbers on the curves indicate the period of incubation in hydroxylamine; electrolyte: phosphate buffer (pH 7.8), 0.05 M; KCl, 0.1 M; hydroxylamine,  $10^{-3}$  M; temperature, 20°; 2% CO<sub>2</sub> in argon;  $4 \cdot 10^4$  ergs·cm<sup>-2</sup>·sec<sup>-1</sup> white light.

Fig. 2. Effect of hydroxylamine on O<sub>2</sub> uptake in the presence of methylviologen in *P. cruentum*. Reaction mixture: phosphate buffer (pH 8.0), 0.05 M; NaCl, 0.25 M; methyl viologen,  $5 \cdot 10^{-4}$  M; NaN<sub>3</sub>,  $10^{-3}$  M; hydroxylamine,  $10^{-2}$  M. Total volume, 3.0 ml.

### Fluorescence transients

Although both DCMU and  $\text{NH}_2\text{OH}$  inhibit the oxygen evolution, chlorophyll *a* fluorescence transients with these poisons differ markedly (Fig. 3). In the normal Porphyridium cells, without any added poison, the fluorescence yield shows an initial fluorescence "O", a shoulder "I", then a temporary decline to dip "D", from which it again rises to a peak "P". From the level P, the well known complex slow decay to quasi-steady state "S" level occurs (see ref. 23). If the only function of  $\text{NH}_2\text{OH}$  in Porphyridium is to replace water as a donor then one would expect a similar transient as that of normal (OIDPS). This is not the case (Fig. 3).

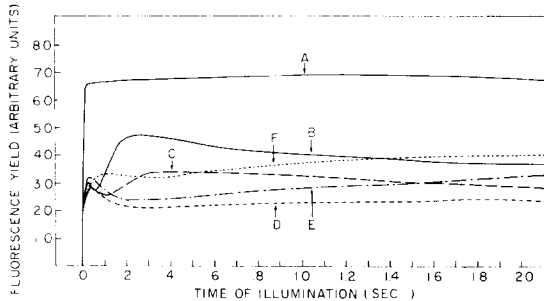


Fig. 3. Fluorescence yield of chlorophyll *a* at 685 nm as a function of time of illumination (0–2.0 sec) in *P. cruentum*. A, DCMU,  $10^{-5}$  M, or DCMU,  $10^{-5}$  M, plus  $\text{NH}_2\text{OH}$ ,  $10^{-2}$  M; B, normal untreated cells; C,  $\text{NH}_2\text{OH}$ ,  $10^{-5}$  M; D,  $\text{NH}_2\text{OH}$ ,  $10^{-4}$  M; E,  $\text{NH}_2\text{OH}$ ,  $10^{-3}$  M; F,  $\text{NH}_2\text{OH}$ ,  $10^{-2}$  M.

On increasing the concentration of  $\text{NH}_2\text{OH}$  from  $10^{-5}$  to  $10^{-4}$  M (*cf.* Fig. 3C and D with B) the time course of chlorophyll *a* fluorescence changes from that of normal: P decreases and O–I rise is slowed down. Two alternate explanations can be given for this effect: (i) hydroxylamine, at these low concentrations, only blocks the reaction between  $\text{H}_2\text{O}$  and oxidized donor Z, but does not feed electrons to Z. As  $Z^+$  cannot be reduced, it, in turn, cannot reduce Q. Assuming Q continues to act as a quencher of chlorophyll *a* fluorescence, the yield will remain low. (ii) the second possibility is that the reduced Q in cells with hydroxylamine is reoxidized at a faster rate than in the normal cells as it happens in the case of cells with added methyl viologen<sup>27</sup>. This implies that hydroxylamine (in its oxidized form), somehow, accepts electrons from some reduced intermediate in the electron transport chain. To distinguish between the two cases, we made the following experiment. We chose the concentration of  $\text{NH}_2\text{OH}$  such that the level of fluorescence was very close to D (a suppressed variable fluorescence as in Fig. 3D). After fluorescence reached this low level, the exciting light was turned off and after one second of dark interval, it was turned on again. No fluorescence transient was observed and the yield remained at the same low level. If  $Q^-$  had been reoxidized at a faster rate, we would expect an immediate reduction of Q when the exciting light was given after a dark interval and, thus, a fluorescence transient. Therefore, the above experiment rules out the possibility of a fast reoxidation of  $Q^-$  by hydroxylamine in intact cells of Porphyridium. As in the normal cells, a long dark period (about 5 min) was necessary to restore the fluorescence transient. We believe that low concentration of hydroxylamine blocks  $\text{H}_2\text{O}$  from donating electrons and this causes the yield of chlorophyll fluorescence *in vivo* to be low, and it has no significant effect on the dark restoration of Q.

However, if the amount of hydroxylamine is raised from  $10^{-4}$  to  $10^{-2}$  M, the fluorescence yield at the S level increases (*cf.* Fig. 3D with E and F). This increase in the yield could be due to the onset of feeding of electrons by hydroxylamine resulting in the reduction of  $Q$  and consequently an increase in fluorescence (destruction of the quencher). It seems that the donation of electrons by  $\text{NH}_2\text{OH}$  occurs at a slower rate than that by  $\text{H}_2\text{O}$  in the normal cells. This could explain why the variable part of the fluorescence transient in the presence of  $10^{-4}$ – $10^{-2}$  M  $\text{NH}_2\text{OH}$  resembles that of normal cells at low intensity of exciting light (light is limiting). Thus, our experiments with Porphyridium suggest that the efficiency of feeding electrons to Photosystem II by hydroxylamine is lower than that by  $\text{H}_2\text{O}$ . This is in agreement with the results on isolated chloroplasts obtained by IZAWA *et al.*<sup>8</sup>. These authors assumed that an increase in the yield of chlorophyll fluorescence in isolated chloroplasts upon the addition of hydroxylamine over the normal was due to the inhibition of back reaction between  $Z^+$  and  $Q^-$ . The experiment described below shows that, indeed, high concentration of hydroxylamine inhibits this back reaction of  $Z^+$  with  $Q^-$  in Porphyridium. Fig. 4a shows that when DCMU is added chlorophyll fluorescence yield rapidly rises from "O" to "I" and remains at a high level (Curve 1). If the exciting light is turned off for a minute, and then turned on again, the original transient is restored (Curve 2). The same experiment was repeated with cells to which both  $\text{NH}_2\text{OH}$  and DCMU were added in the dark. The initial transient (Fig. 4b, Curve 3) is similar to that of Fig. 4a. But when the light was turned off for a minute and then turned on again, the fluorescence yield remained high, *i.e.* it did not show the same O–I rise (Curve 4). Even a longer dark period did not recover the transient. This is because hydroxylamine reduces  $Z^+$  at a faster rate than the back reaction between  $Z^+$  and  $Q^-$ . As DCMU is present,  $Q^-$  cannot be oxidized by A, the adjacent intersystem intermediate. Thus,  $Q^-$  remains reduced in the dark and the fluorescence yield remains high. But in the case

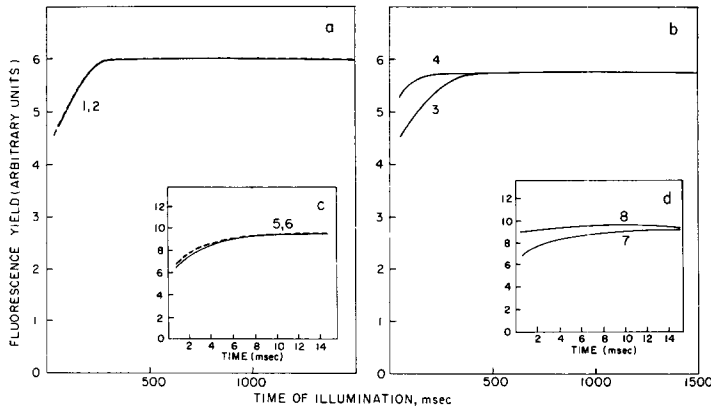
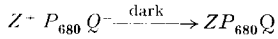


Fig. 4. Recovery of the fluorescence transient in the dark in *P. cruentum*. (main figures: a, b), and in *Chlorella pyrenoidosa* (inserts: c, d). Porphyridium: Curve 1 in (a), initial transient in the presence of  $2 \cdot 10^{-5}$  M DCMU; Curve 2 in (a), the same after 10 min darkness; Curve 3 in (b), initial transient in the presence of  $2 \cdot 10^{-5}$  M DCMU plus  $10^{-2}$  M hydroxylamine; Curve 4 in (b), the same after 10 min darkness. Chlorella (inserts): Curve 5 in (c), initial transient in the presence of  $10^{-5}$  M DCMU; Curve 6 in (c), the same after 1 min darkness; Curve 7 in (d), initial transient in the presence of  $10^{-5}$  M DCMU plus  $10^{-2}$  M hydroxylamine; Curve 8 in (d) the same after 1 min in darkness. (Note the difference in time it takes to complete the transient in Porphyridium and in Chlorella; the intensity of excitation for the latter was higher than for the former.)

of untreated (normal) cells  $Z^+$  can not be reduced by  $H_2O$  as more than one charge have not accumulated on  $Z$ . Hence in this case  $Z^+$  will recombine with  $Q^-$  as:



This leads to the recovery of the original fluorescence transient upon subsequent illumination. We have confirmed this observation with *Chlorella* (Figs. 4c and 4d; Curves 5–8) and isolated chloroplasts from oats. BENNOUN<sup>18</sup> has independently shown this result for *Chlorella* and chloroplasts from spinach. This observation agrees with the earlier findings of BENNOUN AND JOLIOT<sup>10</sup> that, unlike  $H_2O$ , photooxidation of hydroxylamine does not require any storage of positive equivalents on the oxidizing side of Photosystem II (activation reaction). Thus, it is evident that some residual photooxidation of  $NH_2OH$  occurs in the presence of DCMU.

#### Delayed light emission

To further test the validity of our conclusion that hydroxylamine inhibits  $Z^+ Q^-$  recombination, we measured delayed light emission in the 1–10-msec region. Fig. 5

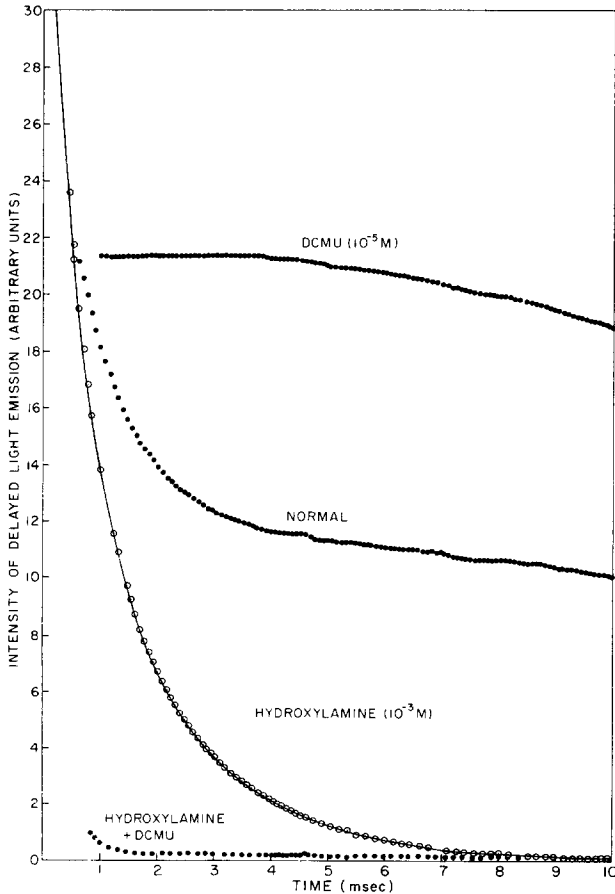


Fig. 5. Intensity of delayed light emission as a function of time in the 1–10-msec region in *P. cruentum*. Square wave excitation ( $\lambda = 632.8$  nm). Details of treatments are shown in the figure.

shows the delayed light emission in 1–10-msec region in normal Porphyridium, those treated with DCMU ( $10^{-5}$  M), with hydroxylamine ( $10^{-3}$  M) and with both DCMU and hydroxylamine. Addition of hydroxylamine to the cell causes the delayed light to have a faster decay than in the normal cells. This decay is not exponential. Addition of DCMU to the cell causes the fast decay to disappear. In the 1–10-msec time range, the delayed light in cells with DCMU decays very slowly and its intensity is higher than that given off by cells with hydroxylamine added. The delayed light of normal cells is intermediate between the DCMU and hydroxylamine cases. Similar results have been obtained for *Chlorella* by STACY *et al.*<sup>14</sup> in the 1–10-msec range. Addition of both  $10^{-5}$  M DCMU and  $10^{-3}$  M hydroxylamine to the cell causes a dramatic decline in the intensity of the delayed light.

If the function of hydroxylamine, at high concentrations, in the cell is to reduce  $Z^+$  as soon as it is oxidized by light so that  $Z^+$  cannot exist as a stable state as in the normal cell, then there should be no chemical back reaction between  $Q^-$  and  $Z^+$  when hydroxylamine is added. In the model of STACY *et al.*<sup>14</sup> delayed light originates from the fusion of triplets, and these triplets are formed due to (1) intersystem crossing during trapping of photons (fast component), and (2) a back recombination reaction between  $Z^+$   $Q^-$  (slow component). The measured decay curve of the delayed light of cells with hydroxylamine added agrees exactly with the theoretical curve calculated with the assumption that there is no chemical back reaction, only intersystem crossing<sup>21</sup>.

In the presence of DCMU alone,  $Q$  is reduced and  $Z$  oxidized by light, but  $Q^-$  cannot be reoxidized to  $Q$  due to a block in the electron transport chain<sup>7</sup>. Therefore, the trapping center is closed and it cannot receive any further energy; this is shown by the increase in fluorescence yield when DCMU is added<sup>31</sup>. With the reaction center in the  $Z^+$   $P_{680}Q^-$  state, triplets will be produced by the recombination of  $Z^+$  with  $Q^-$  at a slow rate. The rate of formation of  $Z^+$   $P_{680}Q^-$  obviously is proportional to the rate of photons absorbed. Using a rectangular pulse of light given 25 times/sec, as used in the present experiments, the rate of producing  $Z^+$   $P_{680}Q^-$  is much faster than its decay to  $Z$   $P_{680}Q$ . Hence, at the steady state number of  $Z^+$   $P_{680}Q^-$  is much greater than  $Z$   $P_{680}Q$ . Therefore, the delayed light emission, when DCMU is added, is due predominantly to the triplets which are produced by this chemical back reaction (LAVOREL<sup>18</sup>; STACY *et al.*<sup>14</sup>; *cf.* refs. 29 and 30).

In the untreated algae, at the steady state conditions, a certain amount of reaction centers will remain in the  $Z^+$   $P_{680}Q^-$  state for a short time because at the steady state some of the electron transport intermediates are reduced so that they cannot reoxidize  $Q$ . Within that time  $Z^+$   $P_{680}Q^-$  can be assumed to decay by a back reaction to form triplet states; hence, in the normal case, triplets are suggested to be produced both by the intersystem crossing and by the chemical back reaction (see refs. 14 and 28).

When hydroxylamine is added alone, there is no significant contribution to delayed light emission from the chemical back reaction. However, when DCMU is added alone, the delayed light is suggested not to originate from the intersystem crossing at the trap but from the back reaction. Addition of both DCMU and hydroxylamine together should then eliminate the delayed light as is shown in Fig. 5.

Our results on the delayed light emission in Porphyridium with hydroxylamine added can best be explained by assuming that hydroxylamine inhibits the back

reaction between  $Z^+$  and  $Q^-$ , and this inhibition of back reaction is due to re-reduction of  $Z^+$  by hydroxylamine. Thus both the fluorescence transient and delayed light emission studies suggest that hydroxylamine feeds electrons directly to  $Z^+$ .

#### Emission spectra at 77°K

There are three main emission bands of chlorophyll *a* at 77°K: F685, F696 and F712 representing emission peaks at 685, 696 and 712 nm (see refs. 16, 32 and literature citations there in). Earlier analyses<sup>24,33</sup> of these emission bands indicate that F685 is emitted mainly from the bulk chlorophyll *a* of System II, F696 is associated mainly with the energy trap of Photosystem II and F712 largely with chlorophyll *a* from the pigment System I. We measured emission spectra at 77°K of normal cells and cells treated with  $\text{NH}_2\text{OH}$  in the presence and the absence of DCMU (Fig. 6). If cells were preilluminated with white light from a 60-W tungsten lamp (for about 5 min) and then quickly frozen, there was a considerable lowering of the F696 band in the case of cells treated with hydroxylamine alone. However, if the cells were not preilluminated, no lowering of the F696 band was observed. (The amount of lowering of this band with respect to F685 varied from culture to culture.) Normal cells with or without DCMU and cells treated with  $\text{NH}_2\text{OH}$  plus DCMU gave essentially similar emission spectra whether the cells were preilluminated or not.

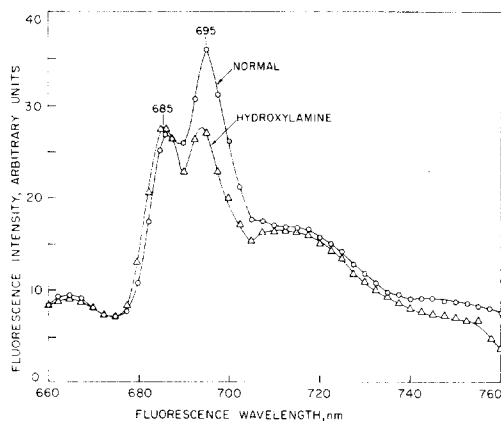


Fig. 6. Emission spectra of normal and hydroxylamine treated *P. cruentum* at 77°K. Curves normalized at 660 nm because we do not expect changes in the yield of phycocyanin fluorescence; samples pre-illuminated at room temperature and then cooled; hydroxylamine,  $5 \cdot 10^{-4}$  M.

It is known that the manganese-deficient cells have lower intensity of the F696 band than the healthy cells<sup>34</sup>. Studies of CHENIAE AND MARTIN<sup>34</sup> indicate that  $\text{NH}_2\text{OH}$  extracts  $\text{Mn}^{2+}$  in dark. We do not believe that the lowering of F696 band in preilluminated hydroxylamine treated cells is due to the loss of manganese as we do not see a decrease of this band in hydroxylamine plus DCMU treated cells, and cells with hydroxylamine that were not preilluminated. In the case of hydroxylamine treated cells, preillumination will cause a steady photooxidation in contrast to all the other cases. Although in the case of cells with both hydroxylamine and DCMU, we expect a residual photooxidation of hydroxylamine, this small amount of photooxidation is not expected to cause a substantial lowering of the F696 band. Thus, we assume that



photooxidation of  $\text{NH}_2\text{OH}$  leads to a quenching of fluorescence at 696 nm. It is, therefore, reasonable to assume that  $\text{NH}_2\text{OH}$  feeds, at least in *Porphyridium*, very close to the System II reaction center as F696 has been suggested to originate in or near the System II trap.

## ACKNOWLEDGMENTS

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