EFFECT OF SYSTEM II INHIBITORS ON LUMINESCENCE IN NH_2OH - PRETREATED CHLORELLA

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Summary

3(3,4-dichlorophenyl)-1,1'-dimethylurea, 3'methyl,3(3,4-dichlorophenyl)-1, 1'-dimethylurea, 3(4 chlorophenyl)-1,1'-dimethylurea, phenyluretan, chlortoluron, cycluron, atrazine, o-phenanthroline elicit the same fast phase ($\tau \cong 5$ µs) in the luminescence decay of *Chlorella* pretreated with hydroxylamine. The induction of the decay pattern during a series of flashes and its dependence upon the flash duration are explained by a competition between recombination of charges (giving rise to luminescence) and a relatively inefficient oxidation of hydroxylamine; the fast phase requires an additional assumption. The inhibitory potency of the System II inhibitors as judged from their effect on the development of the fast phase is in agreement with their known action on photochemical activity. The results suggest a uniform action of these inhibitors on the acceptor side of System II.

INTRODUCTION

In *Chlorella* pretreated with high concentration of NH_2OH, a significant change of pattern of luminescence decay is observed in the 1-100 µs range : a fast phase ($\tau \cong 5 - 10$ µs) is suppressed, while a slower phase ($\tau \cong 50 - 70$ µs) becomes dominant (1). The Z system, which is responsible for the storage of + charges preceding water splitting and oxygen evolution, is disconnected from the System II reaction center by NH_2OH in the same range of concentration (2). Therefore, a reasonable assumption is that the above fast luminescence phase reflects the transfer of + charges from the primary donor Y to the Z system (1). Addition of DCMU (3(3,4-dichlorophenyl)-1,1'-dimethylurea) to such pretreated material elicits another fast phase ($\tau = 5$ µs); it was hypothesized that this phase disclosed the formation of a Q - DCMU complex, providing a parasitic stabilization of the luminescence species C_+^- (or $^+YChlQ^-$) against recombination (1).

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In the present study, we have tested the latter hypothesis looking at the effect of DCMU concentration and comparing the effects of DCMU and other System II inhibitors. The new results have also a bearing on the problem of the sustained luminescence emission under repetitive flash excitation in the NH_2OH + DCMU condition (1), in view of the former finding (3) that in such a system light induces the formation of a photoinactive, non-luminescent state C^- (or $YChlQ^-$).

EXPERIMENTAL

The luminescence intensity L was monitored with the laser-phosphoroscope already described (4). The main difference from the experimental conditions of the preceding report (1) is that we used a 4mW continuous helium-neon laser (CW Radiation, Model S-405 R) allowing variation of the flash duration t_I, instead of the pulsed xenon laser with constant flash duration (0.3 µs). In order to achieve light saturation (as indicated by the fluorescence rise-time), much longer flashes were necessary ($t_I \geq 50$ µs).

Chlorella pyrenoidosa cultivated as previously described (1) was resuspended in fresh culture medium (pH 5.4). Algae were incubated with $3.3\ 10^{-3}$ M NH_2OH during 15 mn prior to measurement of luminescence in the presence of various concentrations of inhibitors. In the standard protocol, we record the average of 10^4 L decays from successive samples of the algal suspension, each receiving 200 flashes. Other protocols will be indicated as required.

CMU and DCMU (technical grade, Dupont de Nemours) were twice recristallized in acetone . DCMU was methylated according to Anderson and Cree (5) and chromatographed on silica plate; purity was checked from NMR spectrum. Chlortoluron and Cycluron (commercial brand) were recrystallized three times in acetone. Atrazine was supplied 99 % pure from Ciba-Geigy. o-phenanthroline and phenylurethane (Prolabo) were not further purified.

RESULTS AND DISCUSSION

The concentration effect of DCMU is shown on Figure 1A. Two important points must be noted : a) 50 µs flashes induce a fast phase (henceforth called DCMU-type phase) of larger amplitude α_1 (see definition on Fig. 3) than previously reported with submicrosecond flashes under otherwise identical conditions (compare this figure and figure 4 of ref. (1)), b) α_1 increases with the DCMU concentration, but the corresponding time constant is invariant ($\tau_1 \simeq 5$ µs), in agreement with previous results (1)); the DCMU-type phase is always followed by the same slower decay (henceforth called NH_2OH-type phase) as in the control, the amplitude α_2 of the latter being complementary to α_1 (this property stems from the invariance of the initial intensity L_o, see ref. (1)).

Table 1

Comparison of pI_{50} of System II inhibitors in their action on luminescence decay in NH_2OH pretreated *Chlorella* and on photochemical activity (according to published data).

Inhibitor	pI_{50} Luminescence	pI_{50} Photochem. activity (1)
3(3,4-dichlorophenyl) 1,1'-dimethylurea	5.65	6.75 [6], 7.5 [7], 6.77 [8]
Atrazine	5.11	6.6 [7], 6.2 [8]
CMU	5.07	6.3 [7], 6.15 [8]
Chlortoluron	4.2(2)	
Cycluron	4.5(2)	5.25 [6]
o-phenanthroline	4.44	4.5 [7]
phenylurethan	3.7(2)	
MeDCMU	< 4(2)	

(1) reference between brackets.
(2) these data were obtained from few points (cf. Fig. 1) and are considered only as order of magnitude.

The first point clearly shows that the DCMU-type phase cannot be accounted for by a stabilization process, as would be required by the DCMU complex hypothesis : briefly, the reason is that a single turnover stabilization step of ~ 5 µs should be completed <u>during</u> a 50 µs flash and, consequently, should not show up <u>after</u> such a flash. The dependency of α_1 on t_I extends to longer flashes : for instance, α_1 is observed to increase by 17 % when t_I is increased from 50 to 95 µs (at 2 10^{-5}M DCMU). This dependency will be discussed below. The second point suggests a mixture of types as a simple formal explanation of the concentration effect, two populations of centers coexist : those which are associated with DCMU and have been blocked in the C^- state as a result of photooxidation of NH_2OH are responsible for the DCMU-type phase and those which are not associated with DCMU or are associated, but not yet blocked continue to decay following a NH_2OH-type phase. Accordingly, monitoring the residual NH_2OH-type phase after completion of the fast DCMU-type phase (*i. e.* at $t_L \geq 50$ µs) yields a measure of the second population; thus α_2 can be used to quantitate the inhibitory potency of DCMU and other similar poisons.

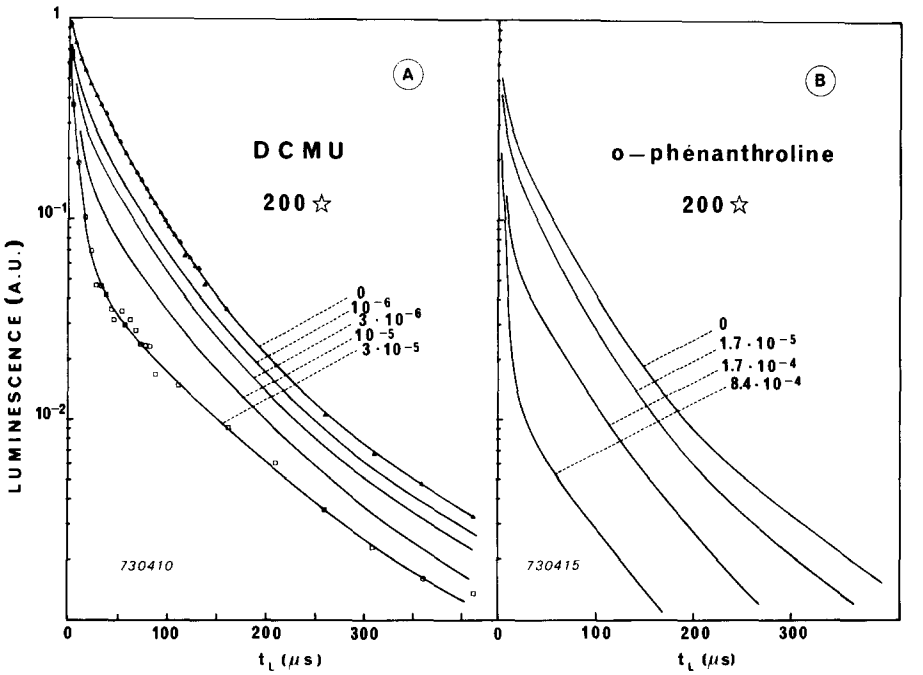

Fig. 1 Semi-log plot of decay of luminescence (L) in *Chlorella* pretreated with 3.3 10^{-3}M NH_2OH in the presence of DCMU (A) or o-phenanthroline (B) at the indicated molar concentrations. Chlorophyll : 50 μg ml^{-1}.Temperature : 20°C.Standard protocol : average of 50 samples X 200 flashes of 50 μs duration; flashing frequency : 20 Hz. For clarity, the experimental points have been indicated for only two curves.

All the other System II inhibitors which we have examined proved to be completely isomorphous with DCMU, regarding the induced change in decay pattern and the lifetimes of both phases; the case of o-phenanthroline is shown as an example on figure 1B. Again, the constancy of lifetime of the DCMU-type phase irrespective of the nature of the inhibitor strongly argues against the original hypothesis : indeed, if this phase directly reflected the kinetics of a Q-inhibitor complex, one would have expected offhand that its kinetic parameters be dependent upon the chemical nature of the inhibitor. As pointed to above, we have used the variation of α_2 with inhibitor concentration in the standard 200 flashes protocol to calculate the pI_{50}'s (-log of molar concentration producing 50 % inhibition) (Table 1). Although the relative order of inhibitory potency matches exactly that of published data, a large systematic deviation in the sense of an apparent lower activity is found. (pI_{50} from published data may be somewhat variable according to the test system -chloroplasts whole cells, algae -, but we are only concerned here with orders of magnitude). The low apparent activity may be seen directly

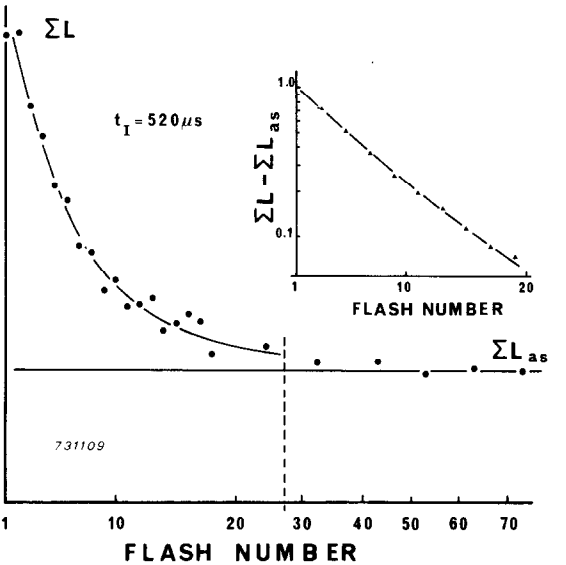

Fig. 2 Induction of luminescence in *Chlorella* pretreated with $3.3.10^{-3}$ NH_2OH, in the presence of 2.10^{-5}M DCMU. Chlorophyll : 25 g ml^{-1}. Temperature : 20°C. Flash duration : 520 µs. Flashing frequency : 20 Hz. Average of 20 samples. Left : direct recording of ΣL, the sum of light taken over the full dark interval (∿ 50 ms); note the non zero asymptotic value. Right : semi-log plot of the variable part of ΣL from left figure.

on Figure 1 as a non-negligible NH_2OH- type phase in the presence of nearly saturating inhibitor concentrations.

This discrepancy was finally resolved when, following the observation by Etienne (9) that in the NH_2OH + DCMU condition a large number of saturating flashes were necessary to bring all centers in the C^- state, thereby exhausting the NH_2OH oxidizing capacity of the sample, we realized that, correspondingly, the amount of luminescence ΣL emitted after each flash of a series decreased exponentially as a function of the flash number to a constant level (Fig. 2). Applying a simple competition scheme (10) to the DCMU (or otherwise) inhibited centers :

$$C \underset{k_L}{\overset{k_I}{\rightleftharpoons}} C_+^- \xrightarrow{k_P} C^-, \qquad (1)$$

where k_I, k_L and k_P are the rate constants for the photochemical reaction, the luminescence recombination reaction and the NH_2OH oxidation respectively, one finds that the concentration of C_+^- after each flash (and thus the L intensity) is indeed an exponential function of the flash number. Obviously, the asymptotic L level is proportional to the concentration of uninhibited centers (however see below for the effect of t_L range on L measurements) ;

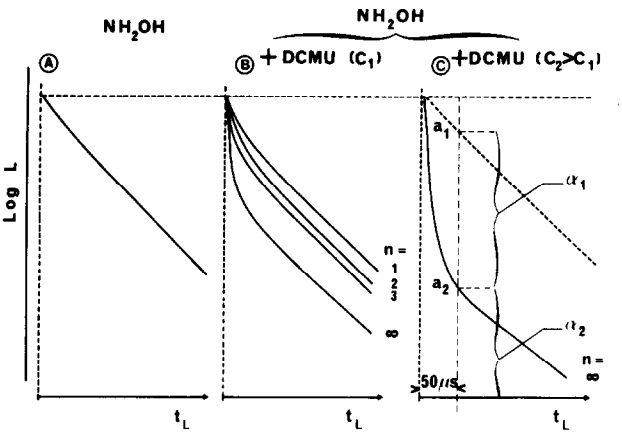

Fig. 3 Schematic of evolution of pattern of L decay (semi-log plot) during induction due to competition between recombination and NH_2OH oxidation (Eq. (1)). A. NH_2OH-type phase. B. Development of the fast DCMU-type phase as a function of flash number n at a DCMU concentration C 1. C. Same as B with a higher DCMU concentration C 2; α_1 and α_2 are calculated from the semi-log plot at t_L = 50 µs, after completion of the DCMU-type phase (dashed line is the pure NH_2OH-type phase) : $a_2 = \log(\alpha_2)$, $a_1 = \log(\alpha_1 + \alpha_2)$.

therefore, pI_{50}'s calculated from this asymptotic levels should be correct. The pattern of decay and its change during a flash sequence according to the above interpretation is schematically depicted in Figure 3. This shows that the average decay recorded during the first 200 flashes in the standard protocol necessarily yields a residual α_2 higher than does the asymptotic decay (n = ∞ in Fig. 3). This interpretation was checked by the following experiments : a) if the inhibitory activity of DCMU is calculated from the asymptotic values of α_2, pI_{50} was found equal to 6.5 (Fig. 4) ; the same value was also found in parallel experiments on inhibition of oxygen evolution by *Chlorella* under low light intensity ; b) comparing the average L decay over flashes no 1 to 200 and 201 to 400 yields the same pattern of decay with α_2 relatively larger in the first than in the second case (not shown) ; c) the induction curve of ΣL (Fig. 2) is terminated after a number of flashes which is not dependent upon the DCMU concentration (not shown ; the parameters in Eq. 1 in effect do not depend on that). The scheme of Eq. 1 readily explains why the α's are t_I dependent (see comment concerning Fig. 1): if $k_L >> k_P$, the + charge in C_+^- has too short a lifetime to be available for NH_2OH oxidation the latter process being relatively inefficient except during the flash when a quasi steady-state of C_+^- is established; therefore, the larger t_I the more C_+^- will be formed per flash and the faster the induction

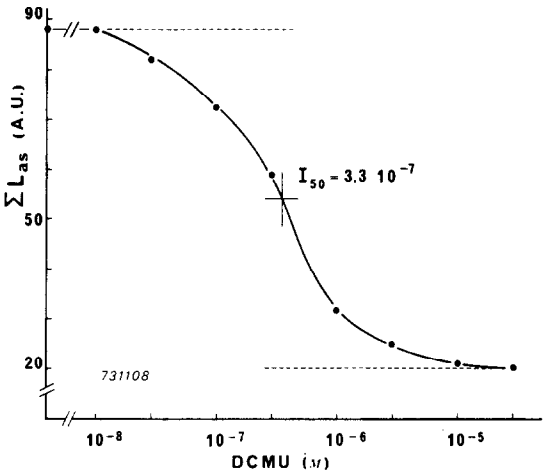

Fig. 4 Effect of DCMU concentration on asymptotic value of α_2 (see Fig. 3) in NH_2OH pretreated *Chlorella*. Conditions as in Fig. 2.

decay of α_2. The complete time integral ΣL (taken from a lower bound close to $t_L = 0$) comprises a DCMU insensitive contribution from the fast DCMU-type phase : this is seen in the induction curve of figure 2 (non zero assymptotic level, in spite of saturating DCMU concentration) and in Figure 4 (lower plateau in the high concentration range).

While the present results clearly disprove the hypothesis that the DCMU-type phase is a direct reflection of the formation (or rearrangement) of a Q-DCMU complex (1) they obviously do not oppose the idea that such a complex may exist. It is only required that, when centers are engaged in this hypothetical association and besides in the inactive C^- state, they are endowed with the property of a very fast photochemical turnover resulting in the DCMU-type phase. Further experimental results and elaboration of ideas concerning this point will be published in a forthcoming paper. The simplicity and similitude of effects of the (chemically) different inhibitors which we have tested and the fact that their activity is not dependent upon the integrity of the Z system is in favor of a single site of action- or a single target - on the acceptor side (Q) of the System II centers.

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