RESEARCH NOTE
Bot. Bull. Academia Sinica **14**: 65-69 (1973)

GRAMICIDIN D ENHANCED FERRICYANIDE HILL REACTION AND FLUORESCENCE INTENSITY DURING ELECTRON TRANSPORT— AN INDICATION OF A POSSIBLE COUPLING SITE ON THE H_2O SIDE OF PHOTOSYSTEM II[1]

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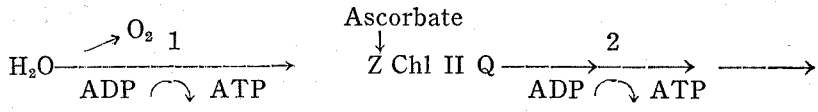
(Received for publication March 30, 1973)

The existence of a coupling site between the water splitting system and the second light reaction has been suggested by several investigators (Böhme and Trebst, 1969; Jacobi, 1963). From their study of photo-oxidation of ascorbate, Böhme and Trebst (1969) have reported that: 1). after substracting the basal rate of electron transport, the H_2O supported electron transport shows a P/2e ratio of 2 (Böhme and Trebst, 1969; Izawa and Good, 1968); 2). in spite of the fact that the coupled electron transport is stimulated by ascorbate, the latter reduces the P/2e ratio to one; this ascorbate-supported electron transport is sensitive to DCMU[3] inhibition and to the energy transfer inhibitor, DCCD[3]; 3). in the presence of unconpler NH_4Cl, ascorbate no longer stimulates the electron flow. Based on these and other evidences, they suggest the following scheme with the location of the two coupling sites on the electron transport chain and the site where ascorbate donates its electron to system II.



Scheme 1. A possible ATP coupling site on the H_2O side of system II.

Renger's finding (1972) that uncouplers do not change the half time of the limiting electron transfer reaction between water splitting system and

(1) This work was supported by the Public Health Service (GM-13470-03), the Center for Naval Analysis (Sub 5-29101) awarded to G. E. Hoch.

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(3) Abbreviations: DCMU = 3-(3, 4-dichlorophenyl) -1, 1 dimethylurea, DCCD = dicylohexylcarbodiimide.

light reaction II seems to put some doubt on such a suggestion. However, without knowing the exact mode of operation of the system II electron transport and the coupled and uncoupled conditions, it is premature to draw any conclusion from experiments conducted under special conditions such as repetitive double flash excitation conditions employed by Renger. Further investigation of the location of coupling sites is still necessary.

According to scheme (1), it is reasonable, as suggested by Cheniae (1970) to expect that uncouplers, which accelerate the electron flow to Q (primary electron acceptor of system II), will stimulate the fluorescence in the presence of Hill reaction. In essence, this is the classical "cross over" approach previously devised and utilized by Chance and his colleagues (1956) in locating the sites of ATP formation in the mitochondrial electron transport chain. In this preliminary report, it is shown that Gramicidin D, as an uncoupler, accelerates the ferricyanide reduction and in the meantime stimulates the fluorescence intensity.

Green house grown oats (12-14 day old) were cut into inch-long pieces and hand ground in mortar prechilled with cold buffer which consisted of sorbital (150 mM), tricine-NaOH(50mM) pH 8. After removal of cell debris by straining through 2 layers of fine muslin, the chloroplasts were sedimented with a table-top clinic centrifuge at its top speed for 45 seconds and resuspended in the same buffer (~2 mg chl/ml). All the procedures (essentially that of Noble's (1967), except the buffer system) were carried out at room temperature. The resuspended chloroplasts were kept in ice and used immediately. Ferricyanide reduction was monitored by observing change at 420 nm. Fluorescence was excited with strong blue light and observed at 680 nm.

Although Gramicidin D increases the Hill reaction rate by more than three fold over the control rate (table 1), it also enchances the fluorescence intensity while Hill reaction is in effect (fig. 1). Figure 1 shows two traces recorded from chloroplat's fluorescence excited by strong light in the presence of ferricyanide. Trace A represents the time course of fluorescence change in the absence of Gramicidin. After an intial spike, fluorescence drops to its minimal intensity and later rises to a maximal intensity as the Hill reaction is finished. Trace B is the recorded fluorescence change in the presence of Gramicidin. The intial spike is lowered and obscured by a higher minimal fluorescence. The maximal fluorescence is reached sooner because of faster Hill reaction rate. The fluorescence change is more pronounced in terms of $\triangle$ fl -- the difference between maximal and minimal fluorescence. The fact that there is little change in the maximal fluorescence indicates that the change in the minimal fluorescence is not due te Gramicidin per se. Rather, it is due to the interactions between Gramicidin and the electron transport process.

Table 1. *Effect of Gramicidin on the rate of the ferricyanide Hill reaction.*

The reaction mixture consists of chloroplast, 6 μg chl/ml.; sorbitol, 150 mM; tricine-NaOH, 50 mM, pH 8; ferricyanide, 0.05 mM in a volume of 2 ml. Wideband interference filter (center at 620 nm) was used.

Addition	Ferricyanide reaction, μ moles/mg chl./hr
$CaCl_2$, 5 mM	185
$CaII_2$, 5 mM+Gramicidin, 4 μM	689

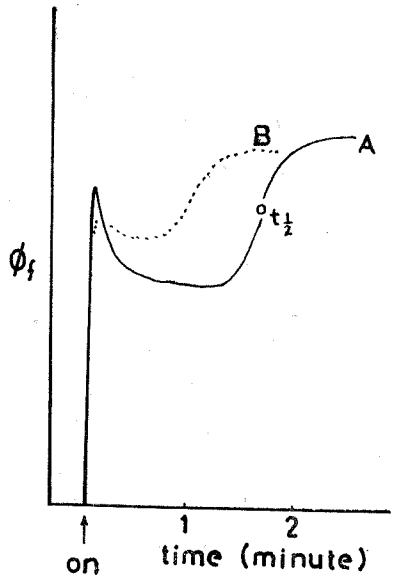

Fig. 1. Effect of gramicidin on the fluorescene induction in the presence of ferricyanide. The reaction mixture consists of chloroplasts, 13 μg chl/ml; sorbital, 150 mM; tricine-NaOH, 50 mM, pH 8; ferrciyanide, 2.8×10^{-5}M in a volume of 1 ml. A. calcium chloride, 5 mM; B. calcium chloride, 5 mM+gramicidin, 4.4 μM. $t_{\frac{1}{2}}$: half time of fluorescence raise, indicated by a circle on trace A. ϕ_f: relative fluorescence intensity. Arrow indicates light on.

During the period of Hill reaction, Gramicidin can produce at least two types of changes in chloroplasts: 1). Change in the equilibrium ratio of Q^-/Q (the reduced and oxidized form, respectively, of system II primary electron acceptor 2). Change in the electro-chemical protential across the thylakoid membrancee. No substantial evidences permit a meaningful disscussion of the relationship between fluorescence change and the membrane potential. We therefore focus our attentions on the change of the ratio of Q^-/Q. The following discussion is based on scheme 1 where a coupling site in between the H_2O system and system II is assumed. In the absence of phosphorylation cofactors or uncoupler, site 1 limits the electron flow. The steady state ratio of Q^-/Q is kept low, hence a low intensity of minimal fluorescence. Upon the the finishing of the Hill reaction, almost all Q is reduced for lack of oxidant and fluorescence rises to its maximum. In the process of Hill reaction, Gramicidin, which accelerates electron flow to Q, therefore, maintains a higher

steady state ratio of Q^-/Q and a higer intensity of minimal fluorescence. The initial spike of fluorescence can be explained by assuming that a small pool of reductants exists in between the site 1 and Q. At the onset of light, this pool supplies the reductants to Q and it quickly diminshed as the continuation of electron flow through site 1 is low, which drives down the fluorescence to its minimum until the completion of the Hill reaction. Gramicidin reduces the height of the initial spike by uncoupling site 2, while the more strongly accelerated electron flow through site 1 keeps a higher minimal fluorescence. Lower spikes were also observed at higher concentrations of ferricyanide.

If the foregoing explanation of the Gramicidin effect on the minimal fluorescence is correct, one expects that in the heat-treated (40°C, 1.5 min.) chloroplasts the H_2O system is inactivated (Böhme and Trebst, 1969), an uncoupler will accelerate the ascorbate-supported electron flow through site 2, hence a decreased minimal fluorescence will be observed. A positive results of this experiment will rule out the supposition that membrane potential play a significant part in the observed fluorescence change.

Another independent observation impling the existing reductant pool in between the H_2O system and Q is provided by the following experiment. The glutaldhyde fixation of chloroplasts prolongs the induction time by about 20 fold in the absence of DCMU. However, in the presence of DCMU, there is only an 30% increase of the induction time, as compared to that of control chloroplasts. This implies that the reductant pool is sufficient to reduce Q but not large enough to reduce pool A, which must be reduced by additional electrons extracted from H_2O (Li, unpublished observation). The proposed site of phosphorylation solves the problem that whether or not there is sufficient potential drop on the electron transport chain between the two systems (Joliot et al 1968). We delibrately omit the location of system 1 to avoid such a difficulty.

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由短桿菌環生素所增進的Hill反應速率和螢光強度——一個和磷脂化並聯結位置有關的現象

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短桿菌環生素可用作磷脂化解結劑 (phosphorylation uncoupler)，將其加入以簡速法，在室溫分離出的葉綠體懸浮液時，它不僅能增進 Hill 反應的速率，且能在此反應進行之際增强螢光的强度。爲能完滿地解釋這一現象，我們可假設在第二光系統反應中心和水裂解系統之間有一磷脂化並聯結 (phosphorylation coupling site)。解結劑能疏暢電子流，使 Q^-/Q 比例值加大，因而增强螢光强度。