# Nanosecond Fluorescence Study of Chloroplast Systems[1]

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*Abstract*

Quinacrine has been employed as a fluorescent probe to study the energized state in chloroplasts by nanosecond fluorescent spectroscopy methods. The fluorescent decay curve of quinacrine consists of two components of lifetimes 4.2 ns and 9.5 ns, respectively. It was found that the well-known decrease in fluorescence intensity of quinacrine in chloroplasts upon irradiation with saturating light was due to a combination of two effects: a decrease in fluorescence intensity of the longlived component without change in lifetime and a change in lifetime of the shortlived component. A comparison of quinacrine fluorescence decay in various pH environments indicated that light-induced changes in pH values of the chloroplast system as a result of proton pumping, is probably not the cause of the observed differences in the quinacrine fluorescence decay curves. Other interpretations will be discussed.

## Introduction

In both photosynthetic and respiratory organelles the electron transport system gives rise to the synthesis of energy storage units in the form of adenosine triphosphate (ATP). In the most accepted model of the process, the chemiosmotic model (8), it is assumed that the electron transport process creates a pH differential between the inside and outside of the organelle membrane and that this $H^+$-gradient drives the synthesis of ATP. Although the detailed mechanism of the coupling of the two processes has been the subject of extensive investigations over the past twenty-five years it has not given rise to any conclusive answers about the nature of the coupling. However, in the photosynthetic system, the classic experiment of Jagendorf and Uribe (5) has clearly shown that the $H^+$-ion gradient artificially induced in the dark can be used as the driving force for ATP production when the chloroplasts are transferred from acidic to basic conditions, still in the dark. That the rate of ATP formation in energized chloroplasts returned to the dark is inversely temperature-dependent has been demonstrated by Kraayenhof et al. (7). In general, the term, "energized state," can be used to symbolize the unknown factors involved in the coupling.

Among the several methods used to investigate the energized state is the technique of incorporating into the system under study a fluorescent probe, such as quinacrine (QC), which uncouples the electron transport system and the synthesis of ATP (9). Changes in the fluorescent output can then be used to monitor the energized state. In the case of QC it is known (6) that the fluorescence intensity decreases when the electron transport system is activated, e.g. by light exposure of a chloroplast system. For this reason, QC is considered a probe of

[1] Supported in part by a small grant from the I.S.U. Research Committee to one of us (T.A.).

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the state of energization of the membrane. However, this interpretation is not unchallenged. It has been suggested that the fluorescence changes of QC are due to binding changes related to the pH differential between the inside and the outside of the activated membrane (4). Also other interpretations of the fluorescence changes have been suggested by Dell'Antone et al. (3).

The emphasis of this investigation has been to begin to clarify the role of QC in chloroplasts in the energized state by use of the technique of nanosecond fluorescence spectroscopy. In this method, the lifetime of the fluorescent probe is measured. In general, this gives more information than a measurement of the corresponding steady-state parameters such as fluorescent intensity and quantum yield. For instance, if a fluorescent probe binds at more than one site to a macromolecule, this can be detected by a lifetime measurement due to the appearance of one specific lifetime for each binding site. A quantum yield measurement would not be able to detect this complexity.

## Materials and Methods

Class II chloroplasts from spinach (*Spinacea oleracea*) were prepared by the method of Vandermeulen and Govindjee (9) in 50 mM phosphate buffer (pH 7.8) containing 10 mM NaCl and 400 mM sucrose. Fluorescence decay measurements were carried out in a reaction mixture consisting of 3.3 mM phosphate buffer (pH 7.8), 3.3 mM $MgCl_2$, 16.7 mM KCl and 0.01 mM phenazinemethosulfate (PMS) as recommended by the same authors. Chlorophyll content, assayed according to Arnon (2), was adjusted to 10$\mu$g/ml in the experimental runs and QC[3] was added to a final concentration of 10$\mu$M.

The fluorescence experiments were carried out on a nanosecond flash apparatus with single-photon counting. The technique has been discussed elsewhere (1, 10). Figure 1 gives a schematic block diagram of the nanosecond fluorescence spectrometer available at Indiana State University. Light from a nanosecond pulse source (an air flash lamp, Ortec 9352) enters a filter or a monochromator (Schoeffel's miniature monochromator) and selected light excites a fluorochrome in the sample. The emitted fluorescent light is analyzed by a second filter or monochromator arrangement and is detected by a single photon counter (RCA 8850 photomultiplier). The time difference between the light source pulse and the arrival of a fluorescent light photon detected at the photomultiplier is converted to a pulse height in the time-to-pulse height converter (TAC). The pulses from the TAC-unit are then registered by a pulse-height multichannel analyzer. In this experiment the excitation light was selected by an absorption filter with maximum transmission at 350 nm. The fluorescent light from QC was observed through an absorption filter with maximum transmission at 510 nm. The sample was contained in a 2.0 ml quartz cuvette mounted for observation of front surface fluorescence and maintained at 0°C during measurement.

[3] QC, quinacrine hydrochloride, is the Sigma Chemical Co. brand name of atebrine.

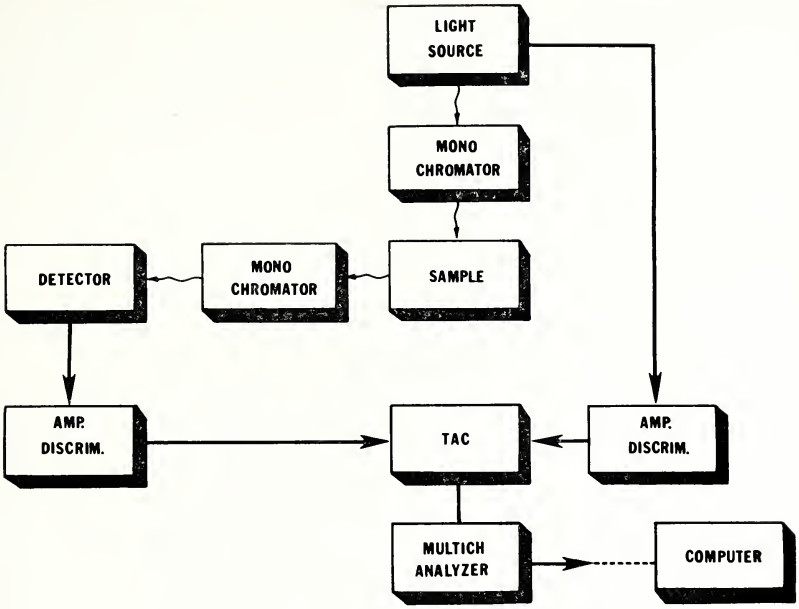


FIGURE 1. *Block diagram of nanosecond fluorescence spectrometer.*

## Results

Figure 2 demonstrates representative decay curves obtained from QC in the complete reaction mixture containing the standardized concentration of chloroplasts.

The curve marked "light" was obtained from a sample which had been preilluminated with saturating white light (5 mw/cm$^2$) for 30 sec. at 0°C outside the fluorescence apparatus. The sample was then introduced into the fluorescence apparatus and decay curves registered for 10 sec. The time interval between the termination of light exposure and the start of fluorescence measurement was approximately 10 sec. This procedure was repeated four to six times with the same sample and results were summed. It is known (7) that the energized state of chloroplasts, under the conditions used in these experiments, has a relaxation time of about 50 sec., so that 10 sec. measurements avoided excessive error due to relaxation. At room temperature the relaxation time is considerably shorter (of the order of 10 s).

The curve marked "dark" represents traces which were identical when obtained from a sample prior to light exposure or returned to the dark for approximately 5 min. subsequent to light exposure.

The "light" decay curve consists of two components with lifetimes of 3.2± 0.2 ns and 9.2± 0.5 ns, respectively. The "dark" decay curve also has two components with lifetimes of 4.2± 0.3 ns and 9.5± 0.5 ns, respectively The total intensity decrease in fluorescence from "dark" to "light" curves is 55%.

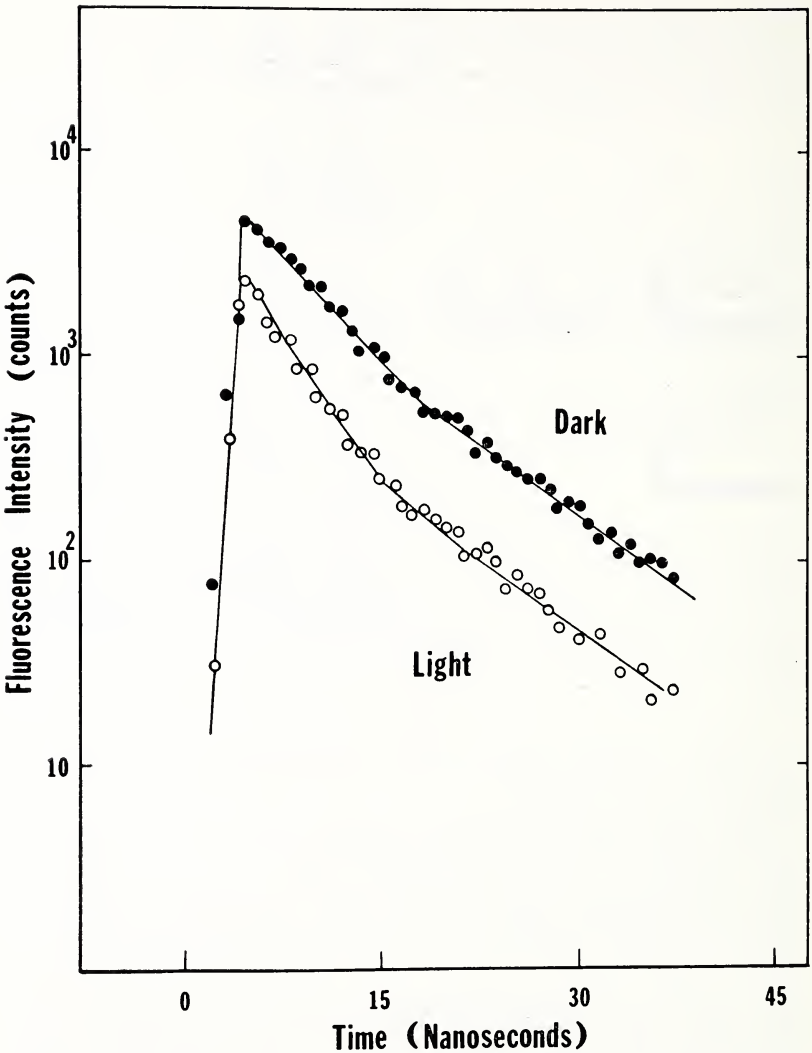


FIGURE 2. *Fluorescence decay curves from quinacrine in chloroplasts. For details see text.*

Since the pH values may be of importance in the systems considered here (4) fluorescence decay curves of QC solutions in various pH environments were also measured. Figure 3 shows the result for three different pH values measured at the same temperature used for the experiments presented in Fig. 2. As can be seen only small changes occur over a relatively broad range (pH 6.5-pH 7.5). A larger change is evident when pH is increased to 9.2.

## Discussion

As shown in Figure 2, the change in fluorescence intensity of QC in chloroplasts upon irradiation with saturating light is due to a combination of two effects: (1) a decrease in fluorescence intensity of the longlived component without change in lifetime and (2) a change in lifetime of the shortlived component. We consider three possible expla-

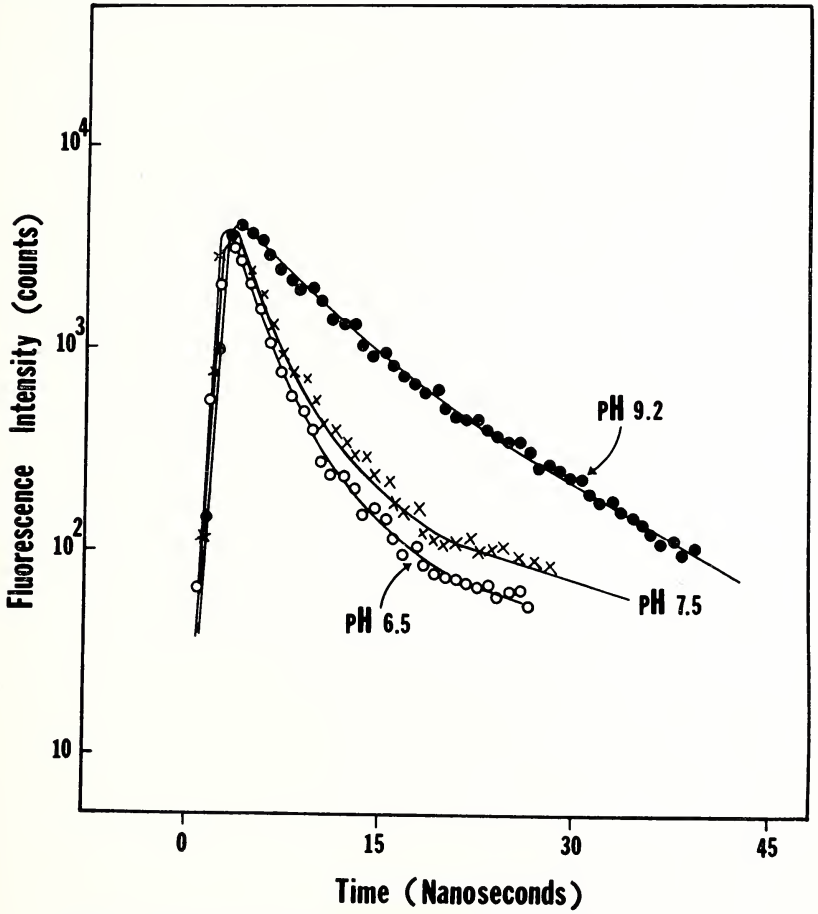


FIGURE 3. *Fluorescence decay curves from quinacrine for three different pH values.*

nations for the observed effect: actual binding of QC to the membranes with subsequent fluorescence decrease, changing environmental pH value, simple screening of the QC dye, or a combination of two or all of the above.

The observed effect as shown in Figure 2 can be explained by assuming that the QC binds at two different places on the membranes. One binding site gives rise to a slight change in the lifetime of the

shortlived component of the decay curve. The second binding site causes a complete quenching of the QC fluorescence. The difference in intensity between the "dark" curve and the "light" curve should then correspond approximately to unbound QC.

As illustrated in Figure 3, the pH value of the environment seen by the QC will influence the shape of the fluorescence decay curve. The maximum possible change in pH value of the membrane environment due to saturating light irradiation is estimated to be 0.3 units under conditions used here (Gross, unpublished results). No change of the fluorescence decay curve of QC can be detected with an environmental pH difference of this magnitude which is obviously too small to explain the measured effect of QC fluorescence decrease in the presence of the chloroplasts; a minor contribution from changing pH environments can not be totally excluded.

The third possible explanation would be that simple screening of the accumulated QC by chlorophyll or other membrane components caused the changes of the fluorescence decay curves. However, experiments with a wide variety of fluorochromes related to quinacrine with different positions of excitation and emission bands have demonstrated that a screening effect is very unlikely in the chloroplast system (7).

The conclusion is, therefore, that the most probable cause of the fluorescence lowering of QC in chloroplast systems exposed to saturating light is due to binding of the probe at two different binding sites, one of which is represented by the shortlived decay and is responsive to irradiation, the other, represented by the longlived decay which is not responsive to irradiation, although there was an intensity decrease upon illumination. A more detailed study is needed, however, in order to reach a firm conclusion and to ascertain the degree, however small, of the involvement of other effects than binding. In addition, further study of the role of QC in the energized state, using the nanosecond fluorescent technique, is clearly desirable in order to elucidate the mechanism of action of this fluorescent probe.

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