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DELAYED LIGHT AND THERMOLUMINESCENCE STUDIES OF ENERGY STORAGE BY THE OXYGEN EVOLVING PHOTOREACTION OF PHOTOSYNTHESIS

by

Susan Lurie

A dissertation submitted to the Graduate
Faculty in Biology in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy, The City University
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1972

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

DELAYED LIGHT AND THERMOLUMINESCENCE STUDIES OF ENERGY STORAGE BY THE OXYGEN EVOLVING PHOTOREACTION OF PHOTOSYNTHESIS

by

Susan Lurie

Advisor: Professor Walter Bertsch

I have investigated the phenomena of delayed light and thermoluminescence as a means of studying the mechanism of energy storage by photosystem II. Thermoluminescence of glow curves exhibited three peaks which appeared to originate in photosystem II. These peaks were 1) absent in algal mutants lacking a functional photosystem II, 2) affected by DCMU, 3) affected by Hill acceptors. The activation energy, frequency factor and half life of these peaks were calculated by two different methods.

I have advanced two different models to explain the connection between the storage states reflected by the glow peaks and delayed light emission in the msec time range. With the present evidence it is impossible to decide between the two methods.

I also investigated the effect on delayed light and thermoluminescence of treatment which inhibit water from serving as the electron donor to photosystem II. The treatments were tris aging, heating, UV irradiation, and chloride depletion. As a result of these studies I have presented a model of how these treatments act to inhibit photosystem II and the means by which electron donors can partially reverse this inhibition.

I have also investigated the effect that uncouplers of phosphorylation have on thermoluminescence and delayed light, in order to understand the relationship between the high energy state leading to phosphorylation and energy storage at photosystem II.

PREFACE

The work described in this thesis was carried out in the Department of Biology, Hunter College, The City University of New York, from January 1970 to July 1972. Except where otherwise stated it is the original work of the author. This dissertation has not been submitted, in whole or in part, for any other degree at this or any other university.

I should like, first, to thank Walter Bertsch for his gentle and skillful guidance and his enthusiasm throughout the course of this research. I should like to thank William Cohen and Dunell Cohn for the numerous productive talks we have had, and the aid these talks were in organizing my ideas. And I am especially grateful to William Cohen for working with me on some of the experiments. In the analytical treatment of the glow curves I would like to thank Ezra Shahn for familiarizing me with some of the mathematical principles, and Peter Stern for helping me to apply these principles. A special thanks, too, is due Peter Stern for friendship and patience during this time. Finally, I would like to thank Kathy Siegel for her expert technical assistance.

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CHAPTER I: INTRODUCTION

A. Chemical steps of photosynthesis

Photosynthesis is the process wherein plants store radiant energy of the sun in a metabolically available form as chemical energy; that is, as compounds with a large free energy of formation (ATP and NADPH) which are used to reduce CO_2. In algae and higher plants photosynthesis converts carbon dioxide and water into carbohydrates, accompanied by the evolution of oxygen. In 1935 van Niel, comparing photosynthesis in green plants and algae on the one hand and in photosynthetic bacteria on the other, found that both required the movement of four electrons for each CO_2 molecule reduced. He expressed this basic theme of all photosynthetic organisms by the formula:

$$CO_2 + 2H_2A \xrightarrow{\text{light}} (CH_2O) + H_2O + 2A \qquad (1)$$

Van Niel suggested that higher plants and algae represent a special case in which water is the oxidizable substrate, or hydrogen donor: a case where H_2A is H_2O and 2A is O_2. This was the first experimental evidence supporting Tunburg's (1923) early suggestion that photosynthesis might involve electron transport.

A parallelism between the photosynthesis of green plants and bacteria was made more convincing by Gaffron's (1942) investigations of hydrogen adaptation in the green alga Scenedesmus. Ordinarily these algae carry on photosynthesis with evolution of oxygen, but they can be adapted, through incubation with increasing amounts of hydrogen, to perform bacterial type photosynthesis in which hydrogen serves as the oxidizable substrate. Under strong illumination the hydrogen adapted algae revert to their normal pattern.

The role that chlorophyll plays in formula (1) is in the primary separation of oxidizing and reducing power. The generally accepted scheme is that photoexcited chlorophyll brings about the transfer of an electron from a high potential donor molecule to a low potential molecule. The general aspects of van Niel's model are still fundamental to modern theories of photosynthesis . Together with the concept of the two light reactions acting in series, it allows us, with a great deal of hindsight, to arrive at the electron transport models of modern theories of photosynthesis in algae and higher plants.

B. Two photosystems *

There were a number of crucial observations which led up to the two photosystem model. Emerson and Lewis (1943) found when examining the quantum yield of photosynthesis in Chlorella that the yield was fairly flat throughout most of the visible spectrum, but dropped precipitously at wave lengths longer than 680 mn, although the absorption of chlorophyll a extended beyond 700 nm. A few years later, Haxo and Blinks (1950) investigated this further and found in red algae that light absorbed by the accessory pigments phycocyanin and phycoerythrin was more efficient in promoting photosynthesis than light absorbed by chlorophyll a. Duysens (1952) found that this same spectral region in red algae was also effective in sensitizing chlorophyll a fluorescence.

Another peculiarity appeared in the form of chromatic transients observed by Blinks and others. If photosynthesis involves only one kind of photochemical act, then all wave lengths of light should have qualitatively the

---

* We use the terms photoreaction and photosystem to denote a complete process in which absorption of light results in production of chemical free energy. The term reaction center denotes a specific site at a chlorophyll-enzyme interface where chemical free energy is delivered to enzyme systems as oxidizing and reducing equivalents; which means that a reaction center is a site where electrons and holes move from the pigment system to the enzyme systems of a photoreaction. The term photosynthetic unit denotes the smallest complete system which can oxidize water with concomitant reduction of NADP and production of ATP

same effect. But when Blinks et al. (1957, 1958) measured oxygen evolution in algae using red light, and then switched to blue light, they were unable to adjust the intensities of the two beams of different wave lengths so as to eliminate a transient disturbance in the time course of oxygen evolution.

Finally, Emerson and his coworkers (1956) found that the low quantum yield in Chlorella observed with far red light (above 680), could be boosted by a 10 times less intense beam of short wave length light. This is the now classic experiment of enhancement. At nonsaturating intensities of light the rate of photosynthesis (measured as O_2 evolution) in a combined beam of short and long wave length light can be nearly twice the sum of the rates observed in the two beams separately (Brody and Emerson, 1959; Meyers and French, 1960; Emerson et al., 1957).

As an explanation of the above data Emerson and Rabinowitch (1960) proposed two separate photosystems, defined by a characteristic absorption spectra, which acted together to produce the overall process of photosynthesis A two photosystem scheme was proposed simultaneously by Hill and Bendall (1960), based partly on the work which Hill and coworkers had done on the unique cytochrome components of the photosynthetic electron transport chain. (Davinport and Hill, 1952; Hill and Scarisbick, 1951) Evidence supporting these models of two photosystems connected by an electron transport chain, was also found by other investigators. Duysens et al. (1961) found that in the alga Porphyridium, cytochrome f is oxidized by far red light and reduced by red light. It was then found by Kok (1961) and Hoch (1963) that in algae and chloroplasts P700 is oxidized by far red light and reduced by shorter wave length light in complete analogy to the behavior of cytochrome f described by Duysens. During the same period Losada and coworkers (1961) were able to separate the evolution of oxygen from the reduction of NADP by using the poison DCMU in combination with artificial electron donors and acceptors in the redox range -300 to +400 mv. Once separated the two processes showed different action spectra, oxygen being promoted better by shorter wave length red light and NADP being reduced efficiently by longer wave length red light.

The two photosystem model is seen as two photosystems cooperating in a series and is generally referred to as the Z scheme (or N scheme by physicists). The process is proposed to operate as follows (see Fig. I-1): illumination of each photosystem caused a primary photoreduction to occur in which a separation of oxidizing and reducing equivalents is effected. In photosystem II an unknown primary electron donor, Z, is oxidized while an unknown primary acceptor, Q, is reduced. Z is rereduced by electrons from water resulting in evolution of oxygen. The primary acceptor which was defined as Q by Duysens and Sweers (1963) is now termed C550 by some investigators (Erixon and Butler,

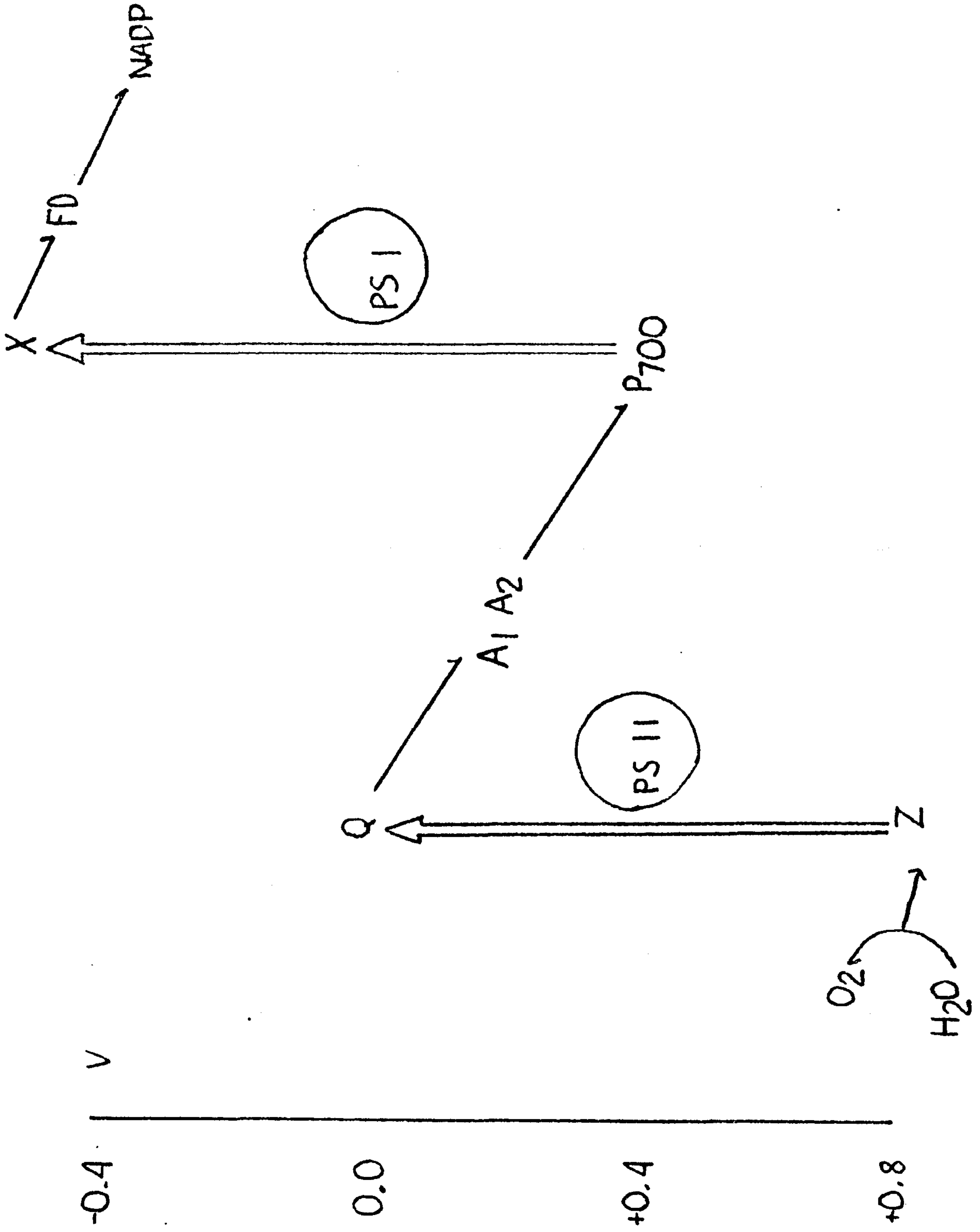

Figure 1-1 The Z scheme.

1971) linking it to an observed light induced absorbancy change at 550 nm. This substance is reoxidized by way of a heterogeneous pool of electron transport components, A_1, A_2, which link photosystem II to photosystem I. In photosystem I the primary donor may be cytochrome f (Chance and Bonner, 1961) or plastocyanine (Gorman and Levine, 1965) and the acceptor may be either a bound iron-sulfur compound, such as a membrane bound ferridoxin (Bearden and Malkin, 1972) or a pteridine of some type (Fuller, et al., 1971). The oxidized donor of photosystem I is rereduced by the components of the electron transport chain-- ultimately by Q and photosystem II-- and the reduced donor is reoxidized by ferridoxin and NADP.

Thus photosystem II raises electrons from a potential of about +800 mv--the potential of the oxygen electrode at pH 7--to about 0 mv. These electrons fall to about -400 mv along the electron transport chain (the reducing potential of an electron is a direct expression of its energy, a change from 0 to +400 mv representing a loss in energy of 0.4 eV or 9 kcal/mole). They are then promoted by photosystem I to a potential around -400 mv, which is sufficient to reduce NADP.

Light dependent phosphorylation of ADP to ATP in isolated chloroplasts was first observed in 1954 by Arnon et al. ATP formation stoichiometrically linked to NADP reduction was seen by the same workers in 1958. The two immediate products of electron flow are therefore NADPH and ATP, and these are used to run the dark reactions of CO_2 fixation, notably the Calvin cycle (Bassham et al., 1954; Calvin and Bassham, 1962; Racker, 1955). Phosphorylation has generally been assumed to be directly coupled to electron flow between photosystem II and photosystem I, but alternative schemes have been proposed by many investigators. The Z scheme is not the only possible model of how the light reactions of photosynthesis function. Gaffron has long advocated openmindedness on this question (Gaffron, 1962). More recently Arnold and Azzi (1968) and Arnon (1970) have put forward other models and reasons why the Z scheme is inadequate.

C. Photosynthetic units and reaction centers

The Z scheme represents the consensus on the chemical processes most closely connected to the photoreactions. It does not, however, say anything about the physical processes of the photoreactions. The generation of one oxygen molecule requires the absorption of 8-12 quanta of light (Emerson and Lewis, 1941, 1949). If every chlorophyll molecule would react photochemically to generate products that are used in photosynthesis, a sufficiently intense flash of light should bring about the evolution of one O_2 for every eight chlorophyll molecules present. This would be the result of exciting every chlorophyll molecule, provided that the 'dark' chemical machinery can cope efficiently with the flood of primary photoproducts released by the flash. In 1932 Emerson and Arnold explored this chemical machinery using Chlorella and measuring the oxygen evolved in response to brief (10^{-5} sec) flashes of saturating intensity. They found that the maximum yield per flash was about one oxygen evolved for every 2400 chlorophyll molecules, and that successive flashes could have their full effect only if they were separated by dark or recovery intervals. At 25°C the 37% recovery time was 0.02 - 0.04 sec for whole Chlorella cells.

Gaffron and Wohl (1936) illuminated cells with a light so weak that it would take years for any one chlorophyll molecule to absorb the quanta required to evolve oxygen. They found that it took only 30 seconds after the beginning of illumination to observe O_2 production, indicating a cooperation among the chlorophyll molecules. Incorporating this finding with that of Arnold and Emerson (1932), Gaffron and Wohl (1936) suggested the existence of a photosynthetic unit involving the transfer of energy among a large number of chlorophyll molecules. The physical mechanism of this transfer of excitation energy has been refined by Forster's theory of slow inductive resonance transfer (Forster, 1947, 1948).

The present picture of the photosynthetic unit is the result of the blending of a number of models of energy transfer (Duysens, 1951; Arnold and Oppenheimer, 1950; Emerson, 1957). Each unit consists of a chemical reaction center served by an antenna of light-harvesting chlorophyll a molecules and accessory pigments. The energy of a photon absorbed by any of the antenna molecules is transferred from one molecule to another by the process of slow inductive resonance transfer. The chances that any exciton will visit a reaction center are high because the transfer rate of the exciton from one molecule to another is very high (less than 10^{12}/sec) compared to the non-photochemical loss rate of the exciton (about 10^{8}/sec). Once the excitation energy is transferred to a reaction center it is trapped and utilized in a photoreaction to produce, ultimately, stable oxidation-reduction products. Photochemical utilization competes well with loss of excitation energy because of the transfer rate from the bulk pigments to the reaction center. It is hypothesized that asymetry in the transfer rates to and from the reaction centers arises from a lower singlet energy level for the trap. (Clayton, 1964; Hoch and Knox, 1968)

The concept of photosynthetic units has been widened to include the two separate photosystems. Each photosystem is now conceived as having its own (250 - 300) antenna pigments, but in view of the comparative constancy of the quantum yield of photosynthesis throughout the absorption spectrum (with the notable exception of the red drop), it has been suggested that transfer of excitation energy occurs between photosystems. The red drop phenomenon indicates that at long wave lengths for energetic reasons any such transfer is largely unidirectional, from photosystem II to photosystem I. Recent results especially in the fields of fluorescence induction and emission spectra show that 'spillover,' possibly in both directions does, however, occur (Murata, 1968, 1969; Bonaventure and Myers, 1969). Brody and Brody (1961) have found that chlorophyll *a in vivo* exists in two main forms, a monomer and a dimer. The monomer has a single absorption maximum, at the red end of the spectrum, while the dimer has two maxima, one on either side of the monomer peak. They suggest that the dimer form is found in photosystem I and the monomer in photosystem II. Preponderance of absorption by the dimer may cause the long wave length decline (red drop). Brody and Brody (1961) measured the peak ratios of the low temperature (-196°C) fluorescence emission bands of the monomer (685 nm) and the dimer (720 nm). In support of their theory they found that organisms having the largest proportion of monomer to dimer showed little loss of photosynthetic efficiency at long wave lengths.

D. *Delayed light*

The current views of the photosynthetic unit visualize reaction centers where the excitation energy is stored until it is utilized by the electron transport chain. If the excitation energy is not trapped in 10^{-9} sec (Brody and Rabinowitz, 1957) it decays by fluorescence or radiationless deexcitation processes. However, Emerson and Arnold (1932) found using *Chlorella* that if the time between flashes was shorter than 20 - 40 msec the oxygen yield/flash was decreased. This is now understood as representing the steady state turnover time of the electron transport chain components. The time after excitation from 10^{-9} to $2\text{-}4 \times 10^{-2}$ sec represents the time during which the reaction center stores the energy in unknown metastable forms. There are various ways to observe and investigate storage of this energy. The phenomenon of delayed light is one which is being intensively studied by a number of laboratories at this time.

Delayed light is emitted by plants from the first excited singlet of chlorophyll a (as is fluorescence), but at a longer time after excitation than the lifetime of the first excited singlet. Delayed light emission in photosynthetic organisms was first reported in algae by Strehler and Arnold (1951). Further investigation (Arnold and Thompson, 1956) showed that it was a universal phenomenon of photosynthetic organisms. An examination of green, blue green and red algae revealed that the emission spectra of the delayed light was that of chlorophyll a, while the action spectrum included light absorbed by accessory pigments as well as chlorophyll a. In his first paper Arnold (1951) suggested that delayed light was a reflection of certain early reactions in photosynthesis which are reversible and can release a proportion of their stored energy through a chemiluminescent mechanism (regeneration of excited chlorophyll).

The origin of most of delayed light emission in algae and higher plants has now been firmly established as coming from photosystem II. Bertsch *et al.* (1967) reported studies on a mutant of *Scenedesmus* lacking photosystem II which emitted approximately 300 times less delayed light than the wild type, while a mutant lacking functional photosystem I emitted 50 times more. Earlier studies on the interactions between photosystem II and photosystem I on delayed emission also implicated photosystem II as the major source of delayed light (Goedheer, 1962). In bacteria the requirement of operative reaction centers for delayed light has been established by Clayton and Bertsch (1965) who found that a mutant of *Rhodopseudomonas spheroides* lacking reaction centers did not emit delayed light. Arnold and Thompson (1956) found that the delayed emission spectrum of *Chloropseudomonas ethylicum* was more characteristic of the bacteriochlorophyll of the reaction centers than the prompt fluorescence, which came primarily from the accessory chlorophyll. This was also found to be true in *Chlorobium* by Clayton (1965). Clayton (1965) also has found that the ratio of delayed to prompt fluorescence is greater in *Rhodopseudomonas viridis* than in most other purple bacteria, probably because the reaction center absorption peak is at a shorter wave length than the bulk pigments; the delayed light excitation therefore escapes from the trapping centers more readily.

Delayed light has been measured from the time range of msec to minutes after a flash of exciting light.

Recently Zankel (1971) has measured delayed light in the time range of 65 - 800 usec. At no time range of investigation is the decay of the emission a simple process. Arnold and Strehler (1957) broadly characterized a fast component (less than 10 msec) and a slow component, while admitting that these were probably compound. A similar classification was used by Bertsch and Lurie (1971). Tollin, Calvin and coworkers (1957, 1958) found the decay curve from 10 msec to seconds made up of three exponential components at room temperature. They found that as their chloroplasts were cooled the curves became simpler and that below -35°C only one component remains. Ruby (1968) and Bonaventura and Kindergan (1971) in examining delayed light in the millisecond range at room temperature found the decay can be expressed as either three or two exponential components, while Zankel (1971) found three components in the usec range. Differential effects of inhibitors on different time regions also indicated the complex nature of the decay (Arthur and Strehler, 1957; Sweetser _et_ _al_., 1961; Bertsch _et_ _al_., 1963)

Arnold's first suggestion of delayed light being a back reaction of the early steps of photosynthesis appears quite accurate. It is generally accepted as reflected a recombination of the separated charge, or, in a solid state system, electrons and holes. A number of people in the 1950's proposed solid state or semiconductor models to explain the mechanism of delayed light production. This was prompted by Arnold's discovery of thermally induced emission (glow curves) from dried chloroplast films. Arnold and Sherwood (1957) and Tollin and Calvin (1957) interpreted the plant as a semiconductor-like unit as did Brugger and Franck (1958). Bertsch (1967, 1969) has proposed a much more detailed model which explains many observations he and others have made of millisecond delayed light.

E. Bertsch's model of the reaction center of photosystem II

Bertsch's model of the reaction center of photosystem II involves several chlorophyll molecules which interact to produce a band structure like that in semiconductor crystals. The reaction center is spatially inhomogeneous due to differing interactions between the chlorophyll molecules and the multi-enzyme systems at the oxidizing and reducing interfaces. These two interfaces, where the chlorophyll is in contact with the primary electron acceptor and the primary hole acceptor, are viewed as being analogous to trapping sites in a semiconductor. The traps in the reaction center are due to interactions at each interface which produce a common energy level that links the chlorophyll to the first carriers which are oxidized and reduced. Excitation is thus separated into a trapped electron and a trapped hole at two different sites in a single reaction center of photosystem II. Electrons and holes left in the reaction center after the initial production of chemical oxidizing and reducing power then can migrate to the opposite traps where further chemical potential is produced. Delayed light can be emitted when opposite charges occupy the same chlorophyll molecule during charge migration. This model is diagrammed in figure I-2.

In this model delayed light would be due to recombination of mobile charge in the pigments. In terms of the band picture, there would be two sources of this mobile charge: 1) non-thermal production of mobile charge which was left in the band system on the original act of charge separation and trapping, and 2) thermal excitation of trapped charge into the band system. At liquid nitrogen temperature one would expect to find only nonthermal processes operative. Calvin and Tollin (1957) as well as Arnold (1966) found millisecond delayed light at this temperature, but it died away after the first few flashes of exciting light. If, after all the traps were filled, no more charge could be separated until traps became available through either thermal untrapping of photochemical usage, delayed light would disappear at liquid nitrogen temperature.

Bertsch _et_ _al_. (1969) observed an increase in the emission of delayed light at one msec upon addition of artificial electron acceptors, such as ferricyanide, to chloroplasts. The increase in delayed light emission at one millisecond upon production of chemical potential could be due to an increase in the nonthermal production of mobile

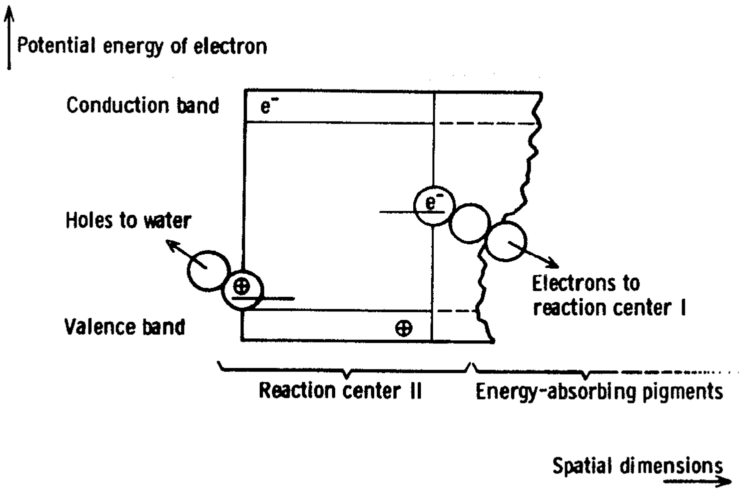

Figure 1-2. Hypothetical band structure for the reaction center of photosystem II. (Bertsch, 1969)

charge caused by additional trapping sites being empty during the flashes of exciting light. This increase in delayed light emission from the functioning photoreaction would be predicted to occur only at times shorter than the turnover time of the electron transport chain, since the additional mobile charge would be swept out of the pigment system as soon as the primary acceptors were regenerated. This explains why the delayed light in the tens of milliseconds decreased in the presence of electron acceptors.

Delayed light at liquid nitrogen temperature may eminate from non-thermal processes. Thermal delayed light emission would be due to the back reactions resulting from thermal excitation of both trapped charge types into the band system. In the presence of DCMU, an electron transport inhibitor, no non-thermal production of mobile charge would occur after the first few flashes of exciting light because all traps would become filled, leaving no sites available for new charge formation. Millisecond delayed light in the presence of DCMU is slowly decaying, reflecting the fact that DCMU blocks charge from leaving the traps to the electron transport chain, thereby reducing the amount to charge available for back reaction (Bertsch, 1963, 1971). Similarly, mutants which have blocks in the electron transport chain or at photosystem I, would show slow decay kinetics in the millisecond delayed light. Bertsch (1967) found this to be the case with a *Scenedesmus* mutant lacking the reaction center of photosystem I.

The model of the reaction center of photosystem II involved the absorption of two quanta to separate oxidizing and reducing power. One quanta would excite an electron to the conduction band with a hole left in the valence band. The electron might be trapped leaving the hole unpaired; a second absorbed quanta would repeat the process allowing the hole of this second pair to be trapped. Once the process has started, the absorption of single quanta for each reaction center would allow it to continue. Supporting evidence for this was found by Jones (1967) using completely dark adapted algae. He found an I^2 dependence of initiation of delayed light emission in these dark adapted algae. Joliot (1971), examining fluctuations in delayed light and oxygen production in flashing light, found that the greatest amount of delayed light came on the second flash.

F. Thermoluminescence

Two properties which Arnold and Sherwood (1957) found dried chloroplast films to have in common with organic semiconductors, thermoconductivity and thermoluminescence, also point towards a solid state process in photosynthesis. Having found energy storage in dried chloroplast films when illuminated, Arnold and Sherwood (1957, 1959) examined thermoluminescence in more detail. They felt that this phenomenon was produced in one of two possible ways.

1) The chloroplasts act like a semiconductor and the energy is stored as trapped electrons. On heating these electrons are released and produce the light. An activation energy E is needed to transfer an electron from the trap to the conduction band.

2) During illumination some high energy compound is formed, that on heating decomposes by a first order reaction, and emits light. E is the activation energy for the decomposition. Either case would be expected to follow the differential equation:

$$dn/dt = -nse^{-E/kT} \tag{2}$$

where n = number of trapped electrons of high energy molecules

s = frequency factor

k = Boltzmann's constant

T = absolute temperature

t = time

This equation has two unknowns, s and E, in the case of chloroplasts, but by using a rate of heating proportional to the reciprocity of temperature Arnold and Sherwood (1959) were able to solve the equation so that E could be calculated separately from s.

The glow curves they observed represented a multiplicity of activation energies. However, if the sample was illuminated and then annealed by heating to 88°C, and then cooling to 20°C before reheating and monitoring emission, then a single peak, representing one activation energy, was present. For this peak Arnold and Sherwood (1959) calculated an activation energy of 0.926 eV and a frequency factor of 2.45×10^9 sec^{-1}. When this frequency factor was assumed for the peaks present in the unannealed sample, values from 0.69 to 0.92 eV were found.

This method gave light emission, but the treatment of the chloroplasts in terms of drying and heating was so severe that it seems unlikely that the peaks represented activation energies in the living system. Arnold (1966), therefore, examined suspensions of whole *Chlorella* cells. The *Chlorella* suspensions were frozen to -196°C after being kept in the dark for five minutes. The samples were then warmed to different temperatures, exposed to light for one minute, refrozen to liquid nitrogen temperature and then heated to 70°C. He found emission profiles using this method quite different from those found in his earlier study. Using the frequency factor calculated earlier for dried chloroplasts (2.45×10^9 sec^{-1}) and the differential equation for a glow curve given by Randall and Wilkins (1945), he got activation energies ranging from 0.57 to 0.47 eV.

Recently Fleischman (1971) has attempted to examine thermoluminescence in photosynthetic bacteria. Glow curves were produced by *Rhodopseudomonas viridis*. Fleischman also calculated trap depths in the range of 0.5 eV for his emission. Interestingly, he was able to correlate the intensity of emission with the extent of reduction of P895, the hypothesized reaction center. He interpreted this as indicating that the glow curves represent energy stored in the reaction center itself.

This thesis will attempt to utilize and correlate thermoluminescence and delayed light in an investigation of the functioning of the reaction center of photosystem II.

In utilizing delayed light I shall first review the findings of previous researchers in examining delayed light of algae and isolated chloroplasts, and the effect of electron acceptors and electron transfer inhibitors on msec delayed light of isolated chloroplasts. I will also examine the thermoluminescence of these different organisms and the effect that electron donors and photosynthetic inhibitors have on this phenomenon. Since thermoluminescence is not a commonly used measurement, I shall examine the effect of various physical parameters (light saturation, temperature of illumination, time of illumination vs. intensity of illumination reciprocity) to attempt to characterize thermoluminescence more fully. I shall calculate the activation energies involved in the thermoluminescence glow peaks by two different methods, as well as the frequency factors and half decay time of these storage states.

I also intend to examine how a number of conditions which affect the functioning of photosystem II alter millisecond delayed light emission and thermoluminescence. There are four treatments (incubation of chloroplasts in

0.8M tris, pH 7.8, heating to 50°C, UV irradiation, and chloride depletion) which inhibit in some manner the ability of water to serve as an electron donor to photosystem II. I shall investigate how these treatments affect thermoluminescence and delayed light with the idea of elucidating the mechanism of action of these treatments and their affect on the reaction center of photosystem II. I shall investigate the effect that uncouplers of phosphorylation have on thermoluminescence and delayed light, in order to attempt to understand the relationship between the high energy state leading to phosphorylation and energy storage at photosystem II as reflected by thermoluminescence and delayed light.

CHAPTER II: EXPERIMENTAL

A. Phosphoroscope

The apparatus utilized for measuring delayed light was a modification of the Becquerel phosphoroscope. Figure 2-1 is a schematic diagram of our instrument. The instrument consisted of a pair of spinning discs mounted on a common shaft, between which the sample was held stationary in a two milliliter cellulose nitrate test tube. In order to provide adequate light baffling, the discs were mounted so that they spun in narrow slots within an aluminum housing. The discs and the slots were painted with flat black paint. The sample was illuminated through two oppositely spaced holes in the front disc, so that the sample received two evenly spaced flashes of exciting light per revolution of the discs. The light emitted by the sample was viewed by a Quantacon photomultiplier placed behind the back disc. The back disc was cut into the shape of a paddle wheel, the blades of which were aligned to coincide with the illuminating holes of the front disc. The back disc thus allowed the cells to be viewed by the photomultiplier for a period between each

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Abbreviations: DCPIP 2,6 dichlorophenol-indophenol, NADP nicotinamide adenine dinucleotide phosphate, FeCy ferricyanide, tris tris (hydroxymethyl) aminomethane, tricine N-tris (hydroxymethyl) methylglycine, TES N-tris(hydroxymethyl)methyl 2 aminoethanesulfonic acid, EDTA ethylene diaminetetraacetic acid

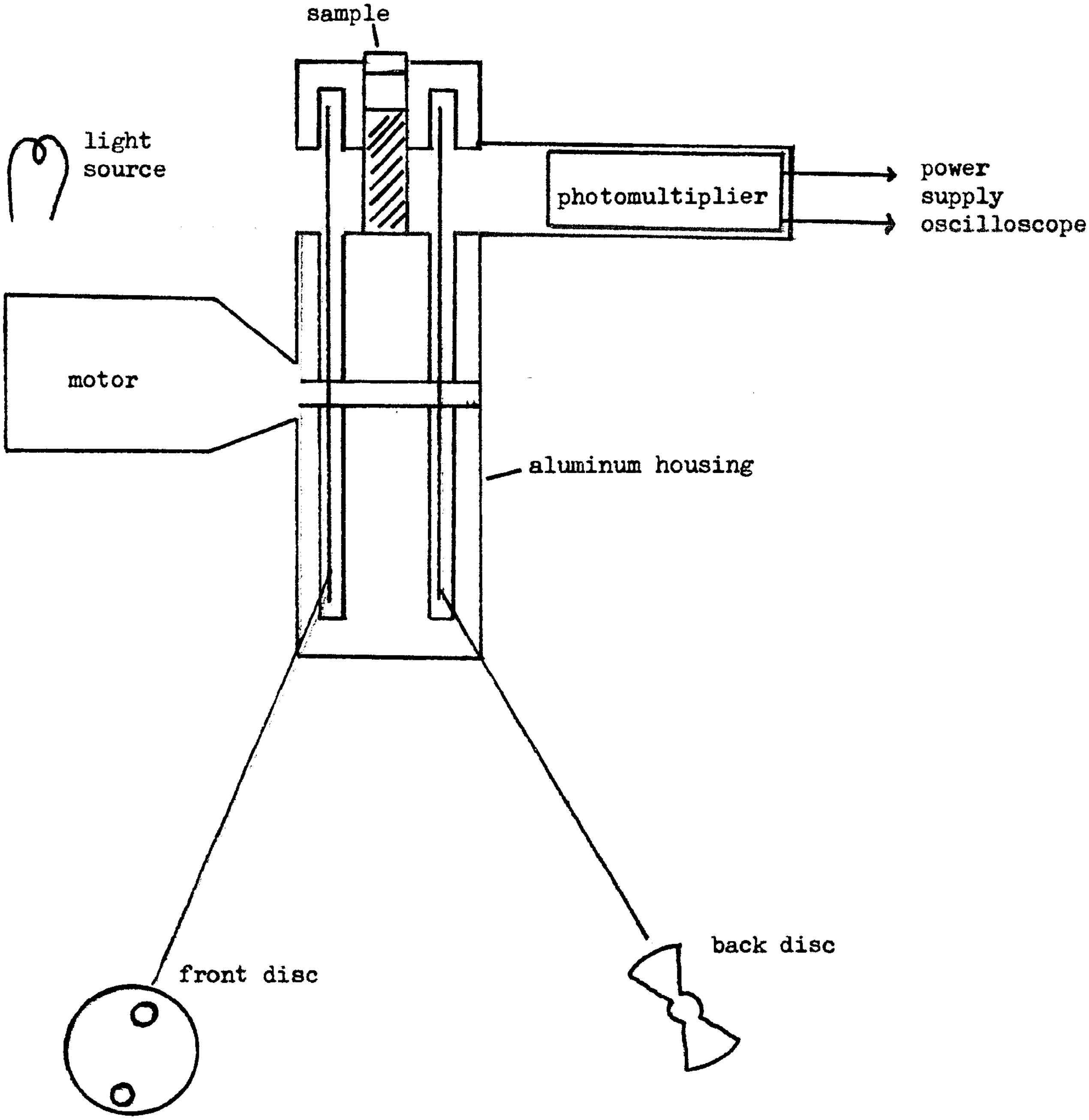

Figure 2-1. Schematic diagram of the phosphoroscope. See text for description.

exciting flash, but for the duration of each flash the blades of this disc protected the light measuring equipment from the bright exciting light. The signal from the photomultiplier was fed into a Tektronix 545A oscilloscope and photographed.

The signal was distorted by the passive properties of the plug-in unit of the oscilloscope (Type D amplifier). This placed a lower limit of resolution on the instrument. At times shorter than 0.7 msec after a flash of exciting light there was an electrical curvature in the signal due to the RC time constant of the plug-in unit. Beyond 40 msec the signal to noise ratio was too low for accurate measurement. Thus observations of delayed light could be made from 0.7 msec to 40 msec after a flash of exciting light.

The delayed light measurements from 1-20 msec were obtained by running the motor at two different speeds. Our decay curves from one to three msec were taken when the reaction mixture received 250 flashes of exciting light per second, whereas the decay curves from 5 to 20 msec were taken when the reaction mixture received 40 flashes per second. The chloroplasts received the same total energy of exciting light per second at both disc speeds, since the duration of each individual flash of exciting light was longer at the slower speed. The flash duration at 250 flashes per second was 0.6 msec, and at 40 flashes per second it was 3.75 msec. Because of this situation, the decay curve was displayed in two segments which were not continuous. Figure 2-2 shows the photographs of the oscilloscope face with traces obtained using isolated chloroplasts at the two different disc speeds, as well as the graph made from combining the two photographs. The dotted line between three and five milliseconds shows where the two measurements do not overlap.

All delayed light measurements were made with the reaction mixture at 25°C. The exciting light was from a Sylvania 650W sun gun tungsten iodide lamp. The white light was passed through a 12 cm water filter and focused on the phosphoroscope opening to give an image of about 1 cm^2. Illumination intensity was 10^6 $ergs/cm^2/sec$, as measured by a Yellow Springs radiometer. To eliminate the effects of induction transients, the reaction mixture was preilluminated for 30 seconds (receiving 250 flashes per second) with 10% of the maximum intensity obtained by inserting a Balzers neutral density interference filter. This preillumination at low intensity was followed by 30 seconds illumination at maximum intensity, after which the decay curve at 250 flashes per second (1 to 3 msec delayed light) was photographed from the face of the oscilloscope. The speed of the motor was immediately reduced to 40 flashes per second (5 to 20 msec delayed light) and within 15 seconds the decay curve at 40 flashes per second was photographed.

In some experiments substances such as electron acceptors, donors, etc. were added to the sample after

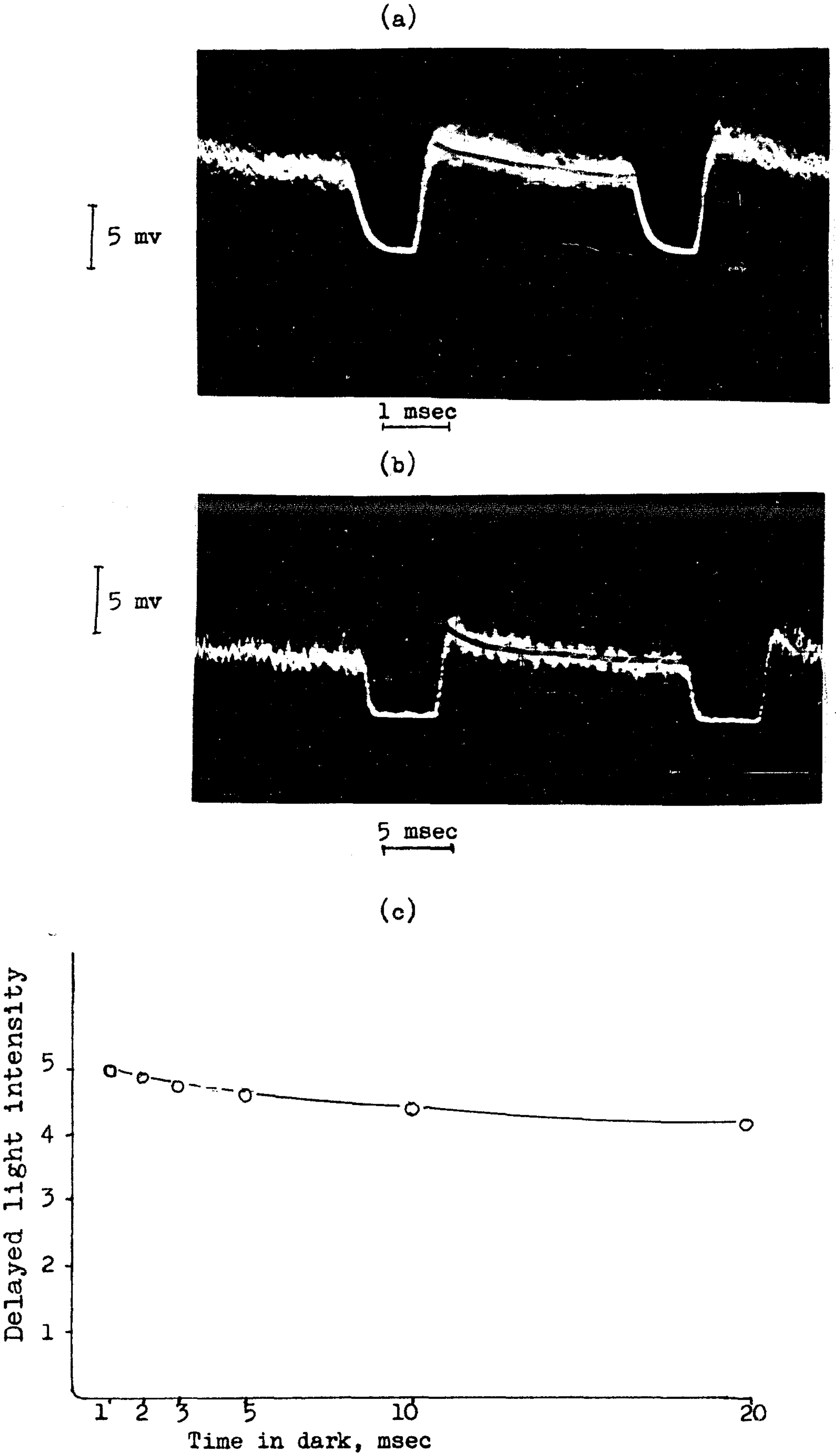

Figure 2-2. Delayed light from isolated chloroplasts. (a) Delayed light from 1 to 3 msec after illumination. (b) Delayed light from 5 to 20 msec after illumination. (c) A plot of the delayed light emission from the two different time periods.

delayed light measurements were taken in the absence of these substances. In these cases after the first two pictures were taken, the motor was increased to 250 flashes per second, and 0.1 ml of a solution containing the substance was introduced into the 2 ml reaction mixture by means of a hypodermic syringe. After addition we waited 30 seconds to eliminate any transient effects on delayed light, and then photographed the two different time spans of delayed light as described above.

B. Thermoluminescence apparatus

To study glow curves from chloroplasts and algae we constructed a thermoluminescence apparatus. Our apparatus is a modified form of that used by Arnold, and is diagrammed in figure 2-3. The sample holder was a copper plug, which gave rapid heat transfer from the sample holder into the sample during heating. The sample had a thermocouple embedded in it which accurately monitored the temperature. The copper plug fitted inside an inconel tube connected to a pair of shutters (see figure 2-3). Shutter A, when open, allowed the sample to be illuminated. When closed, shutter A prevented illumination. Shutter B contained a mirror and when it was in position with shutter A open light was reflected off the mirror and onto the sample. Illumination was from a Unitron 500W microscope illuminator which gave an intensity of 5 x 10^5 ergs/cm^2/sec of white light incident on the sample. When shutter A was closed and shutter B open, no

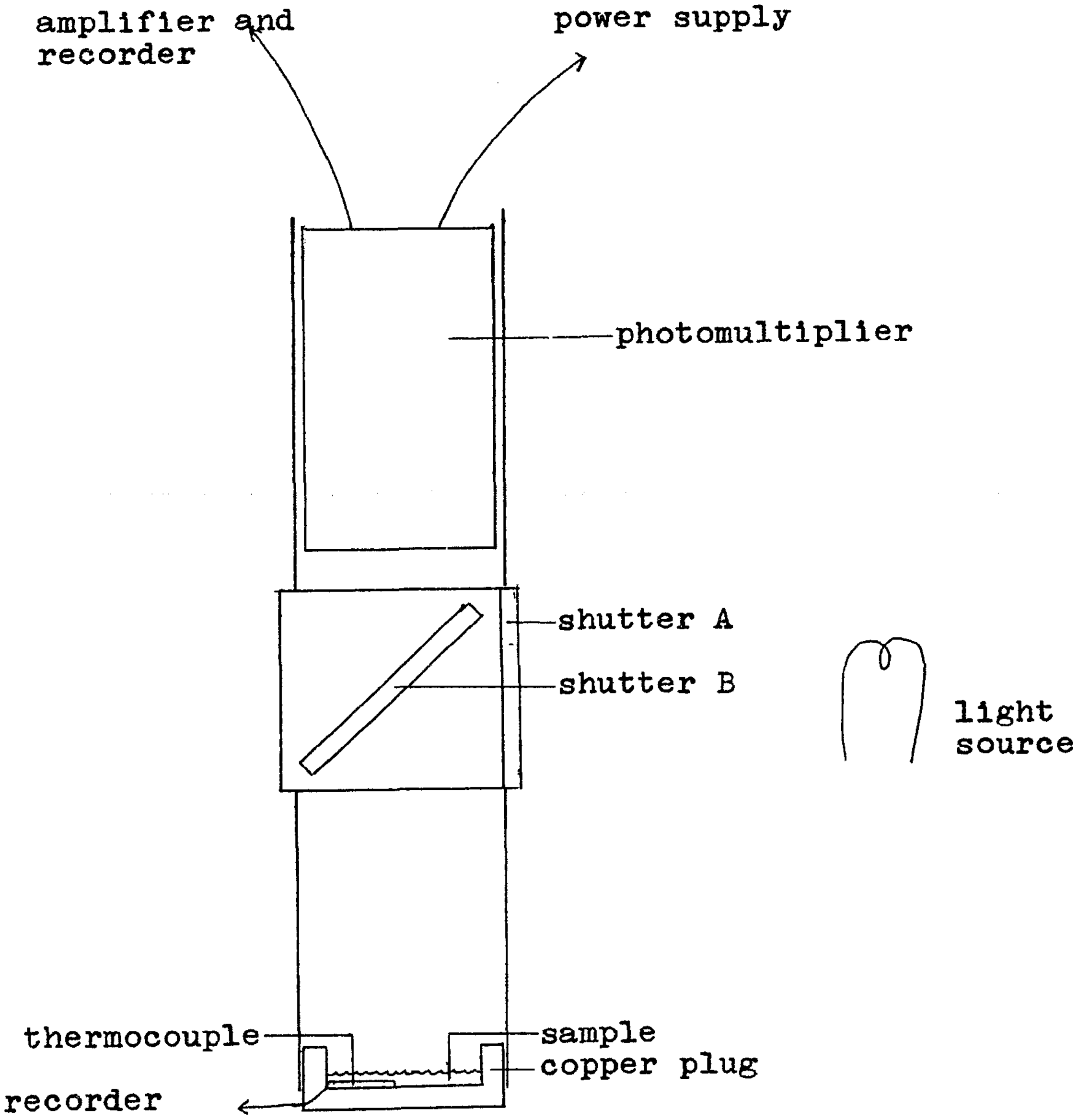

Figure 2-3. Schematic diagram of the thermoluminescence apparatus. The shutters move in and out of the plane of the diagram. Shutter B has a mirror on its underside. When shutter A is open and B is closed the sample receives illumination from the light source, but the photomultiplier is protected. When shutter A is closed and B is open, the photomultiplier views the sample.

light struck the sample, but the photomultiplier monitored any light emitted by the sample. The signal from the photomultiplier (Quanticon) went to a picoammeter (Keithley 417) and from there to a Honeywell Electronik 19 two pen recorder. The picoammeter received 10^{-8} to 10^{-9} amps of signal from the photomultiplier. The other pen monitored the signal from the thermocouple so that the light emitted by the sample and the temperature of the sample were monitored simultaneouly. The sample was cooled by a dewar containing liquid nitrogen which was fitted around the sample holder. It was heated by bringing the copper plug into contact with water at 80°C. To prevent condensation during heating, dry N_2 gas was blown continuously over the face of the sample.

Thermoluminescent glow curves of chloroplasts and algae were obtained with several different protocols. The following standard procedure was adapted for most experiments because it gave maximum emission and best repeatability. A one ml sample containing 50-100 ug chlorophyll with an average thickness of 0.4 mm was frozen to liquid nitrogen temperature, and illuminated with white light while freezing from 0° to -196°C. The total time of illumination was approximately two minutes. Once the sample reached liquid nitrogen temperature illumination ceased, and after 10 - 15 seconds in the dark at this temperature the sample was rapidly heated to 70°C by bringing the sample holder into contact with hot water; this took about 40 seconds. With this procedure the heating rate was 10 - 12°/sec below 0°C and 7 - 9°/sec above 0°C. Use of a relatively thin sample ensured uniform illumination, uniform melting, and prevented large temperature gradients from the top to the bottom of the sample. Variations from this procedure will be indicated where they occur.

The chloroplasts or algae were suspended in an aqueous reaction mixture, which caused a break in the heating rate at the temperature where ice melts (-1°C to +1°C). For this reason we normally indicated the heating rate over the different parts of the temperature range, by reproducing the tracing of the heating rate. Figure 2-4 shows a sample emission profile. The ordinate is intensity of light emitted and the abscissa is time of heating. The rate of heating is indicated by the line above the emission profile. Attempts to obtain a more linear heating rate will be discussed in Chapter IV.

As seen in figure 2-4 there was a glow peak emitted over the temperature range -170° to -130°C. This peak was what Arnold termed the Z peak (Arnold, 1968), and it usually showed a shoulder or second peak on the high temperature side, the main peak coming around -150°C. This peak did not appear to be involved in the functional photosynthetic apparatus and will be dealt with only briefly here. It was present in acetone extracts of chloroplasts; it was filled by blue light and very poorly filled by red light; its emission was in the red region of the spectrum; and it corresponded to a shallow trap depth of 0.1 ev. None of the

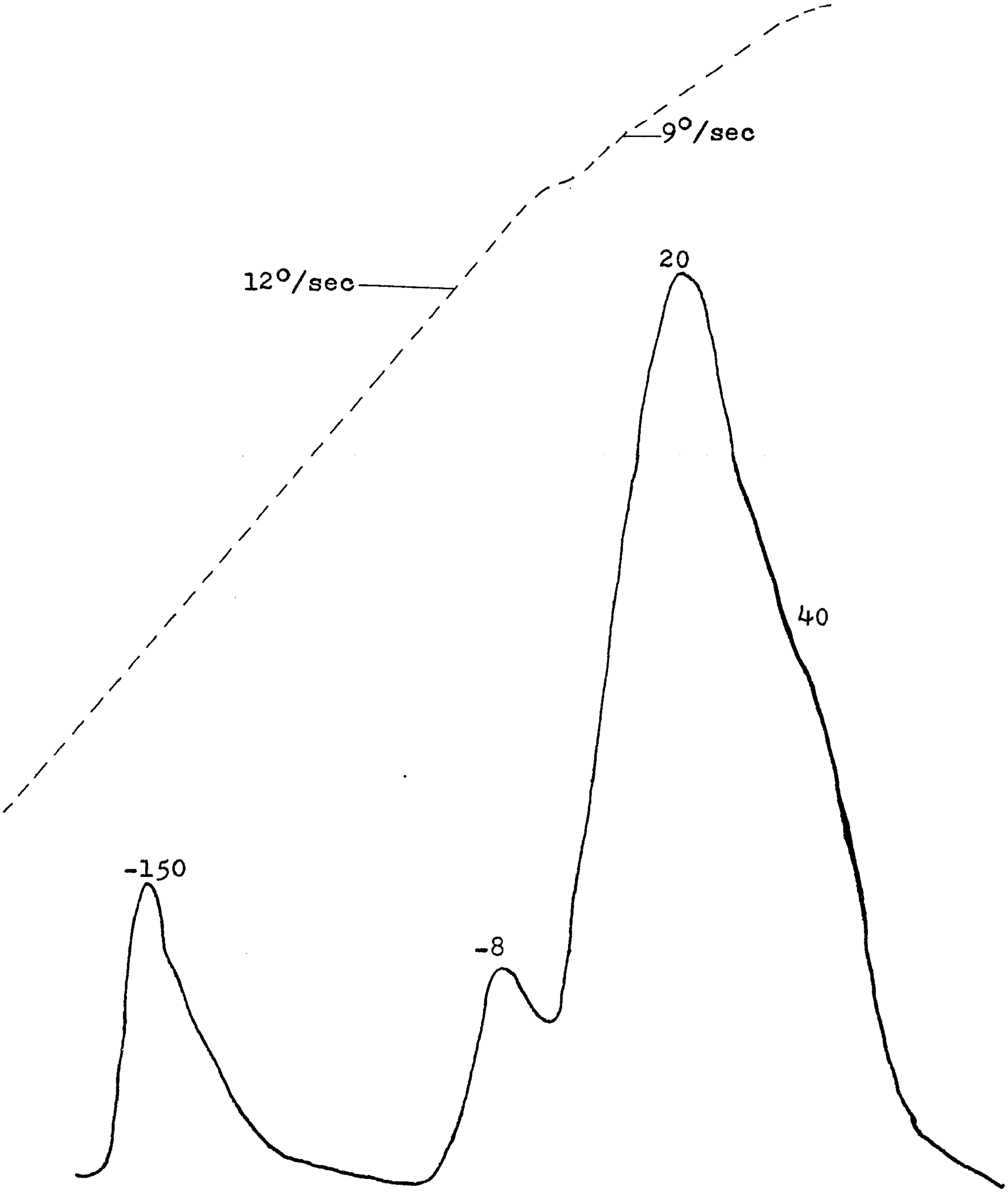

Figure 2-4. Thermoluminescence of isolated chloroplasts. See text for conditions.

treatments examined in Chapter IV affected this peak.

Between -40°C and +60°C a second emission profile was observed consisting of two or three peaks. The low temperature peak we termed peak 1, the main peak was peak 2 and the shoulder on the high temperature side was peak 3 (see figure 2-4). We used the term 'energy storage state' in referring to these individual peaks. The exact temperature of the peaks varied as a function of the heating rate, although the variation was a matter of a few degrees. For this reason the temperature of the peak will be indicated in each figure as demonstrated in figure 2-4. If the heating rate was 6°/sec, the peaks will emerge at a slightly lower temperature than if the heating rate was 8°/sec.

C. Photochemical measurements

Photoreduction of the Hill acceptors, ferricyanide, DCPIP or NADP, was measured spectrophotometrically with an Aminco-Chance Dual Wavelength Spectrophotometer modified to allow for side illumination. For ferricyanide reduction the measuring wave length was set at 420 nm and the reference wave length at 480 nm. DCPIP reduction was measured similarly with the wave lengths set at 590 and 470 nm, while NADP was monitored at 340 nm compared to 375 nm. Red actinic light of 2.2×10^5 ergs/cm^2/sec was obtained with a tungsten lamp and a 675 nm Baird-Atomic interference filter (54 nm half band width). In the ferricyanide measurement the EMI 9524 photomultiplier was blocked from the actinic light with a Corning filter 4303 and a one cm saturated solution of $CuSO_4$; DCPIP reduction used Corning filter 4-76 and a Kodak wratten filter 57 for this purpose; the NADP measurement used Corning filters 5840 and 5970 to block the actinic light.

D. Chloroplast preparation and special treatments

1. Regular chloroplasts

Chloroplasts were isolated from greenhouse grown Good King Henry (*Chenopodium bonicus-henricus*) or market spinach following the method of Jagendorf and Avron (1959). Fifty grams of leaf tissue were suspended in 200 ml of the following ice-cold grinding buffer (0.4 M sucrose, 0.05 M Tricine-NaOH, pH 7.8, and 0.01 M NaCl (STN solution)) and ground in a cold Waring Blender by means of three high speed blending of 5 seconds each. The resulting leaf homogenate was squeezed through 25 u nylon bolting cloth (Tobler and Traber, Elmsford, N. Y.). The filtrate was centrifuged (at 2°C) for five minutes at 8000 x g. The supernatant was discarded and the pellet washed once with fresh grinding medium. The pellets were resuspended in 0.001 M $MgCl_2$, 0.001 M NaCl and stored in ice until use. Chlorophyll was determined according to Arnon (1949).

2. Digitonin subchloroplast particles

Chloroplasts were prepared from market spinach using a modification of the method of Anderson and Boardman (1966).

200 grams of leaves were depetiolated and homogenized in a Waring Blender with 800 ml of the following ice-cold grinding buffer: 0.3 M sucrose; 0.01 M KCl; and either 0.05 M tris-HCl (pH 7.8) or 0.05 M phosphate (pH 7.2). The homogenate was filtered through nylon bolting cloth and centrifuged at 2°C for 10 minutes at 6000 x g. The supernatant was discarded and the pellets washed once with fresh grinding medium.

The chloroplasts were resuspended in 0.01 M KCl; and either 0.05 M tris-HCl (pH 7.8) or 0.05 M phosphate (pH 7.2). 2% digitonin was added to a final concentration of 0.5% digitonin and 250 - 300 ug chlorophyll/ml. The mixture was mechanically stirred for 45 minutes at 4°C. Fractions were then collected by differential centrifugation (Beckman L2 ultracentrifuge). Pellets collected after centrifugation at 10,000 x g for 30 minutes were called D-10; those collected after 144,000 x g for one hour were D-144. The subchloroplast particles were resuspended with the aid of a teflon pestle in 1 M sucrose, 0.002 M tricine-NaOH (pH 8.0).

3. Tris aging

Control and tris treated chloroplasts were prepared by a modification of the method of Yamashita and Butler (1968). Fifty grams of spinach leaves were ground in a Waring Blender for 5 seconds in 150 ml of the following solution: 0.4 M sucrose, 0.05 M tris-HCl (pH 7.8), 0.01 M NaCl. The slurry was filtered through 25 u mesh nylon cloth and spun at

7000 x g for five minutes. Half of the pellet was suspended in 20 ml of the grinding solution (control chloroplasts), and the other half in 20 ml of 0.8 M tris-HCl (pH 8.0) (tris aged chloroplasts). Both resuspensions were incubated at 2°C for 10 minutes and then centrifuged at 12,000 x g for five minutes. The pellets were resuspended in resuspension medium 0.001 M $MgCl_2$, 0.001 M NaCl.

4. Heat Inactivation

Chloroplasts were prepared as in section D. 1. Resuspended, undiluted chloroplasts at a concentration of 1 - 1.5 mg/ml were removed in ½ ml aliquots. These aliquots were placed for various periods of time at 50°C in a constant temperature water bath, and then removed to an ice bucket and cooled.

5. Ultraviolet irradiation

Chloroplasts, prepared as in section D. 1, were suspended in the reaction mixture (0.02 M NaCl, 0.025 M TES-NaOH, pH 7.2, 0.004 M $MgCl_2$) at 100 ug chl/ml and were irradiated in a petri dish (the layer of suspension was around 2 mm thick) at a distance of six cm from a G. E. G8T5 8 watt germicidal lamp (2537Å). The chloroplast suspension was stirred with a magnetic stirrer during the period of irradiation and was maintained at 4°C using an ice water mixture. Control chloroplasts were prepared in an identical manner but were not irradiated.

6. Chloride depletion

Chloroplasts were depleted of chloride using the EDTA washing procedure of Izawa, et al. (1969). Fifty grams of market spinach leaves were ground in 150 ml of ice-cold medium containing 0.4 M sucrose, 0.025 M TES-NaOH, pH 7.4, and 0.005 M Na_2SO_4. The homogenate, filtered through cheesecloth, was centrifuged at 2500 x g for 5 min. The sedimented chloroplasts were resuspended and washed once with a medium containing 0.1 M sucrose and 0.005 M TES-NaOH at pH 7.4, twice with 0.002 M Tricine and 0.0005 M Na-EDTA at pH 7.8 and finally with 0.1 M sucrose, 0.005 M TES-NaOH and 0.005 M $MgSO_4$ at pH 7.4.

E. Reaction mixtures

Unless otherwise specifically mentioned, the same reaction mix was used for delayed light, thermoluminescence and photochemical measurements. The delayed light used a 2 ml sample containing 0.01 M Tricine-NaOH (pH 7.8), 0.02 M NaCl, 0.005 M $MgCl_2$, and 15 - 20 ug chl/ml. A 1 ml sample was used in thermoluminescence containing 50 - 100 ug chl and the same reaction mix as above. In photochemical measurements a 5 ml sample was used with the same components as the delayed light sample but only 9 - 10 ug chl/ml. In measurements with chloride depleted chloroplasts the reaction medium was 0.05 M TES-NaOH (pH 7.4), 0.01 M sucrose, 0.005 M $MgSO_4$. Whenever uncouplers were examined the usual reaction medium was used with the modification that KCl was substituted for NaCl.

CHAPTER III: DELAYED LIGHT

A. Introduction

The delayed light emission from plants reflects, under certain experimental conditions, the storage of metastable energy at photoreaction II. This low intensity light emission, discovered by Strehler and Arnold in 1951, is observed immediately after illumination is terminated and continues for many minutes. The emission spectrum of delayed light from oxygen evolving plants indicates that the emission is from the first excited singlet of chlorophyll a (Azzi, 1966). The action spectra of delayed light emission matches the action spectra of photosynthesis in the organisms studied (Arnold and Thompson, 1955). Lack of delayed light from mutants lacking reaction centers, shows that the emission is associated mainly with a functional reaction center of photosystem II, the oxygen evolving photoreaction in the Hill and Bendall scheme (Bertsch, *et al*., 1967).

Several observations have led to the conclusion that delayed light in the time range of milliseconds is a reliable tool for studying energy storage by photosystem II.

Abbreviations: CCCP carbonyl cyanide 3-chlorophenylhydrazone, DCMU 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCPIP 2,6 dichlorophenol-indophenol, DPC 1,5-diphenylcarbohydrazide, DPSC diphenyl semicarbazide, Fecy ferricyanide, NADP nicotinamide adenine dinucleotide phosphate, tricine N-tris (hydroxymethyl) methylglycine, tris tris (hydroxymethyl)aminomethane, TES N-tris(hydroxymethyl) methyl 2 aminoethanesulfonic acid, EDTA ethylenediaminetetraacetic acid, Fd ferredoxin, ATP adenine triphosphate, ADP adenine diphosphate

This section will first repeat several older observations on the millisecond delayed light characteristics from different photosynthetic organisms and from isolated chloroplasts, as well as repeating the effect of mutations in photosystem I and photosystem II.

In an attempt to determine whether some portion of the delayed light was emitted by photosystem I, we have examined the effect that physical separation of the two photosystems by detergent treatment has on delayed light. The particles enriched in photosystem I did show some millisecond delayed light, but this was found to reflect residual photosystem II activity (Section B). We have also investigated the effect on delayed light of a number of treatments of chloroplasts which affect photosynthesis by inhibiting photochemical processes associated with photosystem II. Photochemistry can be restored after these treatments by substituting an artificial electron donor in the role normally filled by water, and we have compared the delayed light emission of these restored systems to that of untreated chloroplasts (Section C). This investigation also examined the effect that different types of phosphorylation uncouplers have on millisecond delayed light, in an attempt to study interactions between the high energy state leading to phosphorylation with energy storage at photosystem II (Section D).

Whole algae show a rapid delayed light decay in the millisecond time range as shown in figure 3-1 for

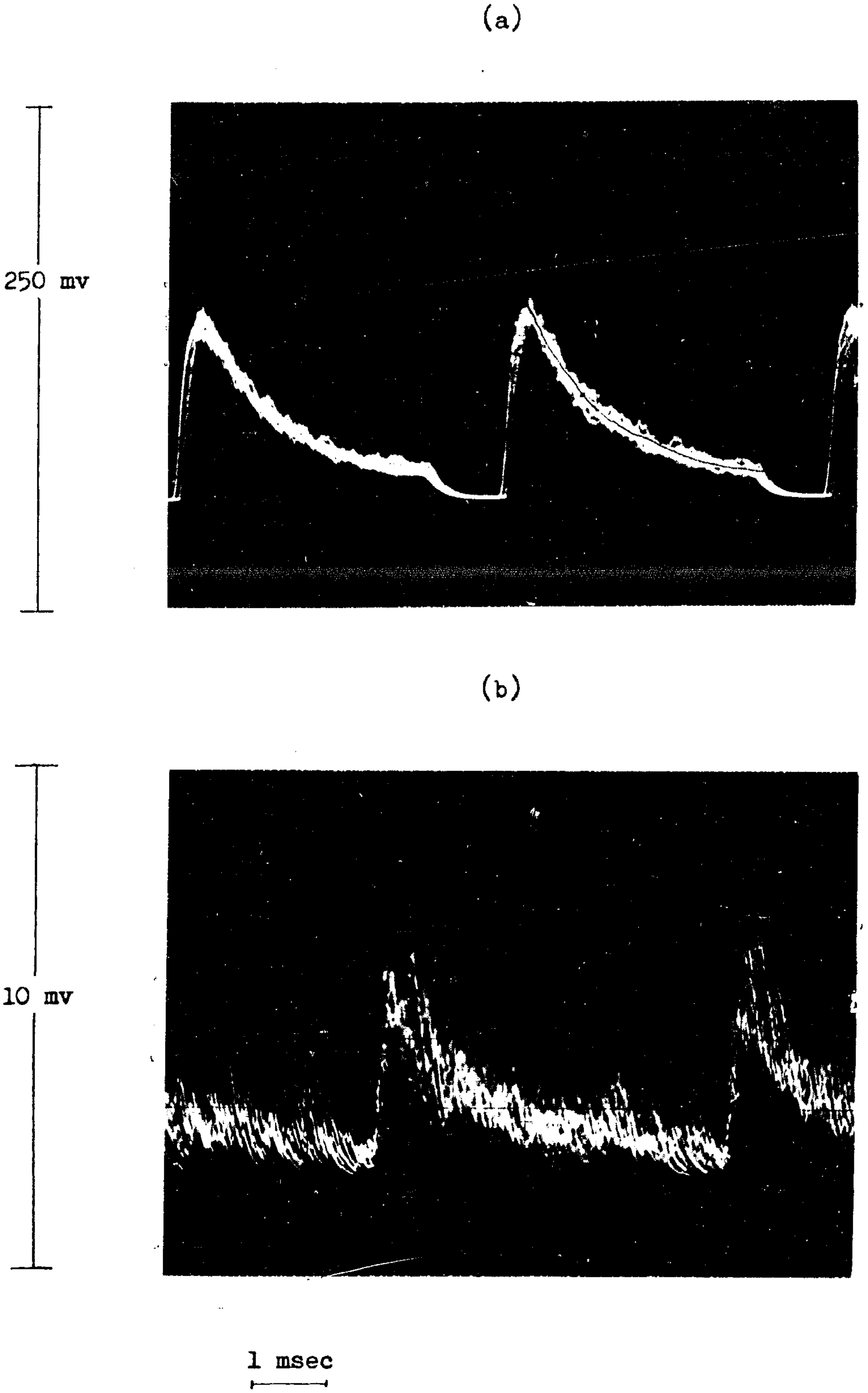

Figure 3-1. Delayed light from the green alga Chlorella pyrendosa (a) and the blue green alga Anacystis nidulans (b). The cells were adjusted to the same chl a concentration (5 ug/ml) and were suspended in their growth media. The cells were in the exponential growth phase. These photographs of the oscilloscope face show delayed light of the cells from 1 to 3 msec after the center of a flash of exciting light.

Chlorella and Anacystis. The blue green alga Anacystis emits only five percent as much light on a per unit chlorophyll basis as green algae such as Chlorella and Scenedesmus. The decay kinetics, however, are similar. When electron transport is inhibited by a mutation which affects one of the photosystems, the delayed light emission is altered. Figure 3-2 compares the emission characteristics of two Scenedesmus mutants with wild type. In mutant 11 photosystem II is nonfunctional, and in mutant 8 photosystem I is nonfunctional.

Mutant 11, in which the oxygen evolving photosystem is nonfunctional, emits very low intensities of delayed light compared with wild type alga. Bertsch et al. (1967) found that when the emission was averaged from 0.75 to 4.2 msec from the center of a flash of exciting light, the wild type emitted 250 times the intensity emitted by mutant 11. The one millisecond emission shown here is also decreased about 250 times when compared with the wild type.

Mutant 8, in which the NADP reducing photosystem is blocked, emits delayed light with different decay kinetics from those of the wild type. In the millisecond time range, mutant 8 emits higher intensities of delayed light and the emission decays more slowly than in the wild type. Poisoning of algal cells with DCMU (Bertsch, 1963) also results in a slow dark decay of emission, implying that this poison blocks energy flow out of the photoreaction which emits the light. DCMU is known to be a specific

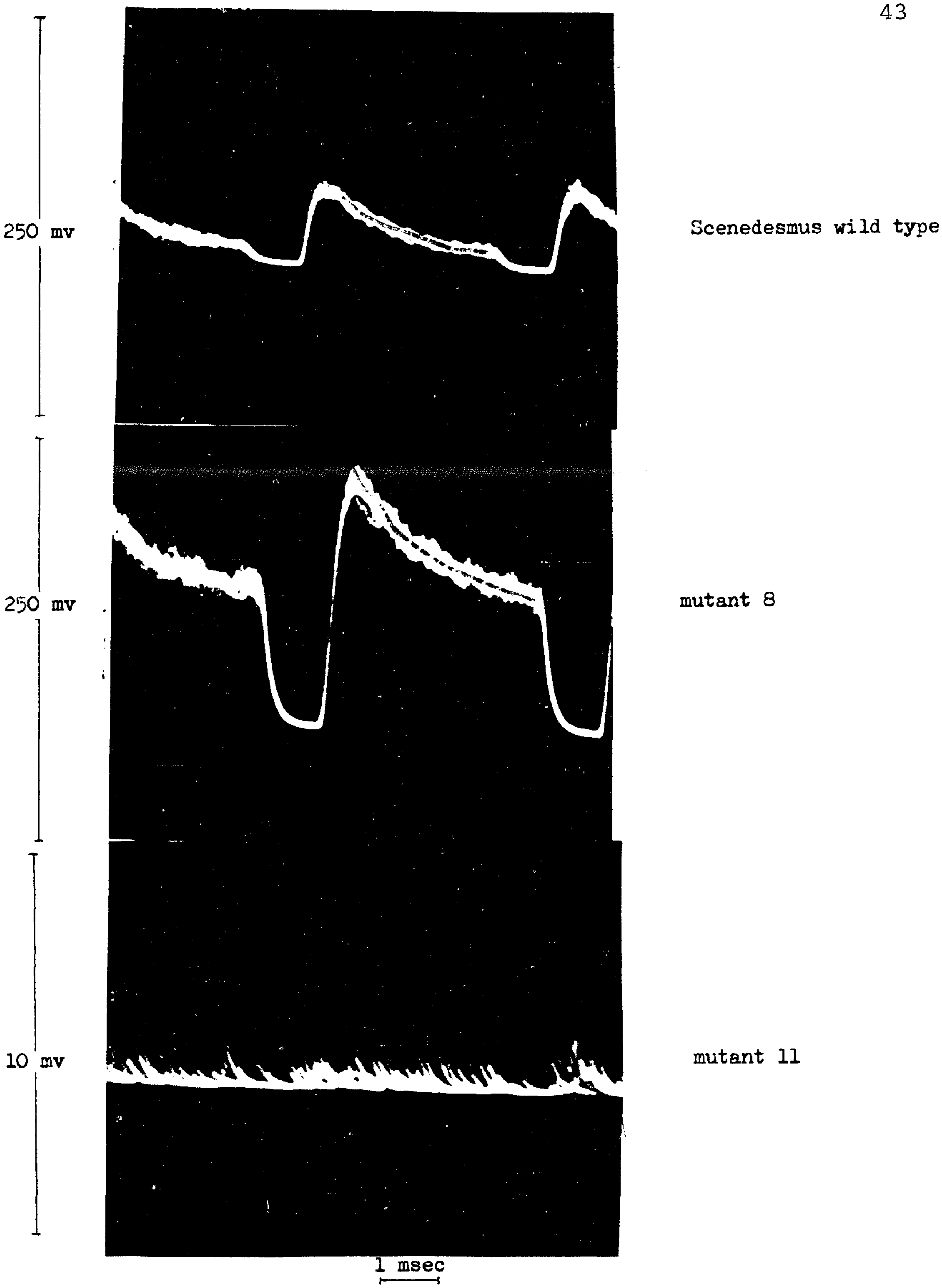

Figure 3-2. Delayed light from Scenedesmus obliquus wild type and two mutant strains, one with a non functional photosystem I (mutant 8) and the other with a nonfunctional photosystem II (mutant 11). The cells were adjusted to the same chl a concentration (5 ug/ml) and were suspended in their growth media. They were harvested in exponential growth phase. The photographs show 1 to 3 msec delayed light

inhibitor of reactions which require photochemical energy from photosystem II.

Figure 3-3 gives the delayed light emission from isolated chloroplasts with and without an added Hill acceptor. The millisecond emission is seen to decay slowly when chloroplasts isolated from leaves are not supplied with electron acceptors. On activation of the electron transport chain by addition of electron acceptors to the chloroplast suspension, the dark decay became more rapid, approaching that of algae or whole leaves.

Figure 3-4 shows a decay curve from 1 - 20 milliseconds after repeated flashes of exciting light, with and without the electron acceptor ferricyanide and in the presence and absence of DCMU. The presence of ferricyanide increases the one millisecond delayed light from 2 to 7 times, depending on the exciting light intensity and the particular chloroplast preparation. DCMU decreases the total magnitude of the delayed light as well as making the decay slower.

Figure 3-5 shows the effect of increasing concentrations of DCMU on one millisecond delayed light in the presence and absence of ferricyanide, and the poison's effect on photochemistry. The decrease in the one millisecond stimulation of delayed light in the presence of an electron acceptor closely parallels the decrease in photochemistry caused by the inhibition of electron flow by DCMU. At the concentration 10^{-6}M, where chemistry is totally inhibited,

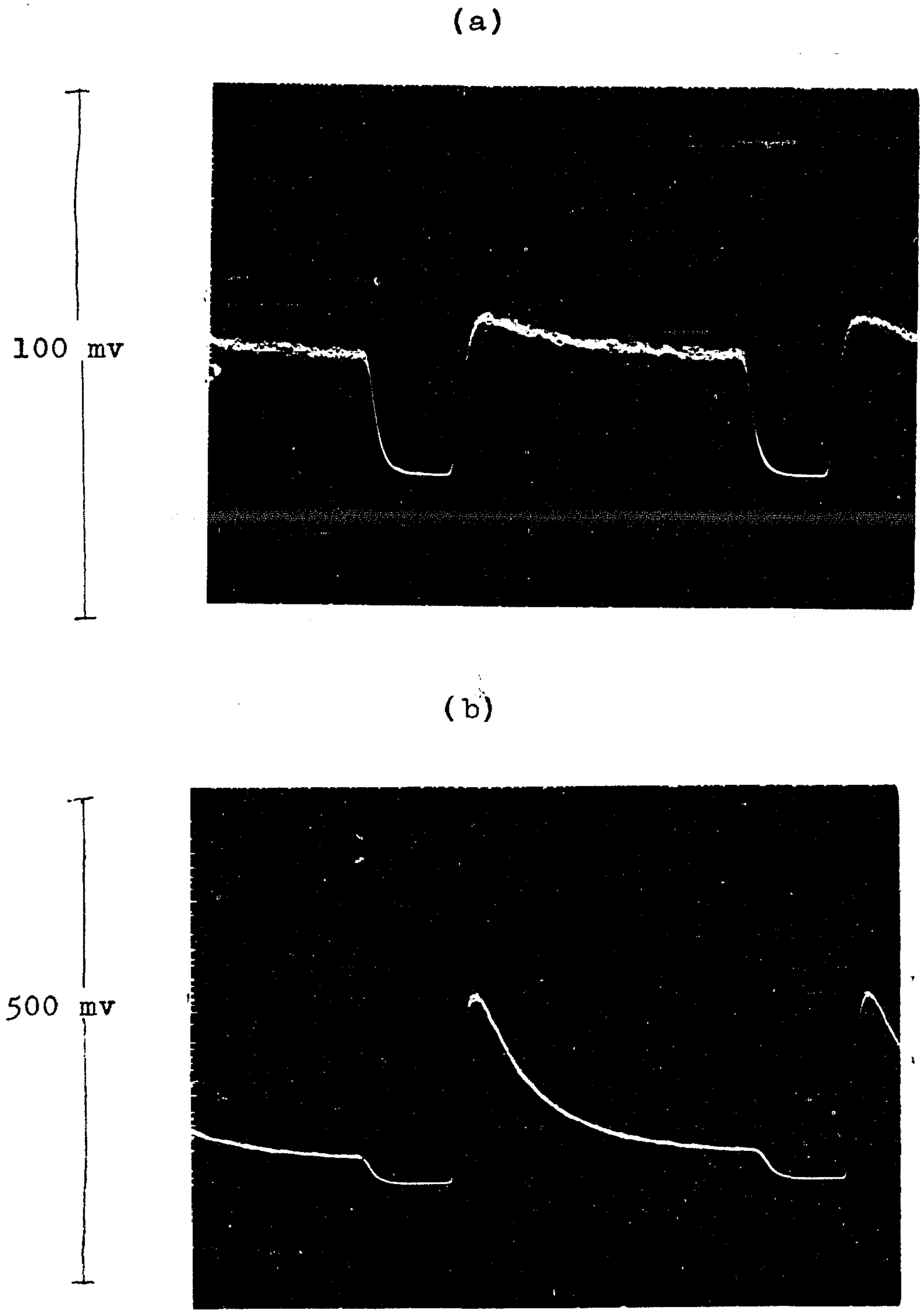

Figure 3-3. Delayed light from chloroplasts in the absence (a) and presence (b) of the Hill acceptor ferricyanide ($5x10^{-4}M$). Photographs of the oscilloscope face show 1 to 3 msec delayed light.

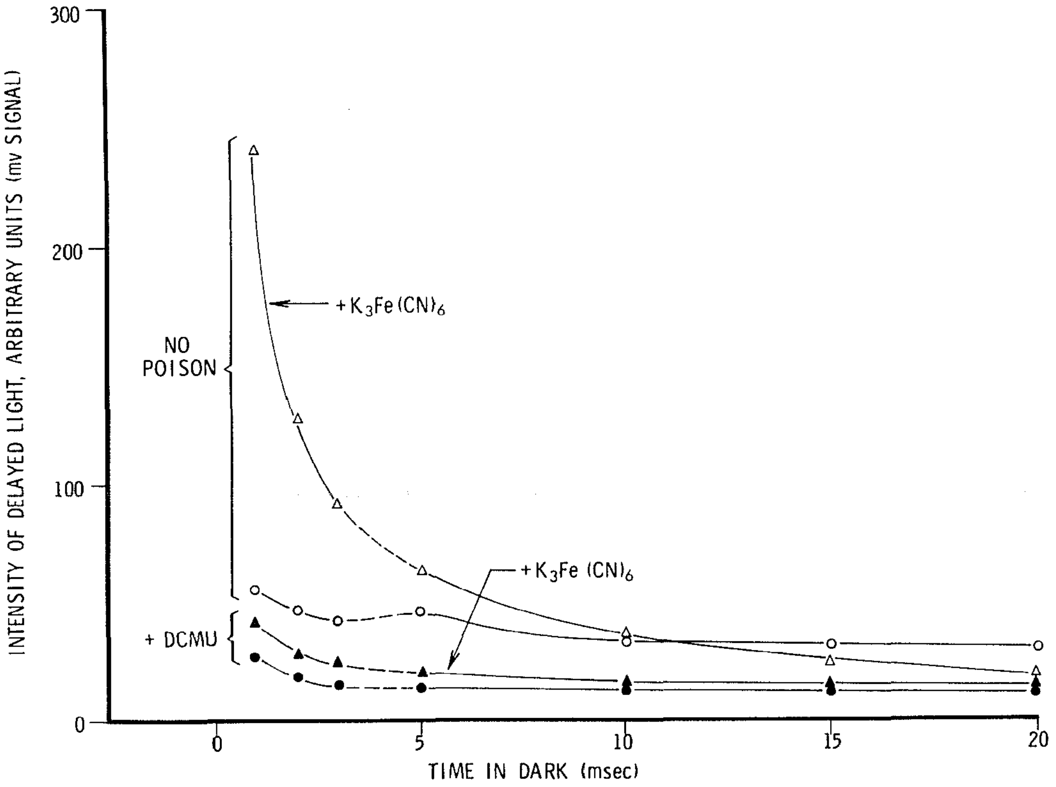

Figure 3-4. Effect of DCMU on the ferricyanide induced increase in delayed light. Where added $5x10^{-4}$M ferricyanide, $3x10^{-6}$M DCMU.

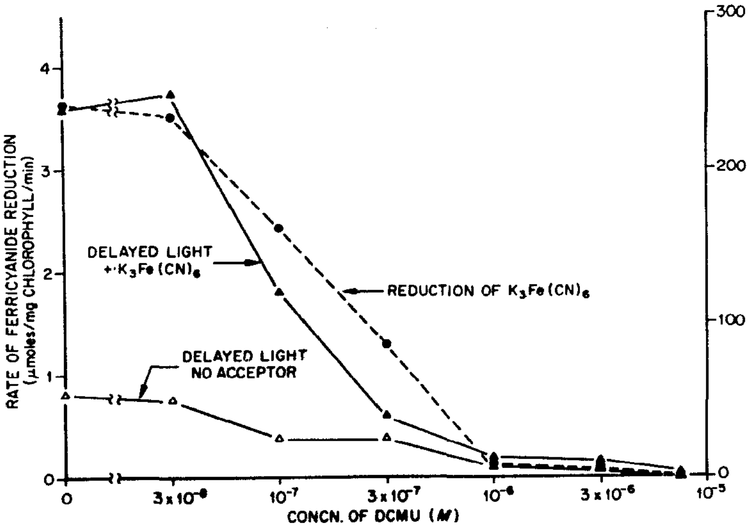

Figure 3-5. The effect of increasing concentrations of DCMU on delayed light emission at 1 msec in the absence and presence of ferricyanide ($3x10^{-4}$M), and the effect of DCMU on ferricyanide reduction.

the one millisecond delayed light increase is also absent, and the intensity of emission is reduced.

All of the data presented above are in agreement with previously published results. The reader is referred to Bertsch, 1969; Bertsch, West and Hill, 1971; Bertsch and Lurie, 1971. The functioning photosynthetic apparatus, either in whole algae or in isolated chloroplasts with added electron acceptors, exhibits rapid delayed light decay kinetics. Slow decays are found in mutant algae blocked in photosystem I (but with a functioning photosystem II), in isolated chloroplasts with no electron acceptor present, and in the presence of the photosynthetic poison DCMU.

When photosynthetic electron transport is occurring the one millisecond delayed light is increased in intensity compared to when photochemistry is prevented. This increased emission at one millisecond reflects a transient increase in energy stored by the functioning photoreaction II at times earlier than 10^{-2} seconds, the steady state enzymatic turnover time. The transient increase in energy storage appears to be due to the mechanisms by which oxidizing and reducing equivalents are donated by photosystem II to the electron transport systems associated with the photosystem. This is substantiated by the fact that the enhanced delayed light at one millisecond caused by the addition of electron acceptors can be selectively inhibited by DCMU. The addition of DCMU, in concentrations which inhibit electron transport, also results in a slow dark decay of delayed light regardless of the presence or absence of the hydrogen acceptor potassium ferricyanide, and the increase in the intensity of emission at one millisecond caused by this acceptor is inhibited by DCMU.

These parameters of delayed light can be understood on the two quanta electron-hole model presented in the introduction. However, from a purely emperical viewpoint we can associate a 'flat', slow msec decay with lack of photochemical activity at photoreaction II. Similarly, a 'steep', rapid msec decay and an increased emission at 1 msec can be emperically associated with performance of chemical work by photoreaction II. We shall use these empirical observations in all our interpretations of millisecond delayed light emission.

B. Subchloroplast fractions from digitonin treatment of chloroplasts

1. Introduction

A number of attempts have been made to separate the two photosystems from each other by treating chloroplast preparations with detergents and by other methods. The criterion for separation has been taken as altered chl a/chl b ratios for the fractions as compared to the original chloroplast suspension. Michels and Michel-Wolwertz (1969) have utilized the French press method and sonication. Vernon, *et al*. (1966) have employed the detergent Triton

X-100. Thornber (1969) used sodium lauryl sulfate, while Anderson and Boardman (1966) as well as Wessels (1965), have employed the anionic detergent digitonin.

Ruby and Brown (1970) using a French press method with the Scenedesmus mutants 8 and 11, reported from their results that photosystem I might contribute to millisecond delayed light emission. In our hands Brown's French press method did not produce sufficient separation of the two photosystems to allow meaningful delayed light experiments. It seemed therefore pertinent to utilize a different method to separate the two photosystems and to examine the resultant fractions for delayed light emission. The method used by Boardman and Anderson (1966) with digitonin combined a good separation with a retention of photochemical activity. This treatment yields lamellar fractions that are enriched in either photosystem I (D-144) with a chl a/chl b ratio greater than 4 or in photosystem II (D-10) with a chl a/chl b ratio less than 2. Delayed light measurements in the millisecond time range, as well as photochemical activities of the two fractions (compared to chloroplasts) were examined.

2. Results

An indication of the degree of separation of the two photoreactions in D-10 and D-144 particles is given by both the chlorophyll a to b ratios, and by the ratio of low temperature fluorescence intensities at 735 and 698 nm (Cederstrant and Govindjee, 1966), which are shown in

Table 3-1. All the fractions examined emitted delayed light. Figure 3-6 shows good agreement between the relative intensity of delayed light emission at 1 msec (either in the presence or absence of ferricyanide), and the relative rates of ferricyanide reduction for chloroplasts as compared to D-10 and D-144. Thus all msec delayed light emission may well come from photoreaction II, and the data support the conclusion that msec emission from the photoreaction I fraction is completely due to contamination with photoreaction II. If one assumes that all of the delayed light in the millisecond time range is emitted by photoreaction II, then the data in Table 3-1 indicate that D-10 particles are enriched in photoreaction II by 40 - 50 percent compared to the original chloroplasts. The D-144 particles contain about 12% photoreaction II contamination, using this same delayed light comparison.

Figure 3-6 shows that both fractions respond to the addition of the electron acceptor ferricyanide in a manner similar to chloroplasts, by an increase in emission at 1 msec coupled to an increase in rate of dark decay of the emission. We interpret this observation as implying that the electron transport chain is functional in all these particles and that photochemistry is occurring (Bertsch, *et al*., 1969).

It has been previously shown that addition of DCMU to chloroplasts completely eliminates the activation of noncyclic electron flow through photoreaction II by

Fraction	Intensity of delayed light at 1 msec (mV)	Concentration of chlorophyl a (μg / ml)	Delayed light per chlorophyl a (mV/μg / ml)	Chl a/Chl b	R^*
Chloroplast	30	7.5	4	3	3.07
D-10 ——	42	7	6	2	1.04
D-144 ——	3.8	8.15	0.46	4.8	8.37

R^* is the ratio of fluorescent intensities at 735 nm and 698 nm measured at -196°C

Table 3-1. Delayed light emission of digitonin subchloroplast fractions at 1 msec. Chloroplasts and digitonin fractions suspended in 0.01 M KCl and 0.05 M tris-HCl (pH 7.8).

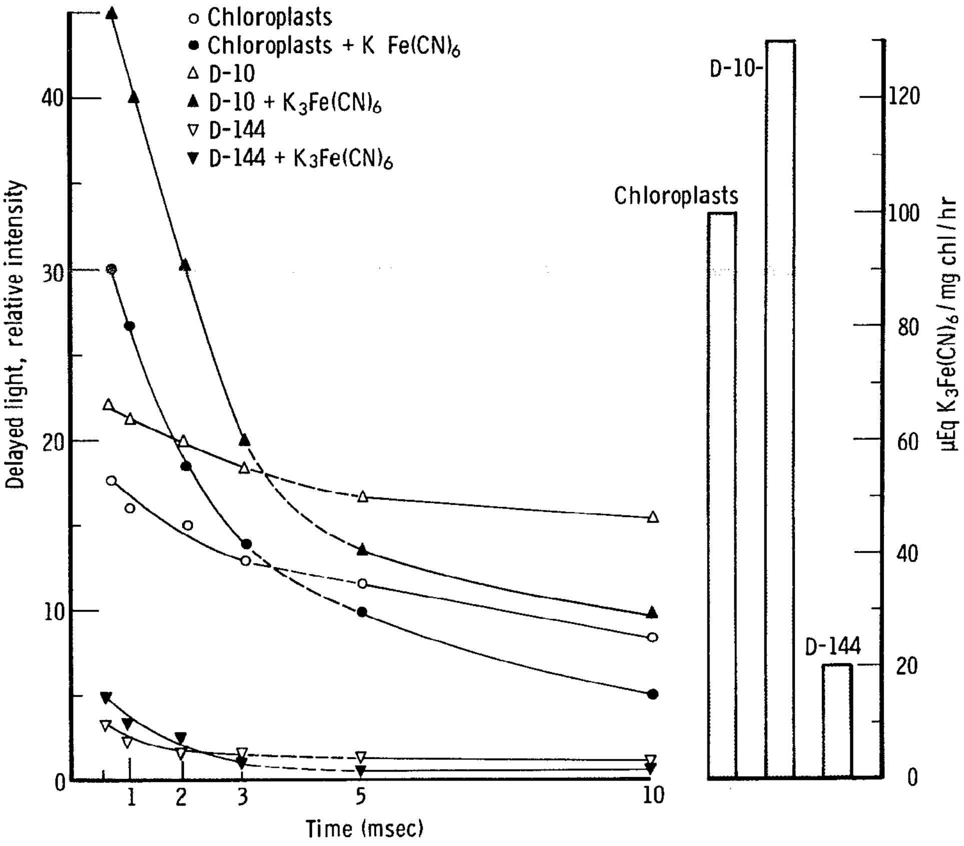

Figure 3-6. Delayed light and photoreduction of detergent fractions and chloroplasts in the absence and presence of ferricyanide. Reaction mix for delayed light: 0.015 M tris-HCl (pH 7.8), 0.025 M NaCl, 0.003 M MgCl$_2$, 3x10^{-4}M ferricyanide, 15 ug chl/ml. Reaction mix for ferricyanide reduction: 0.25M sucrose, 0.03 M phosphate (pH 6.7), 3x10^{-4}M ferricyanide, 4 ug chl/ml.

ferricyanide or NADP$^+$, and concomitantly eliminates the effect of these electron acceptors on msec delayed light (Bertsch, et al., 1971) emitted by chloroplasts or D-10. However, there was no effect of DCMU on the ferricyanide induced changes in msec emission from D-144 (see figure 3-7). Even ten times this concentration of DCMU would not inhibit the ferricyanide effect in D-144. This was apparently the first time DCMU had not been observed to inhibit the activation of noncyclic electron transport through photoreaction II and thereby inhibit the effect of acceptors on msec delayed light. The question thus arises: could ferricyanide be accepting electrons from photoreaction I in the D-144 particles?

If photoreaction I contributes to the delayed light emission, then activation of electron flow through photoreaction I should probably have some effect on the delayed light. Figure 3-8 shows that activation of electron flow from DCPIP-ascorbate to NADP$^+$ had no effect on msec delayed light of either fraction, or of chloroplasts, in spite of the fact that the chloroplasts and D-144 were actively reducing the NADP$^+$. We take this observation as additional evidence that photoreaction I emits a negligible amount of delayed light, since in photoreaction II activation of electron flow causes drastic changes in delayed light.

Since it appears that no previous workers have observed net ferricyanide reduction with D-144 particles,

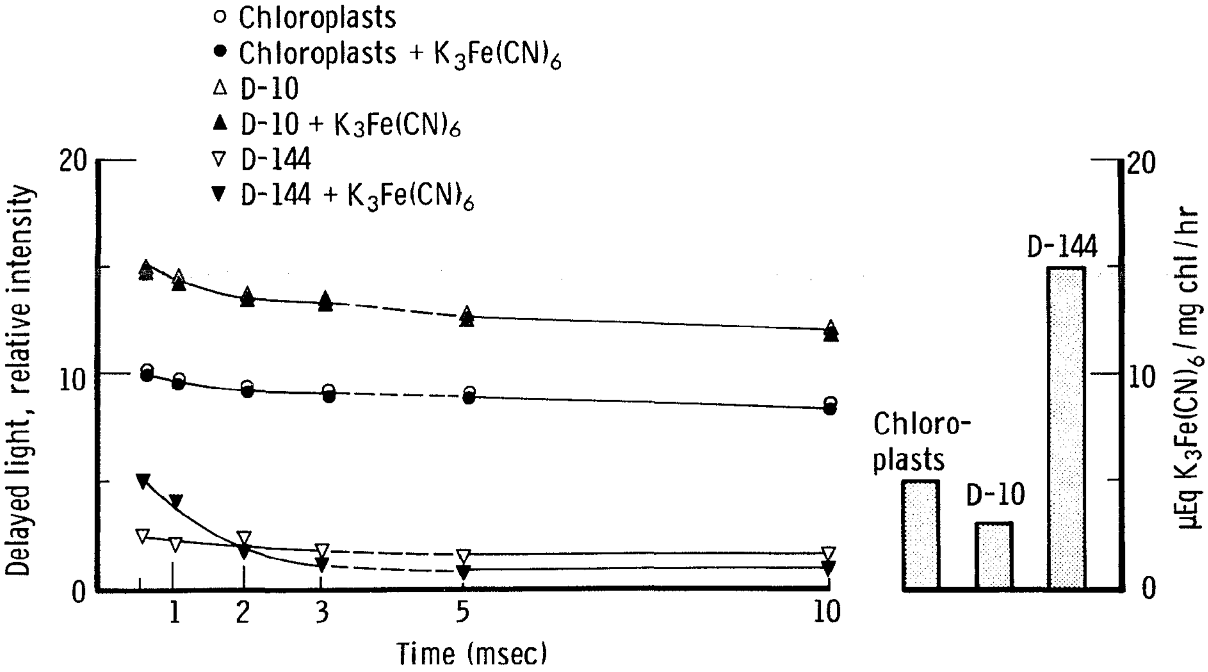

Figure 3-7. Effect of DCMU on delayed light and ferricyanide reduction of detergent fractions and chloroplasts. 10^{-5}M DCMU present in all cases. Absolute values for delayed light and for rates of ferricyanide reduction may be directly compared to figure 3-6. Reaction mixtures as for figure 3-6.

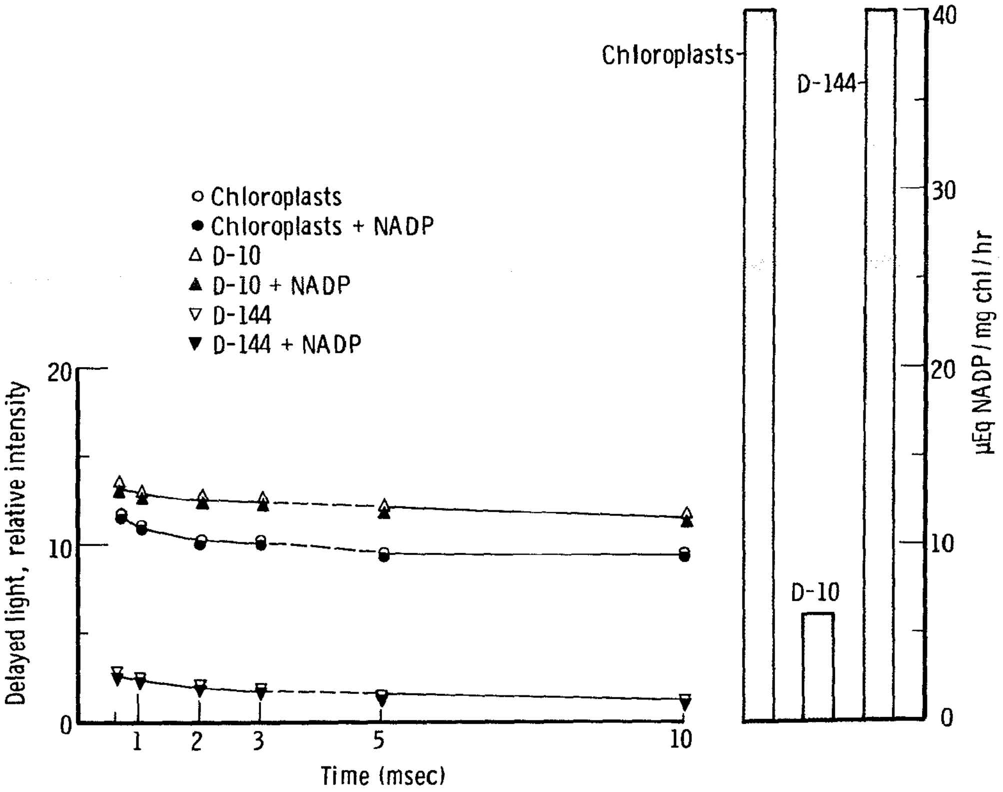

Figure 3-8. Delayed light and photoreduction of detergent fractions and chloroplasts in the absence and presence of NADP$^+$. DCMU, DCPIP and ascorbate present in all cases. Reaction mix: 0.015 M tris-HCl (pH 7.8), 0.02 M NaCl, 0.003 M $MgCl_2$, 10^{-5}M DCMU, 10^{-5}M DCPIP, 0.002 M ascorbate, $3x10^{-4}$M NADP$^+$, saturating amounts of ferredoxin, 15 ug chl/ml.

and since we had not previously observed DCMU-insensitive delayed light in the msec time range, we undertook an investigation of the photochemical properties of these particles. Boardman and Anderson (1966) found that the D-10 fraction photoreduced NADP$^+$ to a small extent and had good rates of ferricyanide reduction and oxygen evolution. These workers found the D-144 particle to have good photoreduction of NADP$^+$ but no measurable photoreaction II activity. However, Vernon and Shaw (1969) have reported the D-144 fraction to reduce DCPIP when DPC is present to supply electrons to photoreaction II in place of water.

Table 3-2 shows oxygen evolution coupled to both DCPIP reduction and to $K_3Fe(CN)_6$ reduction, for both fractions and for chloroplasts. The limits of our resolution were 0.1 ueq/mg chl/hr for both photochemical reductions, and 0.001 ueq/mg chl/hr for oxygen evolution. DCMU inhibited the DCPIP reactions to the limit of our resolution in all but one case, but ferricyanide reduction seemed to be more resistent to DCMU. The clearest case of this is the D-144 fraction, in which DCMU completely inhibited oxygen evolution but only partially inhibited ferricyanide reduction. That is, a residual ferricyanide reduction was observed in the presence of DCMU, but no oxygen evolution remained. It seems clear that the electrons which are accepted by ferricyanide in the presence of DCMU did not originate in the normal oxygen evolving mechanism.

	µEq / mg chlorophyl / hour							
	Oxygen		DCPIP		Oxygen		Ferricyanide	
	-DCMU	+DCMU	-DCMU	+DCMU	-DCMU	+DCMU	-DCMU	+DCMU
Chloroplasts	105	<.001	82	<0.1	114	<.001	100	5
D-10 ——	116	<.001	98	0.3	120	2.0	130	2
D-144 ——	6	<.001	7.6	<0.1	10	<.001	20	15

Table 3-2. Oxygen evolution stoichiometrically coupled to either DCPIP or ferricyanide reduction. Reaction mix: 0.25 M sucrose, 0.03 M phosphate (pH 6.7), 5 ug chl/ml, where added 10^{-5}M DCMU, and either 10^{-4}M DCPIP or $3x10^{-4}$M ferricyanide.

3. Discussion

Our results suggest that D-10 particles are enriched 40 - 50% in photoreaction II activity as compared to the original chloroplasts. Our estimates are based on the assumptions that only photoreaction II evolves oxygen, reduces ferricyanide and emits delayed light, and that the chlorophyll a to b ratios and the ratio of fluorescent intensities at 735 and 698 nm are a reflection of the degree of separation of the two photoreactions. Figure 3-6 and Tables 3-1 and 3-2 show that delayed light emission, reduction of ferricyanide and of DCPIP were all enhanced by about 40% (on a per chlorophyll basis) in D-10 as compared to chloroplasts. On the same basis, D-144 is contaminated with 10 - 20% photoreaction II. We can consider the specific experiment of figure 3-6: D-144 showed 20% of the ferricyanide reduction of chloroplasts, but only 16.7% of the delayed light emission (at 1 msec in the presence of ferricyanide). Thus the amount of msec emission is in good agreement with the amount of oxygen evolution from these 'photoreaction I fragments.' Vernon and Shaw's estimations of the incompleteness of the separation of the two photosystems, which were based solely on dye reduction studies, are in good agreement with ours.

Since the estimates of contamination using delayed light data agree with estimates using photochemical data, the delayed light present in D-144 is presumably the result of incomplete separation of the two photosystems. This conclusion is in agreement with our observation that activation of photoreaction II reactions affects the delayed light emission of D-144 in a manner similar to that observed in other photoreaction II systems.

These results are in conflict with the conclusions of Ruby and Brown (1969), who have presented data which indicate that 20% of the total delayed light at 0.5 msec after a flash of exciting light is associated with photosystem I activity. These measurements were made with French press subchloroplast fractions enriched in photoreaction I, prepared from either wild type of Scenedesmus or from mutant 11. These authors do not indicate whether they have measured photochemical activities of their particles in order to estimate possible residual photoreaction II activity. It is therefore difficult to directly compare our data with theirs.

Since we do not know the source of reducing equivalents for the DCMU-insensitive photoreduction of ferricyanide in our D-144 fraction, we are unable to relate this unexpected reaction to the normal pathway of photosynthesis. We are convinced, however, that water is not the reductant, since we found no concomitant oxygen evolution.

DCMU-insensitive ferricyanide photoreductions have been previously reported. Kahn (1964) reported a ferricyanide photoreduction, which was insensitive to CMU up to 10^{-4} M, to be performed by a chlorophyll-protein complex prepared with Triton X-100. More recently Diner and Mauzerall (1971) using cell free preparations from the blue green alga Phormidium luridum, found that oxygen production coupled to ferricyanide was insensitive to DCMU. These authors suggested that the site of DCMU action had been removed or blocked. However, in our preparation it appears that the site where DCMU acts is present and active, since DCPIP reduction is still inhibited by DCMU, as are the DCPIP-induced changes in delayed light. It thus appears that in our D-144 fraction ferricyanide is able to accept electrons from a site prior to that of DCMU action, perhaps from the reaction center itself.

We do not have a satisfactory explanation for the lack of oxygen evolution during ferricyanide reduction. If ferricyanide is accepting directly from the reaction center, it seems possible that the electron donor might be the bulk, energy harvesting chlorophyll. In this case, chlorophyll bleaching would be observed. We do find an O.D. decrease at 680 nm which is not present when D-144 is illuminated in the absence of ferricyanide. This bleaching indicates an oxidation of bulk chlorophyll which we have calculated to be sufficient in quantity to supply the necessary electrons. However, we feel that this observation alone does not prove that bulk chlorophyll is the source of electrons. Nevertheless, it appears that detergent solubilization may result in production of photochemical competance of a reaction which is presumably absent in normal chloroplasts.

C. Treatments which inactivate photosystem II

There are a number of treatments which have been developed in the last few years which inhibit photochemical processes connected to photosystem II while leaving photosystem I unaffected or only mildly inhibited. The special interest of the particular inhibitor treatments examined here lies in the fact that certain artificial electron donors can 'restore' electron transport, using the donor in place of water in the inhibited systems. The inhibitory treatments include incubating in high molarity tris buffer, heating chloroplasts to 50°C, irradiating with ultraviolet light, and depleting chloroplasts of chloride by washing them extensively in chloride free medium. All these treatments inhibit oxygen evolution and decrease the variable fluorescence yield (which is a measure of the reduction of Q, the primary acceptor of photosystem II).*

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* The fluorescence of algae and of isolated chloroplasts is believed to consist of two components. The first is a luminescence of constant quantum yield, probably emanating from the bulk absorbing chlorophyll, which is not affected by changes in the efficiency of the photochemical reactions. The second is a fluorescence of variable quantum yield which directly relates to the photosynthetic capacity and presumably emanates from the trapping centers of photosystem II (Kautsky, *et al.*, 1960; Duysens and Sweers, 1963). Duysens proposed a mechanism of the variable yield fluorescence based upon the oxidation and reduction of an electron transport component between the two photosystems wherein the fluorescence of chlorophyll *a* associated with photosystem II is quenched by the oxidized form of a hypothetical compound but not by its reduced form. The currently accepted terminology for the hypothetical compound is Q, also thought to be the primary electron acceptor of photosystem II. When Q is reduced it does not quench fluorescence and fluorescence yield is high, in its oxidized form it is an efficient quencher and the fluorescence yield is low. For a more detailed discussion see Bishop (1966).

There are two schools of thought as to what the different treatments are doing to photosystem II. One group of researchers explains the effects as being due to a block in photochemical electron transport on the water splitting side of photosystem II (Yamashita and Butler, 1968, 1969; Katoh and San Pietro, 1967). The other hypothesis is that the treatments set up a cyclic 'short' between the oxidizing and reducing side of the reaction center of photosystem II and prevent normal photosynthetic electron transport from occurring (Bertsch and Lurie, 1971; Hind, 1971). The specific hypotheses and the data supporting them will be discussed in detail for each treatment.

1. Tris aging

Yamashita and Butler (1968) inhibited the Hill reaction by washing chloroplasts with 0.8 M tris-HCl at pH 8.0. They found that artificial electron donors could replace water and reestablish NADP reduction, and these donors also restored the variable fluorescence increase which was reduced in intensity by tris treatment (Yamashita and Butler, 1969). This restored photochemistry was DCMU sensitive. The interpretation of the data by Yamashita and Butler (1969) was that the tris wash induced a block in electron transport between water and photosystem II, and that these donors donated between the block and photosystem II. This does not, however, explain the variable fluorescence data. If the block is on the water oxidizing side of photosystem II it should not prevent the primary acceptor, Q, from becoming reduced. This would lead to high fluorescence yield once charge separation was completed by the reaction center.

We have investigated the effect of tris treatment, and its reversal, on energy storage at photosystem II, by examining the effect on msec delayed light. We felt that this approach might reveal something concerning the mode of action of tris aging which photochemical measurements did not.

Figure 3-9 shows the effect on millisecond delayed light and $NADP^+$ reduction of tris aging. The control chloroplasts show a very slow decay of delayed light from 1 - 20 milliseconds, and addition of NADP/Fd affects them (as does ferricyanide and DCPIP) by increasing the intensity of emission at one millisecond followed by rapid decay of the emission. Tris aged chloroplasts, on the other hand, show an extremely rapid decay of delayed light in the absence of a Hill reagent. We have emperically associated such rapid D.L. decays with the performance of photochemistry. However, the emission from these chloroplasts is not affected by electron acceptors, indicating that normal photochemical pathways are not being utilized.

We used three compounds which functioned as electron donors to tris treated chloroplasts in the absence of ascorbate, as well as a number of compounds which required the presence of ascorbate to maintain them in

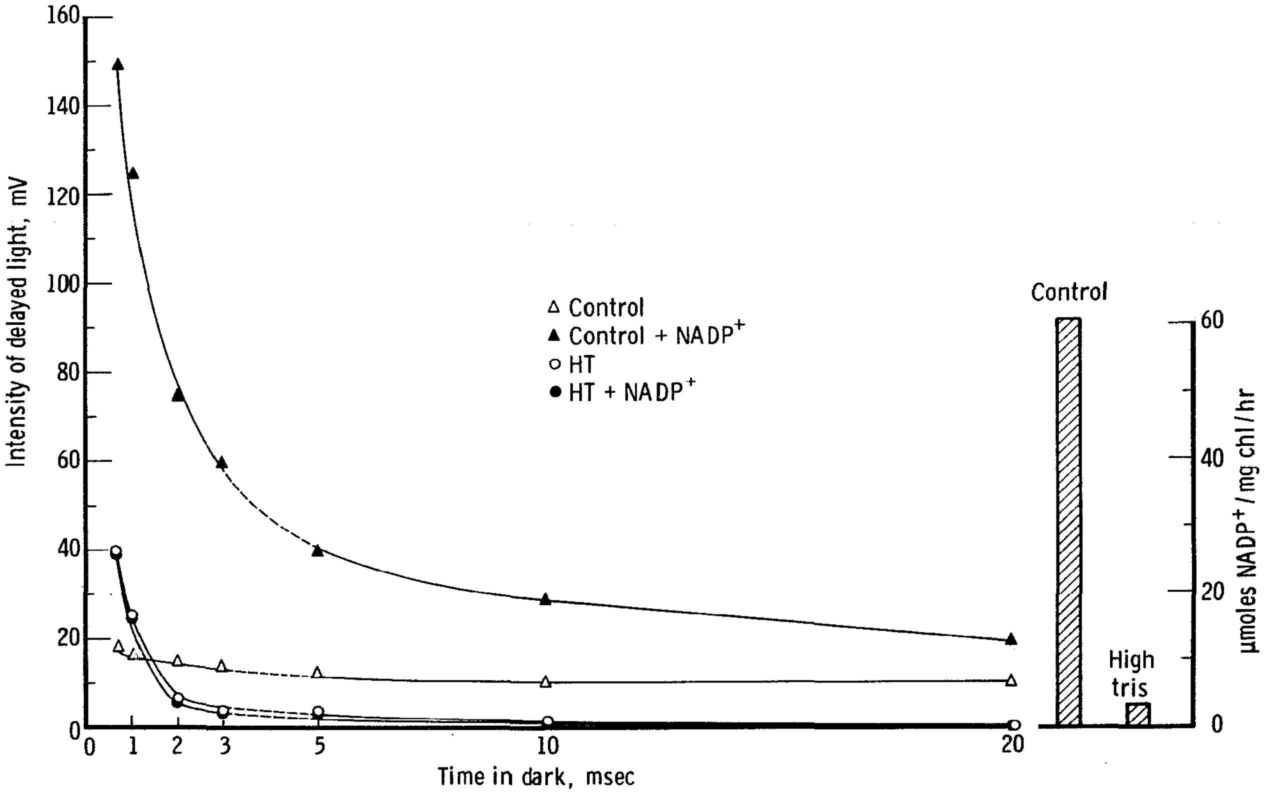


Figure 3-9. Effect of NADP$^+$ on delayed light emission and photoreduction in control and tris treated chloroplasts. 3×10^{-4}M NADP$^+$ and saturating amounts of ferredoxin added where indicated.

their reduced state. Since ascorbate can also function as an electron donor (Katoh and San Pietro, 1967), and since it has effects on delayed light, emphasis was placed on the electron donors which function in its absence: 1,4-diphenyl semicarbazide, 1,5-dephenyl carbohydrazone and manganous ion. Figures 3-10, 3-11, and 3-12 show the effect of these donors on delayed light of untreated and tris aged chloroplasts.

The electron donors have relatively small effects on control chloroplasts, as seen in figures 3-10, 3-11 and 3-12, and they do not substantially change the effect of electron acceptors on delayed light in the untreated chloroplasts. In the tris treated chloroplasts the electron acceptors have no effect unless an electron donor is present. The electron donors themselves cause the rapid decay of the tris aged chloroplasts to become less steep, approaching the slow decay observed in unpoisoned chloroplasts in the absence of electron acceptors. On addition of the electron acceptor to such a slow decaying tris treated system in the presence of a donor, the change in emission is similar (but smaller in magnitude) to that observed on addition of the acceptor to control chloroplasts; the emission decays more rapidly and is more intense at one millisecond. Thus the addition of electron donors to the rapidly decaying tris treated chloroplasts results in a slowly decaying system. The system is then able to reduce $NADP^+$ or ferricyanide and also shows the type of change in millisecond

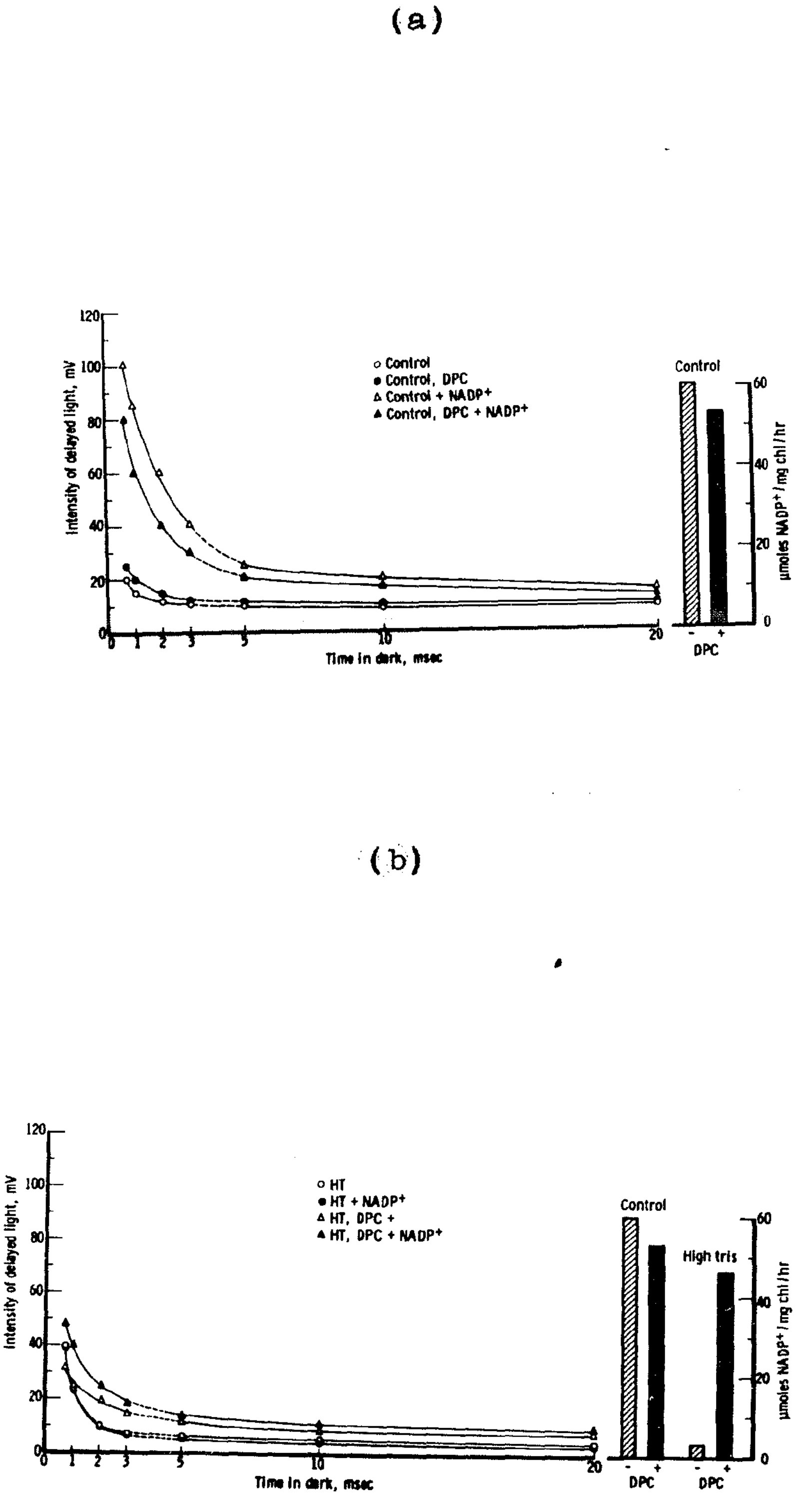
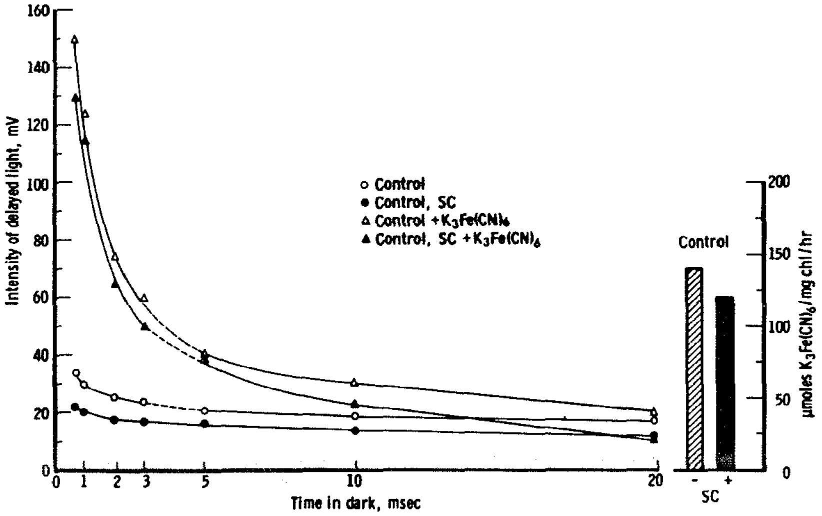


Figure 3-10. The effect of the artificial electron donor DPC on (a) control chloroplasts and (b) tris treated chloroplasts in the absence and presence of NADP$^+$. Where added 5×10^{-4}M DPC, 10^{-4}M NADP$^+$ and saturating amounts of ferredoxin. The bar graphs indicate the rate of NADP$^+$ reduction.

(a)



(b)

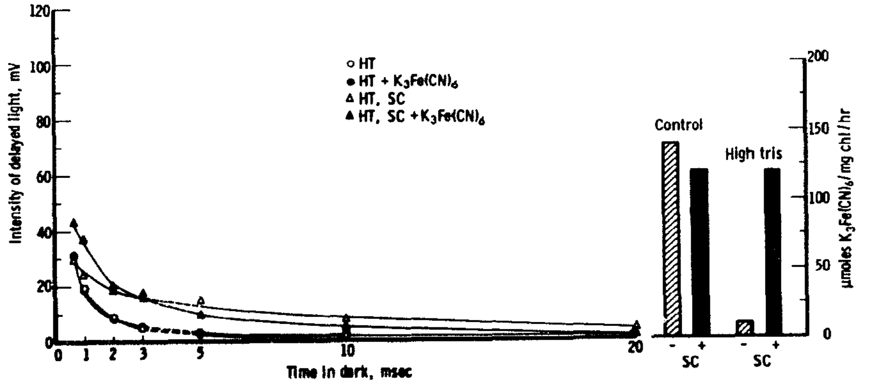


Figure 3-11. The effect of the artificial electron donor diphenyl semicarbazide on (a) control chloroplasts and (b) tris treated chloroplasts in the absence and presence of ferricyanide. 5×10^{-4}M diphenyl semicarbazide and 3×10^{-4}M ferricyanide where added. The bar graphs indicate the rate of ferricyanide reduction.

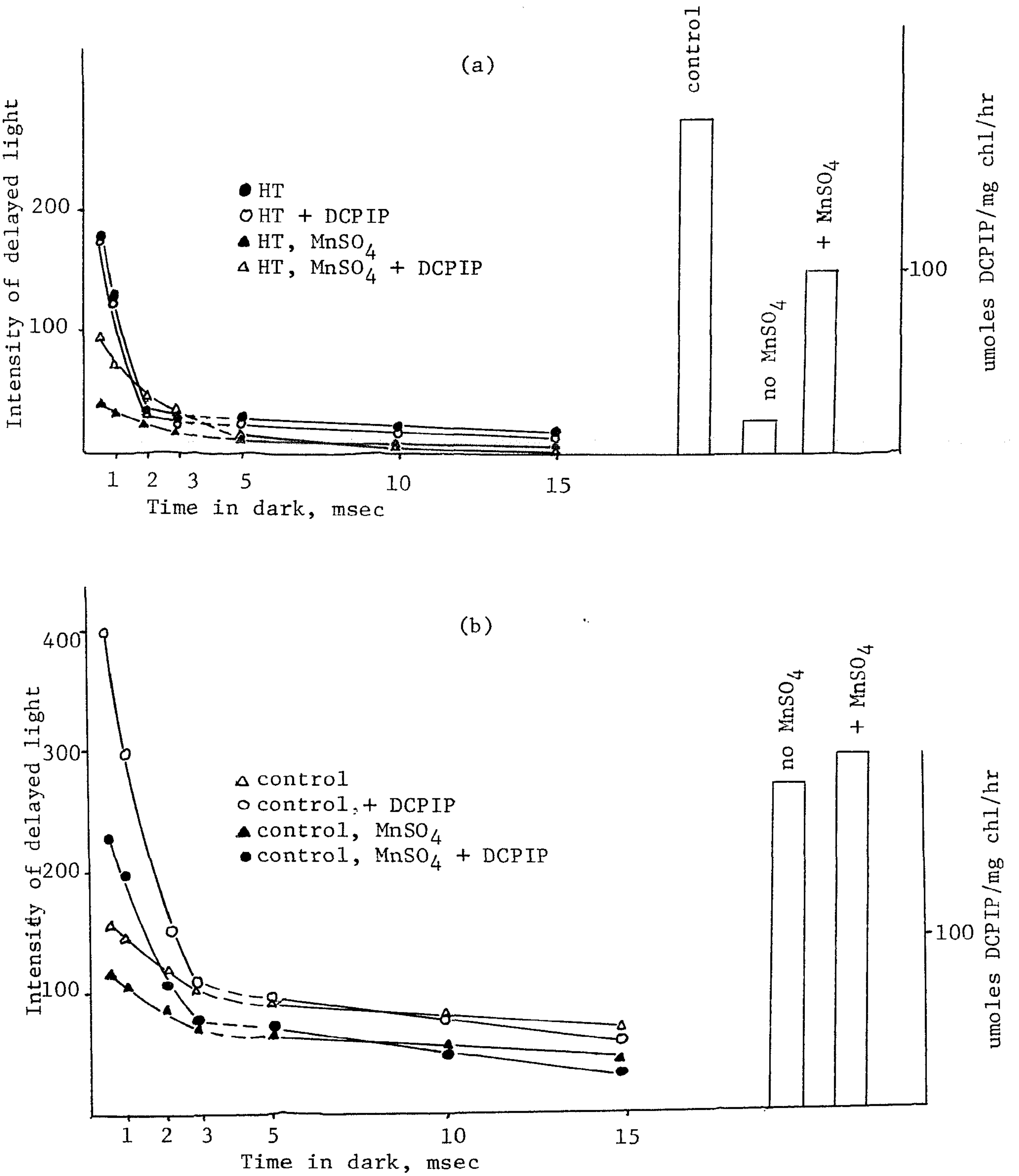


Figure 3-12 The effect of the artificial electron donor $MnSO_4$ on (a) tris treated chloroplasts and (b) control chloroplasts in the absence and presence of DCPIP. Reaction mix: 0.02 M Tricine (pH 7.8), 0.02 M NaCl, 0.005 M $MgCl_2$, 10 ug chl/ml, and where added 0.002 M $MnSO_4$ and 10^{-5}M DCPIP. The bar graphs indiciate the rate of DCPIP reduction.

delayed light which has been previously associated with activation of electron transport.

The idea that tris treatment might induce a side reaction which successfully competes with the normal pathway of photosynthesis led us to examine the saturation curve of the restored system. The delayed light decay is not completely restored by donors to the situation found in control chloroplasts. These findings suggest that photoreduction by the regenerated tris aged chloroplasts occurs in competition with the hypothetical tris induced side reaction. Since the Hill reaction of control chloroplasts involves only photoreduction of the acceptor, photoreduction by the untreated chloroplasts should saturate at lower exciting intensities than photoreduction by the regenerated system. Figure 3-13 shows that this is the case for $NADP^+$ reduction. The Hill reaction using $NADP^+$ saturates at lower light intensity than the reconstituted photoreduction of $NADP^+$ performed by tris treated chloroplasts using DPC as an artificial electron donor.

The studies reported here suggest that the inhibition of photosystem II by treatments of high concentrations of tris is due to induction of a side reaction which operates more rapidly than the normal forward reactions of photosynthetic electron transport. This side reaction possibly represents a cyclic 'short' between electron transport steps on the oxidizing and reducing sides of the

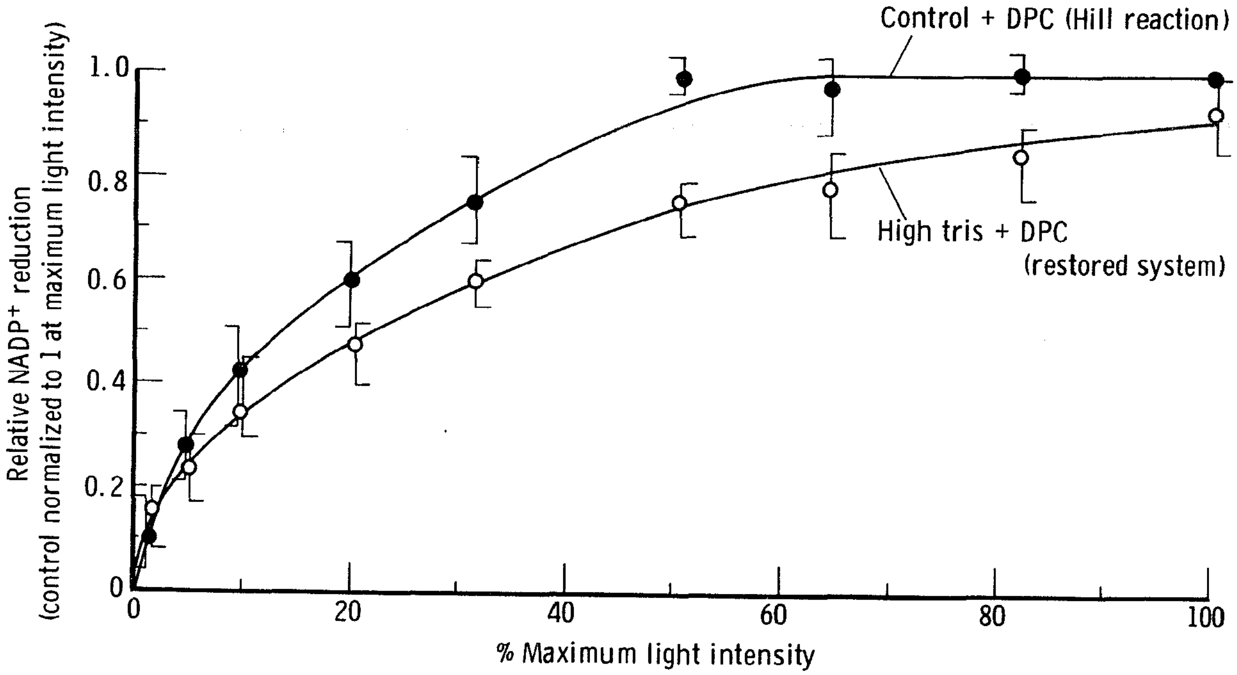


Figure 3-13. Comparison of NADP$^+$ photoreduction with DPC in control and in tris treated chloroplasts at different light intensities. Light intensity was varied by the use of neutral density filters. Points indicate the average of eight separate experiments. The control ordinate was normalized to unity at the maximum light intensity and the other ordinates are expressed as a fraction of this value. The limits of variation at each point are indicated in the figure.

photoreaction. The side reaction operates after Q since Yamashita and Butler (1968) found that DCMU alone restored the variable fluorescence yield. This indicates that DCMU blocks oxidation of Q and allows for its complete reduction. The 'short' would presumably be caused by tris-induced conformational changes in the photosynthetic lamellae, thus altering the normal separation between the two sides of the reaction center. The reversal of tris-induced inhibition by electron donors is due to their ability to donate additional reducing equivalents to photosystem II, so that more reducing power is available for use in the normal photosynthetic pathways. Our results suggest that the tris-induced side reaction functions in the restored system but that the photosynthetic pathway competes successfully with it.

2. Heat

Katoh and San Pietro (1967, 1968) found that mild heating of Euglena chloroplasts inhibited their ability to photoreduce $NADP^+$. This could be restored by ascorbate (acting as an electron donor) and the restored activity was inhibited by DCMU. Yamashita and Butler (1968) found that heat caused the same inactivation in spinach chloroplasts, and that the electron donors they used to restore tris washed chloroplasts were also able to donate in the heat-inactivated system. The variable fluorescence showed a similar response to that of tris washed chloroplasts;

the treatment reduced the yield and electron donors restore it. One difference between heat and tris aging was that DCMU, which restored the variable fluorescence in tris washed chloroplasts did not restore the variable fluorescence in heated chloroplasts. This indicated, although Yamashita and Butler (1968) did not comment on it, that whatever side reaction was leading to fast reoxidation of Q (and thus low fluorescence) was before the DCMU block. Otherwise DCMU would have restored variable fluorescence as it did in the case of tris aging.

As was shown in the section on tris treated chloroplasts, the three electron acceptors, $NADP^+$, ferricyanide and DCPIP had similar effects on delayed light. Likewise, the three electron donors, DPC, DPSC and $MnSO_4$ all restored photochemistry to a limited extent and flattened the steep delayed light kinetics of tris treated chloroplasts without affecting delayed light emission from untreated chloroplasts substantially. We, therefore, chose to employ the electron donors DPC and $MnSO_4$ and the electron acceptor DCPIP in the examination of the remaining treatments.

We investigated the effect of different times of heating at 50°C on millisecond delayed light and DCPIP photoreduction (figure 3-14). This treatment, unlike tris aging, had the advantage that it was possible to examine the progression of inhibition due to heating for different times. As figure 3-14 indicates, the degree of steepness

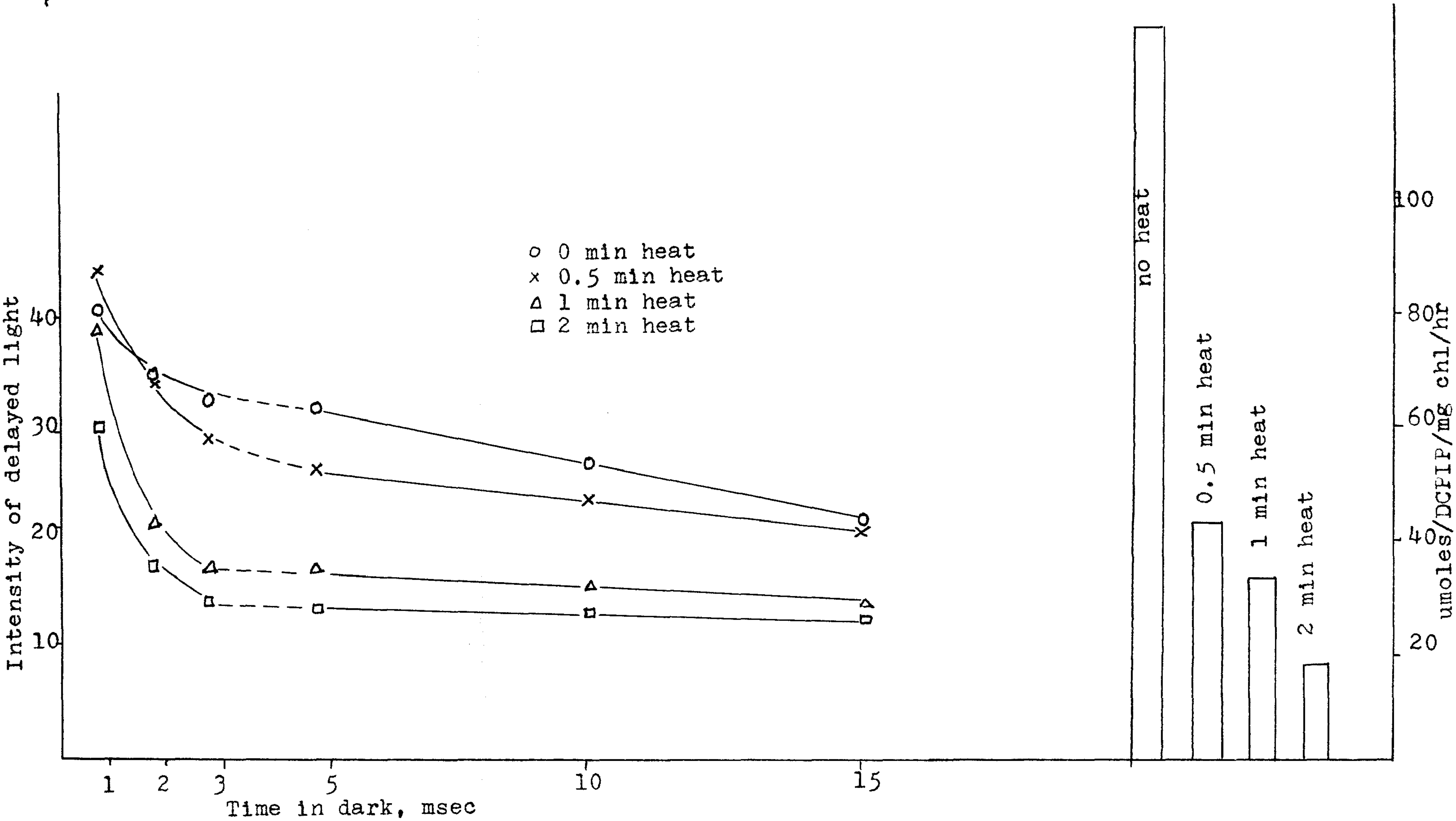


Figure 3-14. Delayed light of chloroplasts after varying times of heating. The bar graphs show the extent of photochemical inhibition caused by the different times of heating.

of decay of the delayed light emission increases as the time of heating is increased. Figure 3-15 shows that the ability of electron acceptors, in this case DCPIP, to increase the emission at one millisecond decreases with increased time of heating. An increase in one millisecond delayed light emission in the presence of electron acceptors is an indication that photochemistry is occurring, and as figure 3-15 shows, the extent of this increase diminishes parallel with the inhibition of photochemistry caused by heating.

Table 3-3 also reflects the steepness of the decay of delayed light as heat progressively inhibits the chloroplasts, and the inhibition of the one millisecond increase. We have used the ratio of delayed light at 0.8 msec to that of 2.8 msec as a measure of the steepness of the emission decay. Control chloroplasts are not perfectly flat in their decay, but they decay slowly (ratio of 1.4) compared to treated chloroplasts (ratios of 3 to 8). Electron donors, in this case $MnSO_4$, were found to give restoration of chemistry and slow the rapid decay. Donors do not, however, completely restore either electron transport or delayed light to that of untreated chloroplasts.

The second ratio indicates the extent to which the addition of an electron acceptor increases the intensity of the one millisecond delayed light. It is the ratio of 1 msec delayed light in the presence of an acceptor to 1 msec delayed light in the absence of an acceptor.

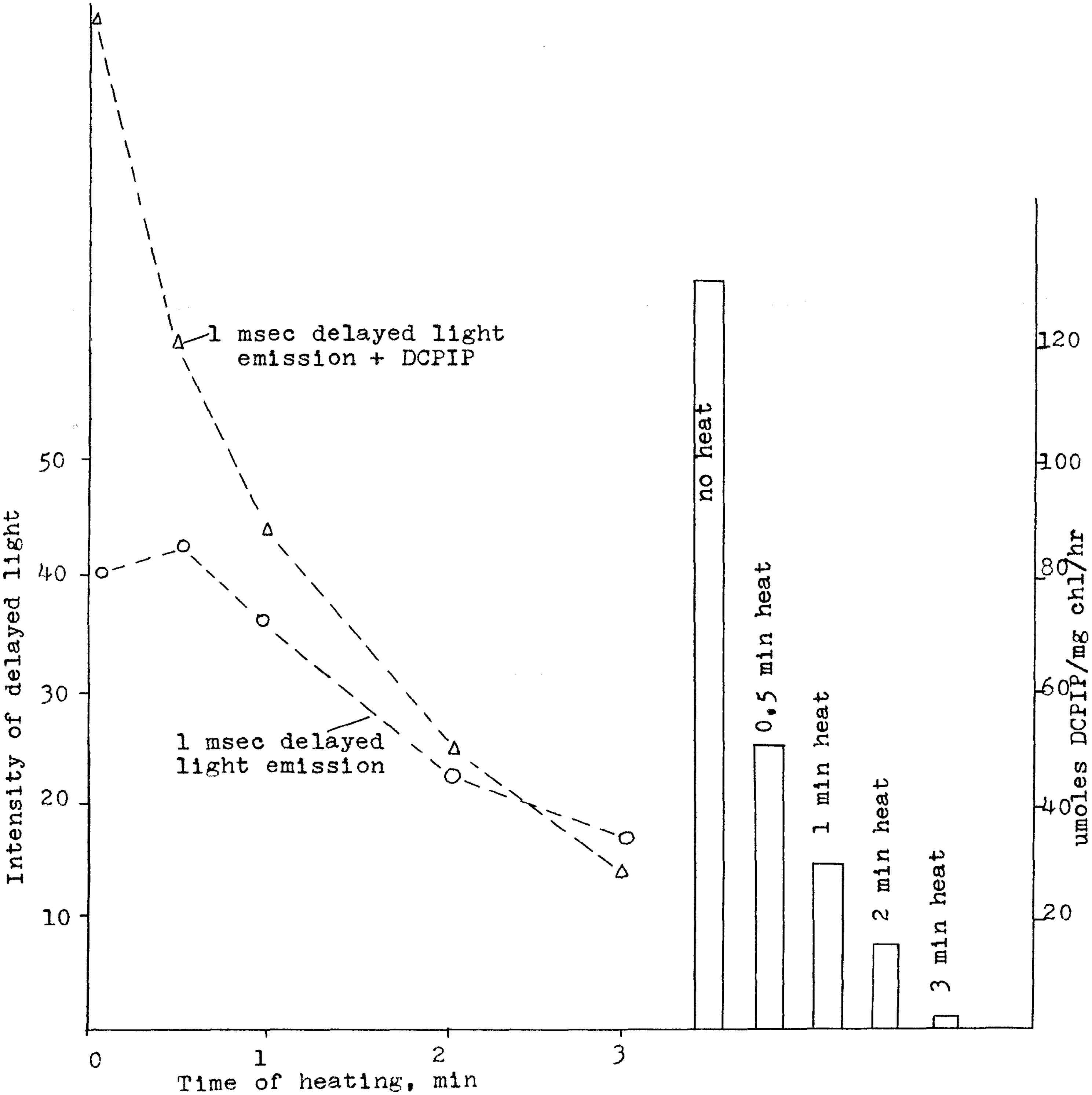


Figure 3-15. The effect of varying times of heating on DCPIP reduction and on 1 msec delayed light emission in the absence and presence of DCPIP.

Condition	$\frac{0.8 \text{ msec}}{2.8 \text{ msec}}$	$\frac{1 \text{ msec + DCPIP}}{1 \text{ msec - DCPIP}}$	ueq DCPIP/ mg chl/hr
unheated	1.4	6.3	163
unheated + 2mM $MnSO_4$	1.4	5.5	150
heated 90 sec	3.0	3.0	82
heated 90 sec + 2mM $MnSO_4$	2.0	4.0	115
heated 120 sec	5.0	1.0	30
heated 120 sec + 2mM $MnSO_4$	2.5	2.4	60

Table 3-3. The effect of heating on delayed light emission and DCPIP reduction. The first ratio compares the intensity of light emission (in the absence of DCPIP) at 0.8 msec to that at 2.8 msec. A large number indicates a rapid decay. The second ratio compares the delayed light emission at 1 msec in the presence of an electron acceptor to that in the absence of an acceptor. A number greater than 1 indicates that an increase in emission at 1 msec occurred with the addition of an electron acceptor. The third column is the rate of DCPIP reduction.

Untreated chloroplasts show a sixfold increase while increasing heat treatment inhibits this increase in a dramatic fashion. Addition of $MnSO_4$ to heat treated chloroplasts restores the one msec increase to some extent. This is seen both in Table 3-3 and figure 3-16. The restoration of photochemistry in heated chloroplasts was never as complete with electron donors as it was in tris aging, as can be seen from comparing figure 3-10 with Table 3-3.

These data indicate that heat, like tris aging, induces a competitive side reaction around photosystem II. The steepness of the delayed light decay after heating indicates that electrons are being removed from the reaction center in a manner which is faster than that of normal photosynthesis. The fact that variable fluorescence (Yamashita and Butler, 1968) is decreased can be understood as reflecting the fact that the reduced Q is reoxidized faster than normal and the greater concentration of oxidized Q quenches the fluorescence.

Although cycles are induced by the two treatments, they are apparently not the same cycle. DCMU restores variable fluorescence in tris treated chloroplasts but not in heated chloroplasts. This suggests that the side reaction induced by tris is on the reducing side of the DCMU block while in heated chloroplasts it is on the oxidizing side, assuming DCMU blocks on the reducing side of RCII. Additional evidence that the two treatments act

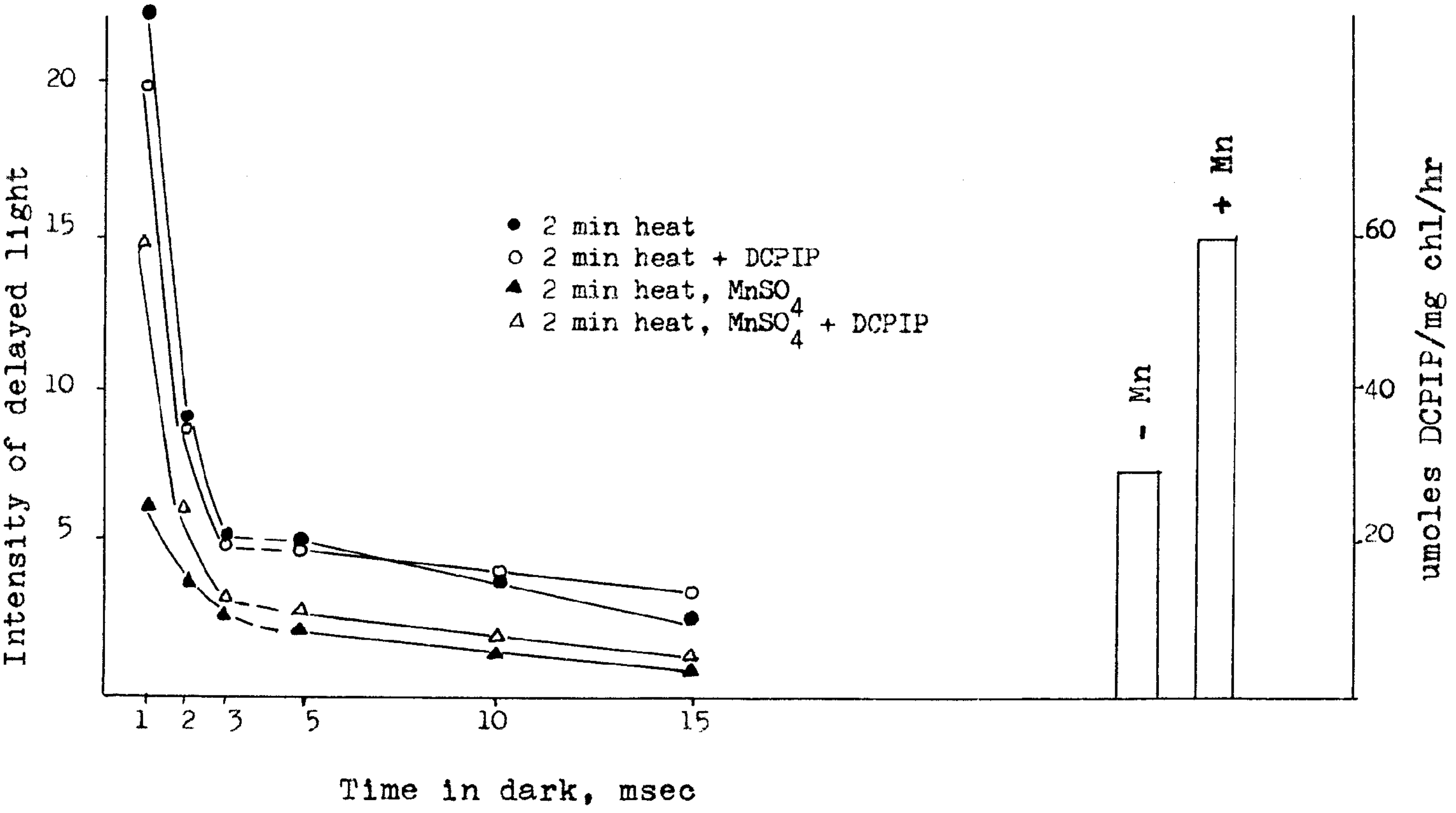


Figure 3-16. Effect of manganese on heated chloroplasts in the absence and presence of DCPIP. Where added 0.002 M $MnSO_4$, 10^{-5}M DCPIP. The extent of DCPIP reduction of heated chloroplasts with and without the artificial donor $MnSO_4$ is shown on the bar graphs.

differently is that electron donors do not restore photochemistry as completely in heated chloroplasts as they do in tris aged ones, nor do they flatten delayed light completely. It is possible that heating is a more radical procedure which causes a number of changes in the state of photosystem II, not all of which are reversible.

3. Ultraviolet irradiation

The mechanism of UV inhibition has been the subject of a number of investigations. Bishop (1961) showed that UV irradiation destroyed plastoquinone in chloroplasts, and found a correlation between the degree of inhibition of the Hill reaction and the extent of destruction of plastoquinone. The apparent parallelism between plastoquinone destruction and loss of Hill reaction activity was confirmed and extended by Shavit and Avron (1963) and also Trebst and Pistorius (1965). However, these researchers noted that the addition of unirradiated plastoquinone to irradiated chloroplasts did not restore the Hill reaction, and they concluded that UV irradiation inactivated other components as well as plastoquinone.

Jones and Kok (1966) localized the UV inhibition to a photosystem II reaction by showing that the photoreduction of $NADP^+$, with the DCPIP-ascorbate couple as the electron donor, was not inhibited by a UV treatment which completely inhibited the water to $NADP^+$ Hill reaction. Jones and Kok found, as had others (Krogman and Olivero, 1962), that heptane extraction of chloroplasts removed plastoquinone and inhibited the Hill reaction. The photochemical activity was restored when plastoquinone was added back to the chloroplasts. However, exposure to UV light of chloroplasts from which plastoquinone was extracted rendered them incapable of reactivation by the readdition of plastoquinone or nonirradiated heptane extracts. These results could be explained either by incomplete extraction of plastoquinone, or by multiplicity of the action of UV light. They suggested that a second site of action might be the reaction center of photosystem II, since a linear relationship was observed between the decay of O_2 evolution activity and of the variable fluorescence component. However, Yamashita and Butler (1968) found that electron donors which donated on the water side of photosystem II were able to restore UV irradiated chloroplasts, an indication that the photosystem II reaction centers were still functional.

Bishop and coworkers (1967, 1970) reexamined the effect of UV damage on chloroplasts. It was found that cyclic phosphorylation and the 515 nm absorption shift were inhibited at the same rate as the Hill reaction. This suggested that UV caused a disruption of the lamellar membranes which affected both electron transport and phosphorylation. In agreement with Jones and Kok (1966) they found that UV irradiation decreased the variable yield fluorescence. The digestion of chloroplasts with lipase (which breaks up membranes) decreased fluorescence in a manner similar to

UV irradiation. Three possible interpretations were advanced to explain the fluorescence data: 1) an increased efficiency of drainage of energy occurs from the trapping centers of photosystem II (assumed to be the variable fluorescence yield emitter), even though electron flow through the normal pathway is blocked; 2) actual destruction of the fluorescence emitter results; or 3) increased internal quenching of fluorescence caused by a collapse of chloroplast membranes. Yamashita and Butler (1968) found that restoration of a DCMU sensitive $NADP^+$ photoreduction (with hydroquinone-ascorbate as the artificial donor couple) did not restore variable fluorescence. The decreased yield of the fluorescence was also not reversed by the addition of dithionite. Bishop _et_ _al_. (1970) suggested that the apparent anomalous behavior between the capacity to restore electron transport, but not the variable yield fluorescence, is due to an alteration of the lamellar structure of chloroplasts which results in an increased and irreversible internal quenching of the fluorescence.

This treatment is one which, like heat, can be applied progressively. We exposed the chloroplasts to various times of UV irradiation. Figure 3-18 shows how increasing time of UV irradiation affects the delayed light emission and DCPIP reduction. Photochemistry is progressively inhibited by longer times of UV exposure, although a brief exposure (one minute) uncouples electron flow slightly. The millisecond delayed light also becomes

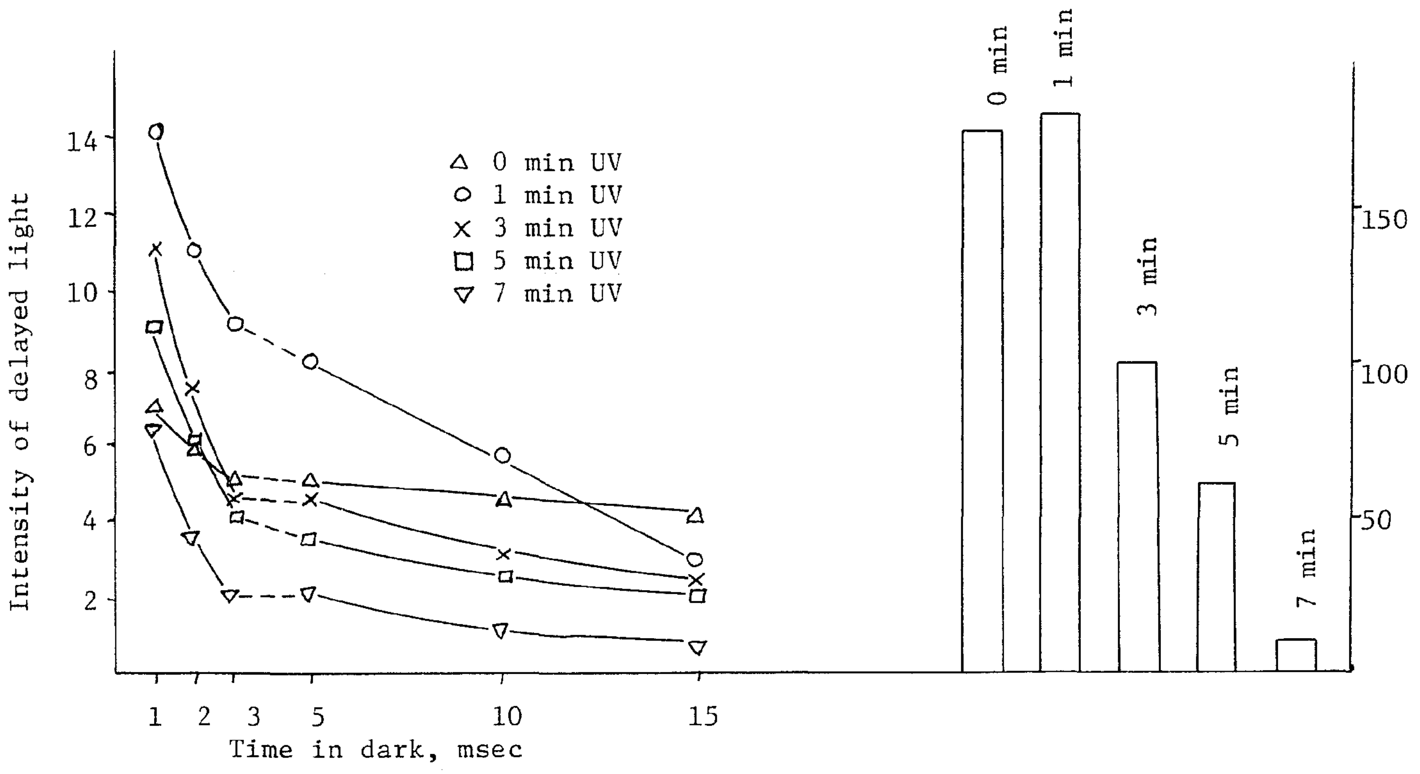


Figure 3-18 Delayed light of isolated chloroplasts after varying times of UV irradiation. The bar graphs show the extent of photochemical inhibition caused by the different times of UV irradiation.

increasingly steeper, but not to the extent that was observed after heat treatment. The ratio of 0.8 to 2.8 msec delayed light for these different times of irradiation is shown in Table 3-4. As figure 3-19 indicates, the increase in emission at one millisecond upon addition of electron acceptors also disappears with UV treatment. This is demonstrated also in Table 3-4.

Figure 3-20 shows the effect that artificial electron donors have on delayed light of UV treated chloroplasts and on DCPIP reduction. It is interesting that electron donors acting on the water side of photosystem II will restore DCPIP photoreduction to 50 - 70% of the control without flattening the delayed light decay kinetics or restoring the one millisecond increase; both indications of a return to normal photosynthetic energy storage and electron transfer conditions. This is also reflected in Table 3-4. It appears that although from the rapidity of the delayed light decay a side reaction is being effected by the irradiation, it is of a different nature, since normal delayed light kinetics are not restored while photochemistry which is sensitive to DCMU is partially restored.

4. Chloride depletion

Hind, Nakatine and Izawa (1968) developed a procedure to deplete chloroplasts of chloride. It had been known for a long time that photosynthetic oxygen evolution required the presence of chloride, although its

Conditions	0.8 msec / 2.8 msec	1msec + DCPIP / 1msec - DCPIP	ueq DCPIP/ mg chl/hr
(a)			
0 min UV	1.4	5.0	175
1 min UV	1.6	3.6	180
3 min UV	2.2	2.2	100
5 min UV	3.0	1.1	60
7 min UV	3.5	0.5	10
(b)			
untreated	1.5	7.5	148
untreated + .5 mM DPC	1.8	5.7	135
UV for 5 min	4.3	1.0	11
UV for 5 min + .5 mM DPC	4.9	1.0	40

Table 3-4.a. The effect of UV irradiation for varying times on delayed light emission and DCPIP reduction. b. The effect of diphenyl carbazide on delayed light and DCPIP reduction of control and UV irradiated chloroplasts. The first ratio is the delayed light emission (without DCPIP) at 0.8 msec compared to that at 2.8 msec. The second ratio is delayed light at 1 msec with DCPIP compared to delayed light emission at 1 msec without DCPIP

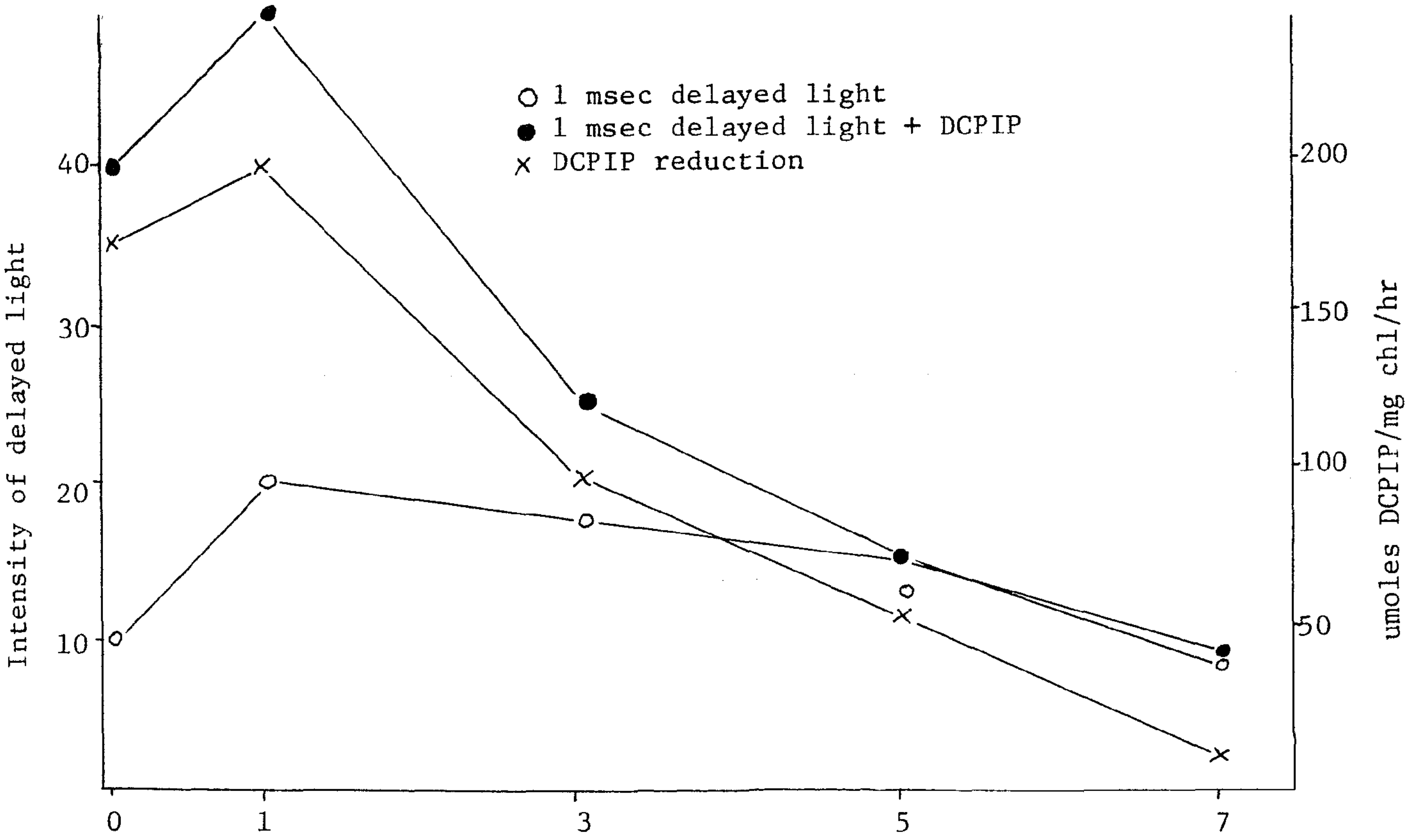


Figure 3-19 The effect of varying times of UV irradiation on DCPIP reduction and on 1 msec delayed light emission in the absence and presence of DCPIP. Reaction mix: 0.025 M Tricine (pH 7.8), 0.02 M NaCl, 0.005 M $MgCl_2$, 10 ug chl/ml, and when present $2x10^{-5}$M DCPIP.

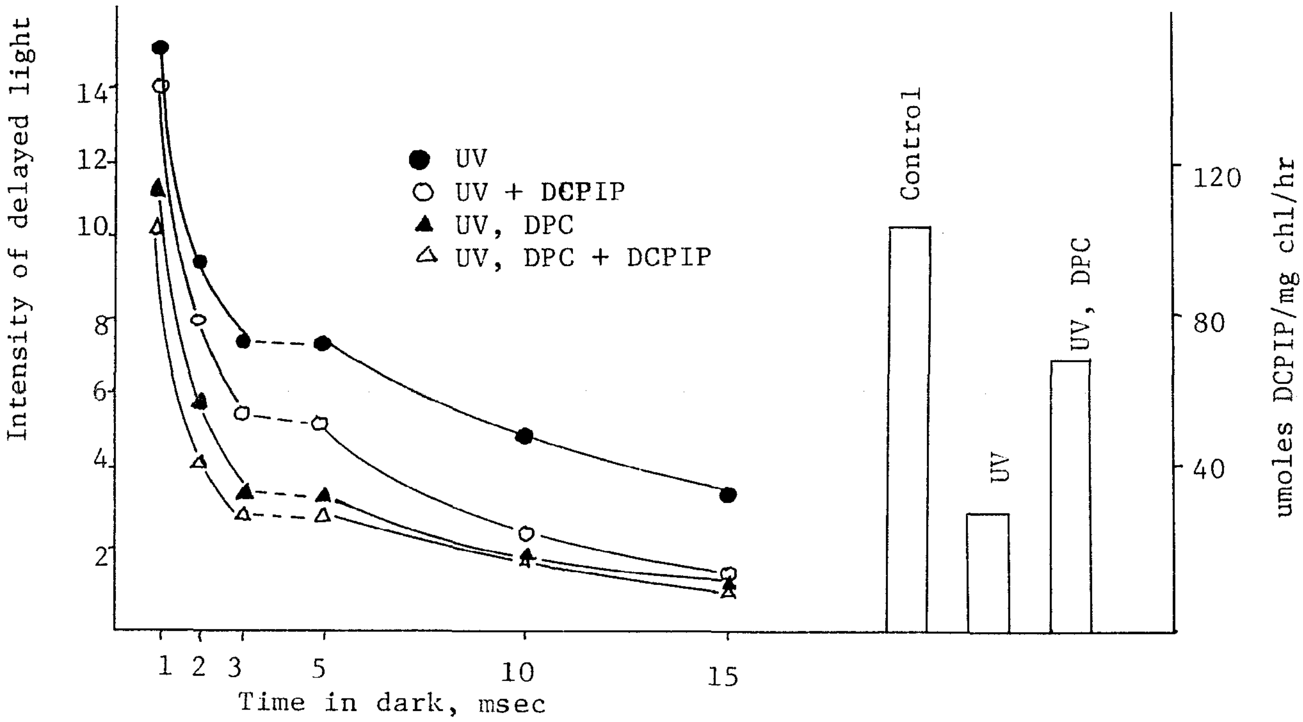


Figure 3-20 Delayed light of UV irradiated chloroplasts in the absence and presence of the electron donor DPC and the electron acceptor DCPIP. The bar graphs show DCPIP reduction of control chloroplasts, UV irradiated chloroplasts, and UV irradiated chloroplasts plus DPC. Reaction mix: 0.025 M Tricine (pH 7.8), 0.02 M NaCl, 0.005 M $MgCl_2$, 10 ug chl/ml, and when present 2 x 10^{-5}M DCPIP and 5 x 10^{-4}M DPC.

function was unknown (Warburg and Luttgens, 1946). Hind and coworkers (1968) found that whole chloroplasts showed no chloride requirement until the internal chloride was released by some treatment which damaged the outer membrane and promoted swelling. Once isolated chloroplasts had been washed and resuspended in chloride free medium, the inhibition of oxygen evolution was promoted by gentle aging (holding the chloroplasts at room temperature for fifteen minutes), or alkalai treatment.

Heath and Hind (1969) examined the fluorescent properties of chloride deficient chloroplasts in an attempt to locate the functional site of the chloride action. The fluorescence yield in the absence of chloride was lower, due to a decrease in the variable fluorescence at saturating light intensity and a decrease in quantum efficiency. It was found that the fluorescence yield kinetics in the absence of chloride resemble more closely those obtained with added methyl violegen, which speeds electron transport, rather than those obtained with DCMU or orthophenathroline (which block the oxidation of the quencher by photosystem I). They concluded that chloride deficiency increased electron flow, but not in the normal photosynthetic pathway.

The addition of substrate amounts of chloride to chloride-depleted chloroplasts increased the ability of these chloroplasts to photoreduce DCPIP from two to six times (restoring photochemistry to over 90% of untreated chloroplasts), and restored oxygen evolution. Other donors to the water side of photosystem II also enhanced dye reduction (without restoring O_2 production). These reactions were inhibitable by DCMU. Hind et al. (1969) concluded that the site of chloride involvement was on the oxidizing side of photosystem II and that the absence of chloride decreases the electron flow from water to photosystem II. They explained the lower quantum efficiency of the variable fluorescence as being due to a cycle, induced by the chloride deficiency, in which the primary acceptor Q^- recombined with the primary donor Z^+ instead of donating electrons to the electron transport chain.

This conclusion is very similar to that drawn by us from examination of the tris-treated system. It was therefore of interest to examine energy storage by the reaction center of photosystem II in these Cl^- deficient chloroplasts. The delayed light showed a faster decay in the millisecond range as would be predicted by a side reaction, indicating that stored charge is being removed from the reaction center at short times. Figure 3-21 compares delayed light from chloride depleted chloroplasts and control chloroplasts with and without DCPIP. The control chloroplasts and the chloride deficient chloroplasts have been washed with EDTA which uncouples phosphorylation. Their decay is faster than isolated chloroplasts because of this treatment, and the ramifications of this will be discussed in the next section. The chloride depleted chloroplasts show no one millisecond increase in delayed light when an electron acceptor is added. Figure 3-21 shows the effect of adding back chloride to the chloride depleted chloroplasts. The delayed light decay is flattened, though not as completely as that of tris aged chloroplasts, and the one millisecond increase in the presence of an electron acceptor is restored to an extent. Also shown in figure 3-21 is the amount of DCPIP reduction of chloride depleted chloroplasts and the restored chloride chloroplasts.

The results shown here are in agreement with Hind's hypothesis that chloride depletion leads to a wasteful side reaction which removes electrons by other pathways than the electron transport chain. The steep decay kinetics of delayed light are an indication that charge is being pulled out of the reaction center at times shorter than photosynthetic electron transport. NaCl restores photochemistry, flattens the delayed light curve and restores the one millisecond increase in the presence of an electron acceptor, all signs that photosynthetic electron transport is successfully competing with the side reaction.

5. Discussion

One possible hypothetical scheme for the site of action of the various treatments and for the site of electron donors interaction with the electron transport chain is presented in figure 3-22. All the treatments examined here appear to create a cycle around photosystem II

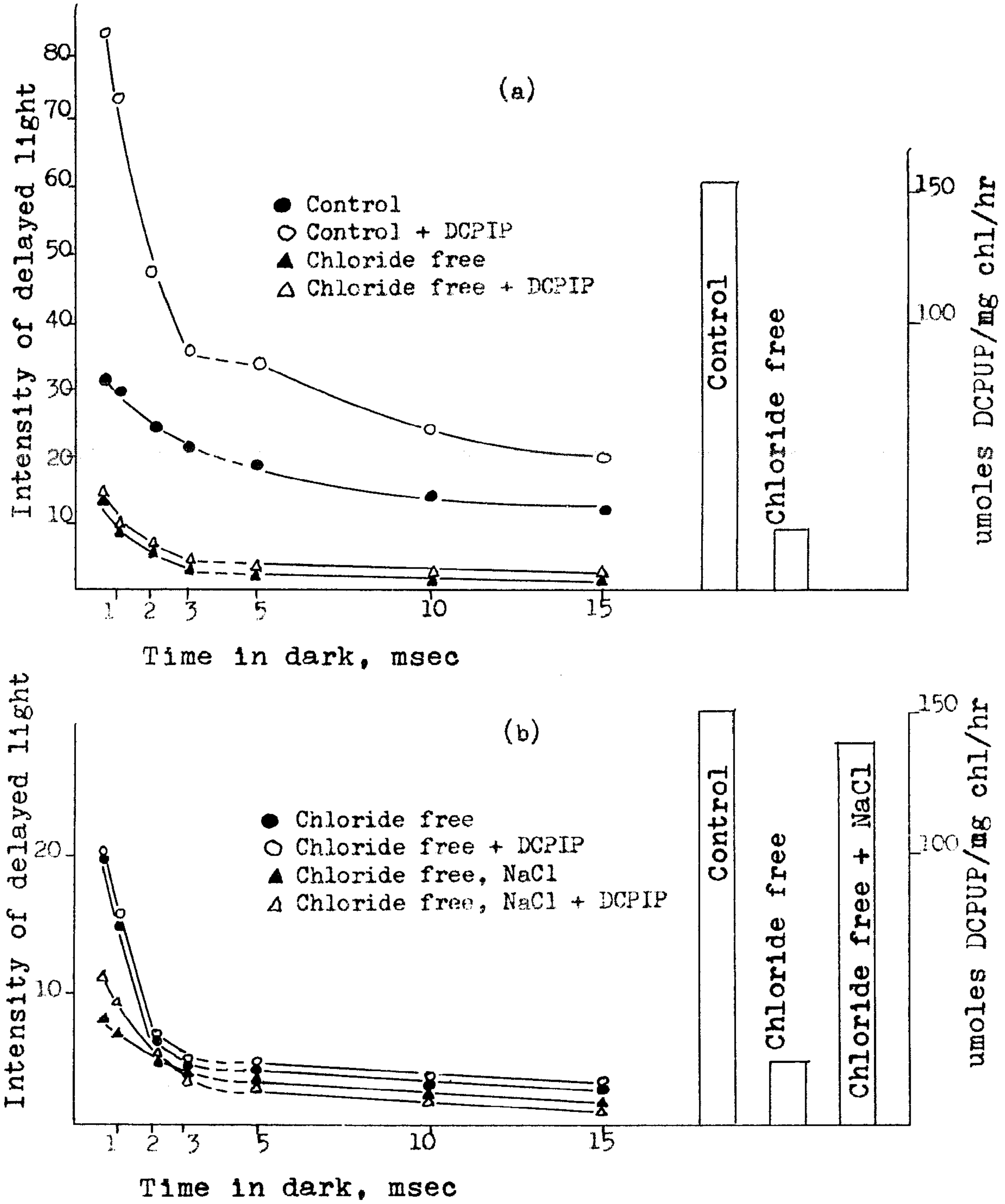


Figure 3-21. Delayed light of chloride depleted chloroplasts. (a) untreated chloroplasts ± 10^{-5} M DCPIP compared to chloride depleted chloroplasts. (b) The effect of adding back chloride on delayed light and photochemistry. Concentrations were 10^{-2} M NaCl, 10^{-5} M DCPIP.

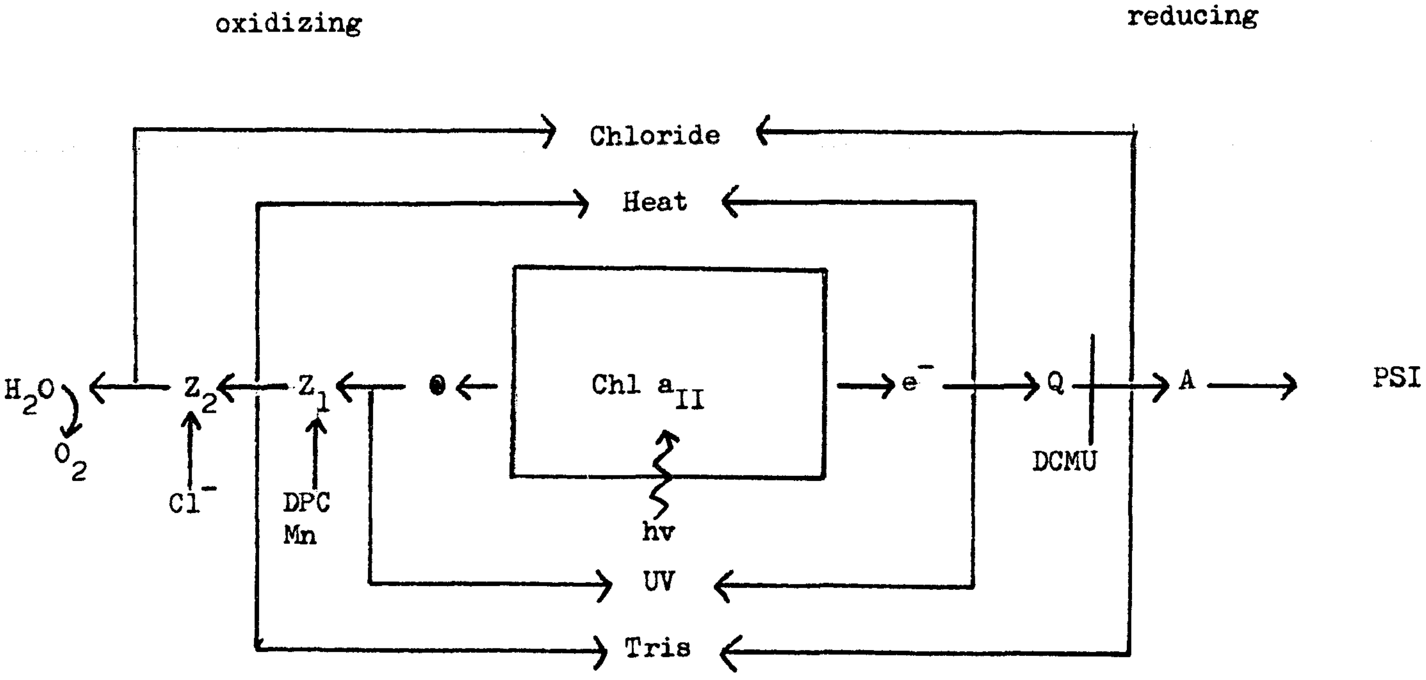


Figure 3-22 Model of the action of treatments which inactivate photosystem II. See text for description.

allowing for a recombination of the charge separated by the reaction center. Concomitantly with the induction of the cycle water is prevented from acting as an electron donor. We have not established a causal relationship between the two events, but both may be linked to conformational changes in the membrane caused by the treatments.

Each cycle has some attributes which indicate that it is different from the others. The cycles induced by tris and heat are very similar. Both manganous ions and DPC can restore photochemistry and the increase in delayed light at 1 msec as well as slowing the rapid decay kinetics of delayed light (see Table 3-5). From the light saturation study of the restored tris system (figure 3-13), it appears that donors do not completely block the cycle but allow greater numbers of electrons to enter photosynthetic electron transport. However, the two cycles differ with respect to restoration of the variable fluorescence; this is restored by DCMU in tris treated chloroplasts and not restored by DCMU in heated chloroplasts. This indicates that on the reducing side of photosystem II the tris cycle operates on the side of Q towards photosystem I and the heat cycle is on the other side of Q (see figure 3-22).

Ultraviolet irradiation is thought to be a more complicated story. Previous researchers suggested that more than one site was affected by this treatment. Our studies can be interpreted in a simpler manner (figure 3-22).

Condition	DCPIP reduction % of untreated	0.8 msec DL / 2.8 msec DL	1 msec + DCPIP / 1 msec - DCPIP
untreated	100	1.5	2.2
" + NaCl	100	1.5	2.6
" + DPC	90	1.8	1.3
" + $MnSO_4$	95	1.5	2.0
tris treated	8	3.2	0.9
" + DPC	50	1.8	1.2
" + $MnSO_4$	33	2.0	1.1
tris treated	12	5.6	0.9
" + DPC	68	2.7	1.1
" + $MnSO_4$	40	2.5	1.0
heated	26	5.2	1.0
" + DPC	45	4.0	1.2
" + $MnSO_4$	47	2.2	1.4
heated	31	5.6	1.0
" + DPC	46	3.0	1.1
" + $MnSO_4$	64	2.8	1.3
UV irradiation	4	3.5	0.9
" + DPC	32	4.3	0.9
" + $MnSO_4$	24	2.5	0.9
UV irradiation	35	3.0	1.0
" + DPC	75	3.0	1.0
" + $MnSO_4$	47	2.5	1.0
chloride deficient	30	4.0	1.0
" + NaCl	100	2.5	1.1
" + DPC	66	3.2	1.1
" + $MnSO_4$	50	2.3	1.1
chloride deficient	40	5.0	0.9
" + NaCl	100	2.1	1.4
" + DPC	55	3.0	1.0

Figure 3-5. Effect of electron donors on delayed light emission and DCPIP reduction in untreated chloroplasts, and chloroplasts treated to inactivate photosystem II. Representative experiments were chosen in each of the treatments to show the variation in photochemical inhibition and delayed light decay kinetics, as well as the consistency with which electron donors partially reversed the photochemical inhibition and slowed the steep delayed light decay kinetics.

The decay kinetics of the delayed light indicate that a cycle is established by the irradiation. However, while the donors can partially restore photochemistry they do not seem to affect the delayed light. One explanation may be that more than one cycle is induced, although the electron donors can restore normal photosynthetic electron transport to a limited extent, the cycle closest to the reaction center is still operative and determines the delayed light kinetics. However, a simpler explanation is that a cycle is induced closer to the reaction center than the site of electron donation. While not preventing electron donation, this cycle will determine the delayed light kinetics as well as the variable fluorescence.

The action of chloride in figure 3-22 has been placed at a different point in the chain between water and photosystem II. This is because of the fact that chloride ion itself will restore chloride deficient chloroplasts but not affect any of the other types of treated chloroplasts. The placement does not necessarily denote a different site of action; chloride deficiency may work on the same site in the chain but in a different manner. Additionally, DPC and manganese can serve as donors for this system (see Table 3-5), so the site cannot be closer to the reaction center of photosystem II than the place of these donors entrance into the chain.

Figure 3-17 shows how an increase in the steepness of the delayed light decay curve, in this case heat inactivation, correlates well with inhibition of photochemical reduction of DCPIP. However, the percentage of restoration of chemistry by different artificial donors is not directly correlated with their ability to flatten the delayed light kinetics, as shown in Table 3-5. Manganous ions slowed the fast decay kinetics of the delayed light emission to the greatest extent in all the treatments, although in individual experiments DPC sometimes had a greater effect on the kinetics. But except in heated chloroplasts DPC consistently restored DCPIP reduction better than manganous ion. In chloride depleted chloroplasts NaCl restored DCPIP reduction better than either of the artificial electron donors, and slowed the rapid delayed light decay kinetics as well as manganous ion did.

Table 3-5 also demonstrates the effect of the artificial electron donors on untreated chloroplasts. Photochemistry was either not affected by their presence or showed a maximal effect of 10% difference in dye reduction. DPC and manganous ion sometimes enhanced DCPIP reduction slightly and at other times inhibited it 5 to 10%. NaCl had no effect on photochemistry. The only electron donor which had an effect on the decay kinetics of acceptorless delayed light in untreated chloroplasts was DPC which steepened the decay slightly. Manganous ion and NaCl had no effect. DPC and manganous ion decreased the extent of one msec increase in delayed light caused by the addition of an electron acceptor, but did not abolish the increase.

From the data in Table 3-5 it appears that although slowing of rapid delayed light decay indicates the restored ability for chloroplasts (except UV treatment) to do normal photochemistry, the extent of the flattening of the decay curve is not an accurate indication of how much normal photosynthetic ability has been restored. This is a qualitative rather than a quantitative measurement. Also, although photochemistry may be almost completely restored (as with NaCl in chloride depleted chloroplasts and DPC in UV irradiation) the delayed light decay is never restored to that of untreated chloroplasts. It appears that neither the artificial electron donors nor NaCl completely break the cycle set up by the treatments, and this is reflected better by the delayed light decay curves than by measuring photochemistry. Alternatively, the treatments may have a secondary effect on photosystem II which does not necessarily interfere with photochemistry, but which causes some alteration in the reaction center reflected in the above delayed light measurements.

Conceivably, some conformational or structural change occurs which is irreversible but does not interfere with partial operation of the photosynthetic electron transport chain (restoration is generally 45 - 75%). It does not, however, allow for optimal charge separation and storage, and this is reflected in the delayed light.

D. Phosphorylation uncouplers

There has not been a great deal of investigation of the possible interactions between delayed light and photophosphorylation. The possibility that such an interaction exists arose when Mayne (1966) demonstrated that post illumination emission of light (stimulated luminescence*) could be induced by acid base transitions. This experiment showed that if chloroplasts were illuminated in an acid medium and then in the dark quickly transferred to a basic medium light was given off by the chloroplasts. A similar experiment was done using a basic medium containing ADP and inorganic phosphate and led to ATP formation in the dark, indicating that some high energy state was formed which could be used to make ATP (Jagendorf and Uribe, 1966). This light emission, or stimulated luminescence, caused by an acid base transition was decreased by phosphorylation uncouplers such as ammonia amines, CCCP,** and by the electron transport inhibitor DCMU.

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* The term stimulated luminescence, or stimulated light emission, is used here to mean light emitted by chloroplasts due to sudden change in the ionic strength of pH of their environment. Preillumination is necessary for this light emission. The light emitted has the same emission spectra as delayed light (first excited singlet of chl a), and is emitted by photosystem II.

** The proposed methods of action of the uncouplers used here are given following the Mitchell (1962) chemiosmotic hypothesis of coupling between electron transport and phosphorylation. Mitchell envisions the precursor to the formation of ATP as being the development of an electrochemical

In 1968 Mayne examined the effect of these uncouplers on four msec delayed light and found the emission to be inhibited by these same substances. From these experiments it was suggested that the pool of metastable energy used for delayed light emission was located in the coupling mechanism. Clayton and Fleischman (1968) examined the effects of phosphorylation uncouplers on 4 msec delayed light and the 515 nm absorption shift (a measure of the membrane potential of chloroplasts or chromatophores, Witt (1969)) in the photosynthetic bacteria Rhodopseudomonas spheroides. They found that amine uncouplers, CCCP and gramicidin inhibited 4 msec delayed light emission and the carotenoid band shift.

Stimulated light emission similar to that observed by Mayne in acid base transitions, was first seen by Miles and Jagendorf (1969) in sudden shifts of salt concentrations. Preillumination was necessary for light emission. Kraan and coworkers (1970) extended these observations and measured

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and pH gradient across a membrane. The dissipation of this gradient through an anisotropic ATPase located in the membrane provides the energy requirement for ATP synthesis. Ionized NH_4 and amines act by entering the thylakoid in an unprotenated form and becoming protenated on the inside, thereby destroying the pH gradient. EDTA treatment removes the coupling factor (ATPase) which leaves holes in the membrane. CCCP makes the membrane permeable to ions. Valinomycin makes the membrane preferentially more permeable to K ions. Nigericin exchanges K ions for protons, and gramicidin makes the membrane permeable to cations. All of these substances destroy the pH gradient or membrane potential and therefore the 'high energy intermediate' leading to phosphorylation. See Mitchell's books (1966, 1968) or Grenville's review (1969).

fluorescence and stimulated light emission simultaneously during acid base and ionic strength transitions. The stimulated light emission caused by these treatments was not paralleled by a significant increase in prompt fluorescence. This indicated that the stimulation is due to an increase in the rate of back reaction of primary photoproducts of photoreaction II which is not caused by an increase in concentration of reduced electron acceptor brought about by 'reversed' electron flow.

By adding different concentrations of permeable and impermeable salts Barber and Kraan (1970) found differential effects on stimulated light emission. They interpreted their results as indicating that the different permeabilities of the cations and anions establish a diffusion potential across the thylakoid membrane which is positive on the inside with respect to the outside and this effects stimulated light emission. This interpretation was supported by the fact that the detergent Triton X-100 (which destroys membrane integrity) abolished stimulated light emission.

These studies on stimulated light emission have established that it is induced by conditions which establish a pH gradient or membrane potential and decreased by substances which dissipate or prevent the establishment of the pH gradient and membrane potential. On the basis of the Mitchell model of phosphorylation this light emission appears to be an alternate means of dissipating the high energy state other than by forming ATP. Four msec delayed light examined in chloroplasts by Mayne (1968) and in chromatophores by Clayton and Fleischman (1968) is decreased by the same phosphorylation uncouplers which decreased stimulated light emission. It appears that the high energy state has an effect on millisecond delayed light.

The relationship between the high energy state and delayed light has been examined using delayed light induction by Wraight and Crofts (1971). Wraight and Crofts studied the kinetics of the induction of delayed light from the onset of illumination, and the effect of uncouplers on this rise. When the intensity of delayed light measured one msec after a repeating flash was observed during the first few seconds of illumination in a phosphoroscope, there were two distinct phases to the initial rise--a fast phase ($t_{1/2} < 0.1$ sec) followed by a slow phase with a half rise time of about 0.35 sec. Wraight and Crofts observed that when the slow phase of the delayed light rise was replotted with the intensity on a logorithmic scale, the curve appeared more nearly monophasic, and had a rise time the same as proton uptake.

From the sensitivity of delayed light induction to ionophores and other uncoupling agents they concluded that most of the delayed light was dependent on the pH gradient across the chloroplast membrane, and the slow rise of the millisecond delayed light emission reflected the onset of the pH gradient as a result of the hydrogen ion uptake. The initial fast component of the rise was inhibited by valinomycin, and was attributed to an effect on the membrane potential. While nigericin and amines abolished only the slow component of the rise (by dissipating the pH gradient), CCCP or nigericin plus valinomycin inhibited both fast and slow components (by affecting both membrane potential and pH gradient).

Itoh and coworkers (1971) also examined delayed light induction and found it sensitive to both uncouplers and electron transport inhibitors. Their findings were the same as those of Wraight and Crofts.

There has been research on the effect of phosphorylation uncouplers on four msec delayed light and on millisecond delayed light induction. However, very little work has been published on the effect of the high energy state on steady state millisecond delayed light kinetics. Wells, Bertsch and Cohen (1972) have examined the effect of phosphorylating conditions (presence of ADP and Pi) and the uncouplers NH_4Cl and CCCP on millisecond delayed light kinetics. The following is an extension of their study.

1. Effect of ionophorus uncouplers

Millisecond delayed light was examined in the presence of the substances nigericin, gramicidin and valinomycin. The latter substance does not act as an uncoupler in the steady state but does change the characteristics of the membrane with regard to ion flux. Valinomycin is thought to create channels for the movement of potassium ions (Lardy, et al., 1967). In the steady state where the main component of the high energy state is the pH gradient valinomycin has no effect, but in flash experiments the main component is the membrane potential (Junge and Witt, 1968). In this situation valinomycin's ability to make the membrane permeable to potassium ions prevents the establishment of the membrane potential.

Figures 3-23 and 3-24 give the effects on millisecond delayed light of these three substances in the presence and absence of the ferricyanide Hill reaction. All three additions reduced the emission intensity 50 - 75% in the absence of electron transport. Table 3-6 shows that the slow decay kinetics which obtain in the absence of electron flow from photosystem II were not significantly affected. Activation of noncyclic electron flow resulted in the typical increase in emission at one millisecond, accompanied by rapid decay kinetics. Gramicidin and nigericin reduced the absolute emission intensity in the presence of noncyclic electron flow. As seen in Table 3-6 they did not have a large effect on the kinetics of decay in the presence of ferricyanide, although the uncouplers increased the rate of electron flow two to three times as seen by the amount of ferricyanide reduction in their presence and absence.

Conditions	- FeCy $\frac{0.8 \text{ msec}}{2.8 \text{ msec}}$	+ FeCy $\frac{0.8 \text{ msec}}{2.8 \text{ msec}}$	$\frac{1 \text{ msec} + \text{FeCy}}{1 \text{ msec} - \text{FeCy}}$	FeCy redn
no additions	1.6	4.3	5.9	120
+ gramicidin (10^{-6}M)	1.7	4.5	3.3	250
+ nigericin (10^{-6}M)	1.7	4.0	3.2	175
+ valinomycin (10^{-6}M)	1.5	4.0	9.5	100
NH_4Cl (10^{-2}M)	1.6	4.5	3.9	180
methylamine (10^{-2}M)	1.8	4.0	3.4	210
CCCP (10^{-6}M)	1.9	3.8	3.5	200

Table 3-6. The effect of uncouplers on delayed light and ferricyanide reduction. The first ratio is that of 0.8/2.8 msec delayed light without ferricyanide present. The second ratio is that of 0.8/2.8 msec delayed light with ferricyanide. The third ratio is of delayed light emission at 1 msec with ferricyanide present over delayed light emission at 1 msec without ferricyanide. The last column is the ueq/mg chl/hr of ferricyanide reduced.

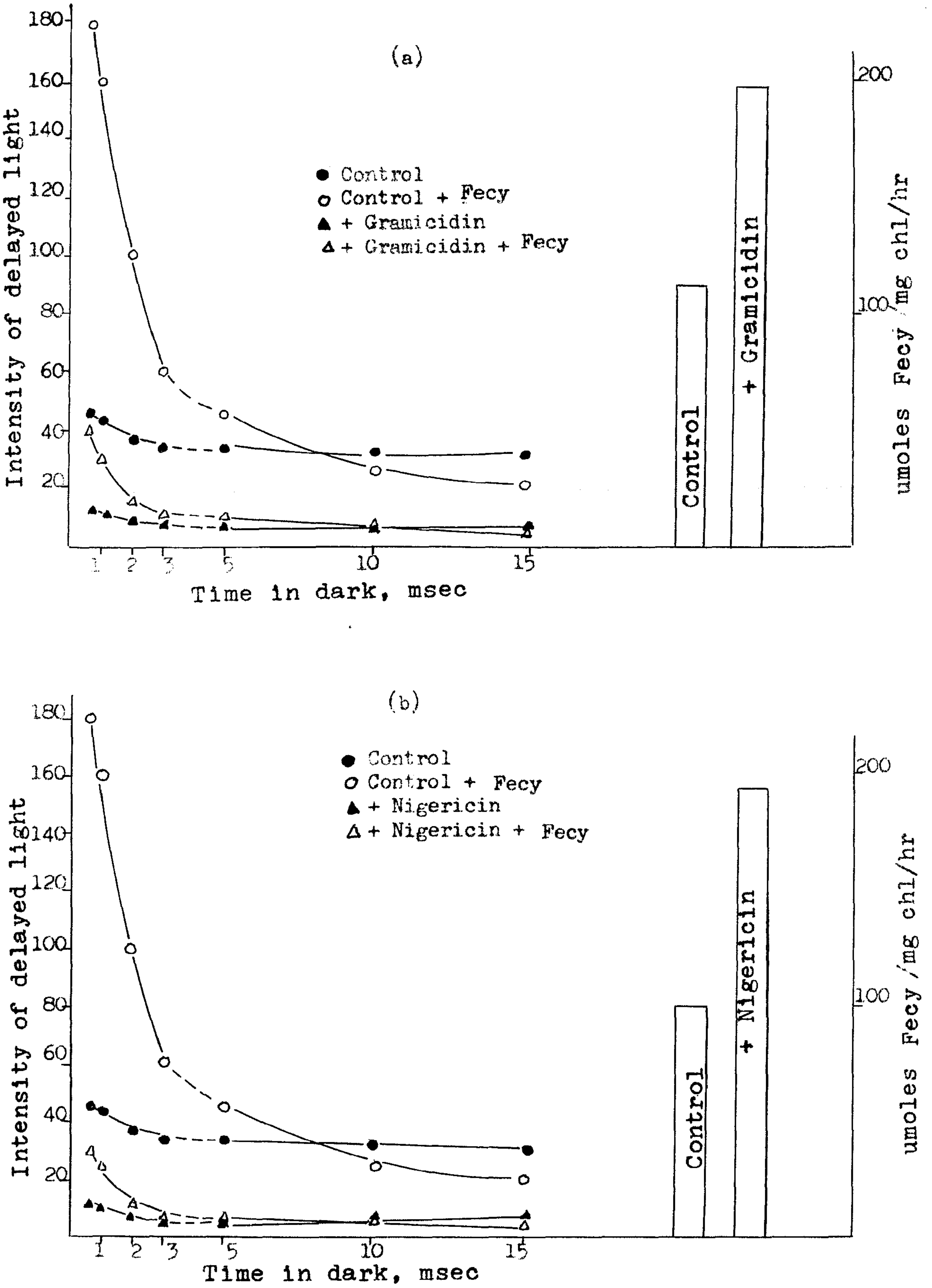

Figure 3-23 a) Delayed light emission from 1 to 15 msec of chloroplasts with 10^{-6}M gramicidin in the absence and presence of ferricyanide (3×10^{-4}M) compared with control chloroplasts. b) Delayed light emission from 1 to 15 msec of chloroplasts with 10^{-6}M nigericin in the absence and presence of ferricyanide compared with control chloroplasts.

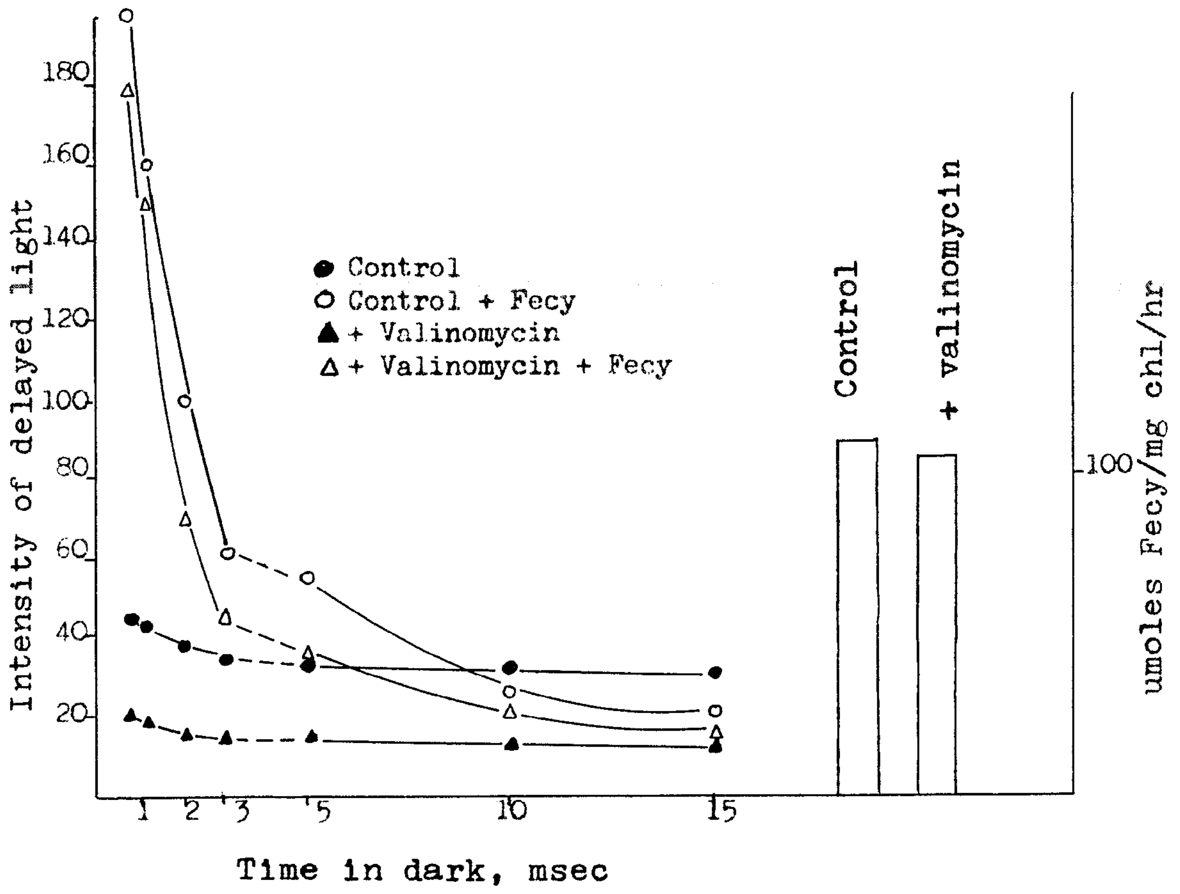

Figure 3-24. Delayed light emission from 1 to 15 msec of chloroplasts with 10^{-6}M valinomycin in the absence and presence of ferricyanide ($3x10^{-4}$M) compared with control chloroplasts.

Valinomycin, which does not act as an uncoupler in the steady state, is an interesting contrast to nigericin and gramicidin. It decreases the delayed light in the absence of an acceptor to the same extent as the two uncouplers, but affects the delayed light in the presence of ferricyanide very little. It slightly decreases the rate of electron flow as measured by ferricyanide reduction. It may be that the decrease in the acceptorless delayed light caused by these compounds is associated with their membrane modifying properties, while the extent of one millisecond increase in the presence of an electron acceptor has to do with their effect on electron flow. This will be developed further in the discussion. Figure 3-25 shows the effect of increasing concentrations of gramicidin on one msec delayed light in the presence and absence of ferricyanide. The delayed light at 1 msec is decreased over the same gramicidin concentration range which enhances electron flow. The effect on one msec delayed light correlates with the degree of uncoupling as seen by the increased rate of ferricyanide reduction.

2. Effect of amines

Figure 3-26 shows the effect of NH_4Cl and methyl amine on delayed light in the presence and absence of an acceptor. These substances are thought to act by entering the thylakoid and becoming protonated on the inside by the excess of hydrogen ions, thus destroying the pH gradient between the inside and outside of the thylakoid

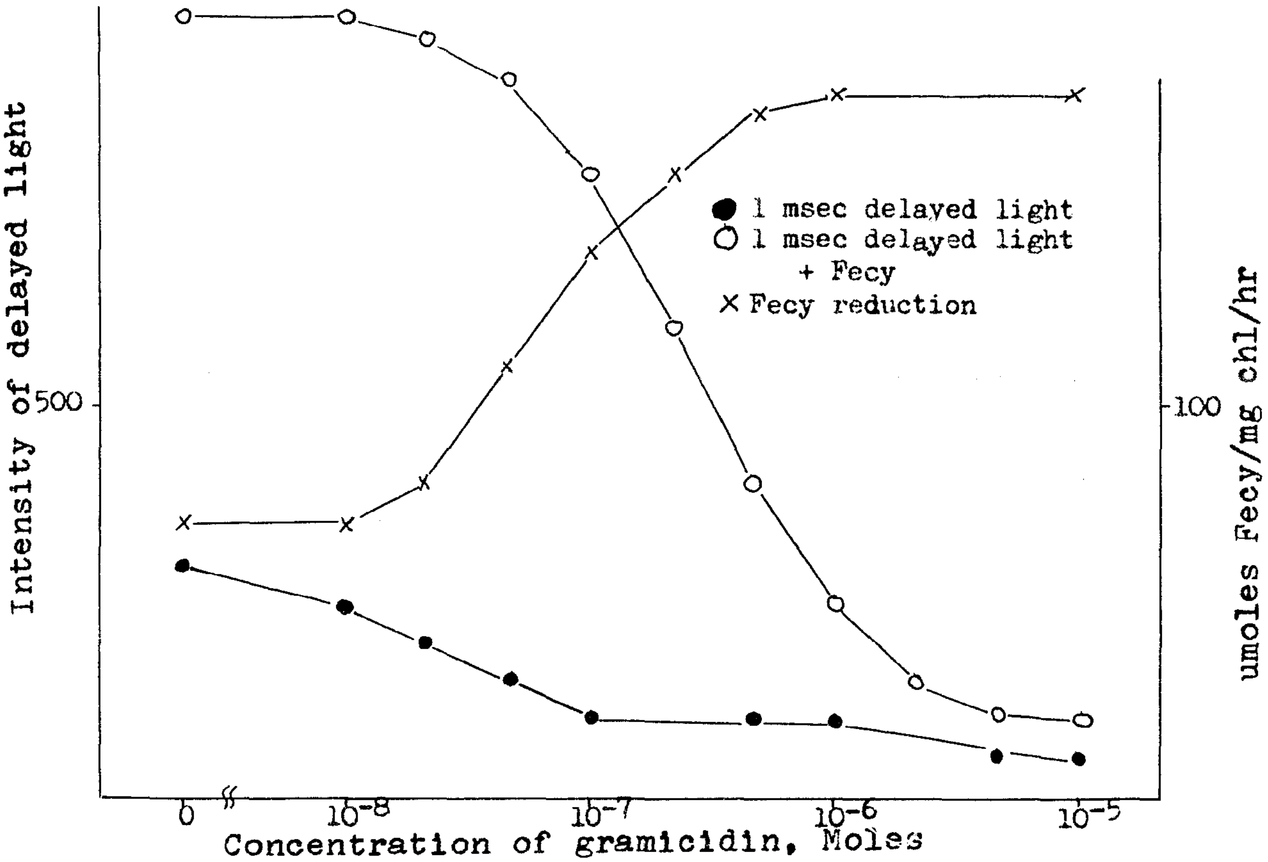

Figure 3-25. The effect of increasing concentrations of gramicidin on delayed light emission at 1 msec in the absence and presence of ferricyanide (3×10^{-4}M) and on ferricyanide reduction.

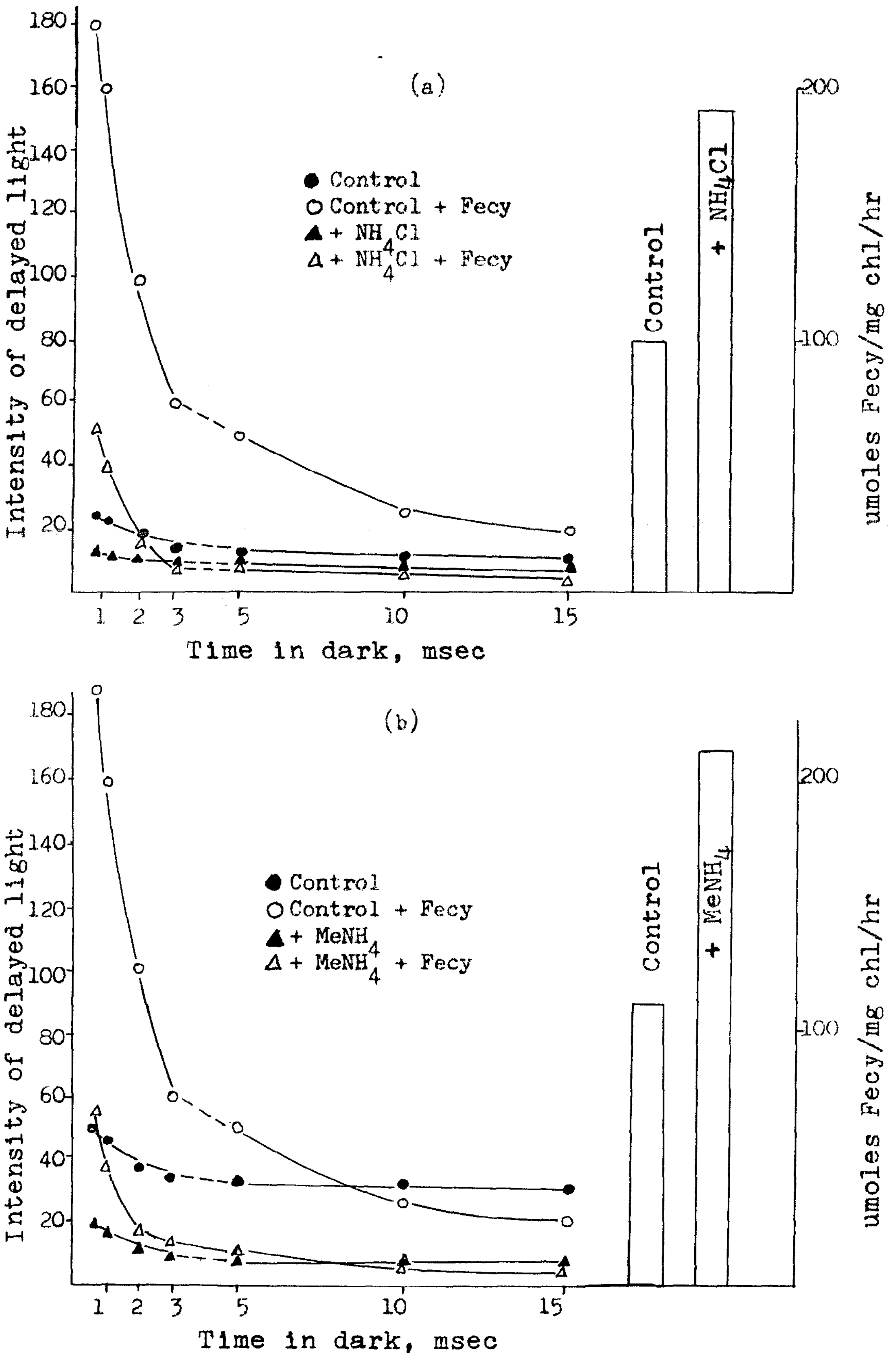

Figure 3-26. a) Delayed light from 1 to 15 msec of chloroplasts with 10^{-2}M ammonium chloride in the absence and presence of ferricyanide (3×10^{-4}M) compared to control chloroplasts. b) Delayed light from 1 to 15 msec of chloroplasts with 10^{-2}M methyl amine in the absence and presence of ferricyanide (3×10^{-4}M) compared to control chloroplasts.

(Crofts, 1968). Their action is accompanied by swelling of the thylakoid vesicles. The effect of NH_4Cl and methylamine on millisecond delayed light is the same as that of the ionophorous uncouplers nigericin and gramicidin. The acceptorless delayed light is decreased about 50% in the presence of these uncouplers and the increase caused by ferricyanide at one millisecond is smaller in magnitude than the control chloroplasts. As seen in Table 3-6 the additions did not have a large effect on the decay kinetics with or without the electron acceptor present, but did reduce the relative increase at 1 msec on addition of acceptor.

3. Effect of CCCP

CCCP appears to act as an uncoupler by making the thylakoid membrane permeable to the movement of ions (Haytler and Prichard, 1963). Unlike gramicidin (which acts as an uncoupler over several orders of magnitude of concentration) CCCP has a narrow concentration range where it acts as an uncoupler. Maximal uncoupling is from 1 to 10 x 10^{-6} M. Below that concentration coupled electron flow is unaffected, and above that concentration CCCP inhibits electron transport. It was, therefore, interesting to observe that delayed light was affected by this compound at concentrations where the rate of electron flow was unaltered. Figure 3-27 shows the effect of an uncoupling concentration (10^{-6} M) of CCCP on delayed light in the presence and absence of DCPIP. Of all the uncouplers examined CCCP is the most

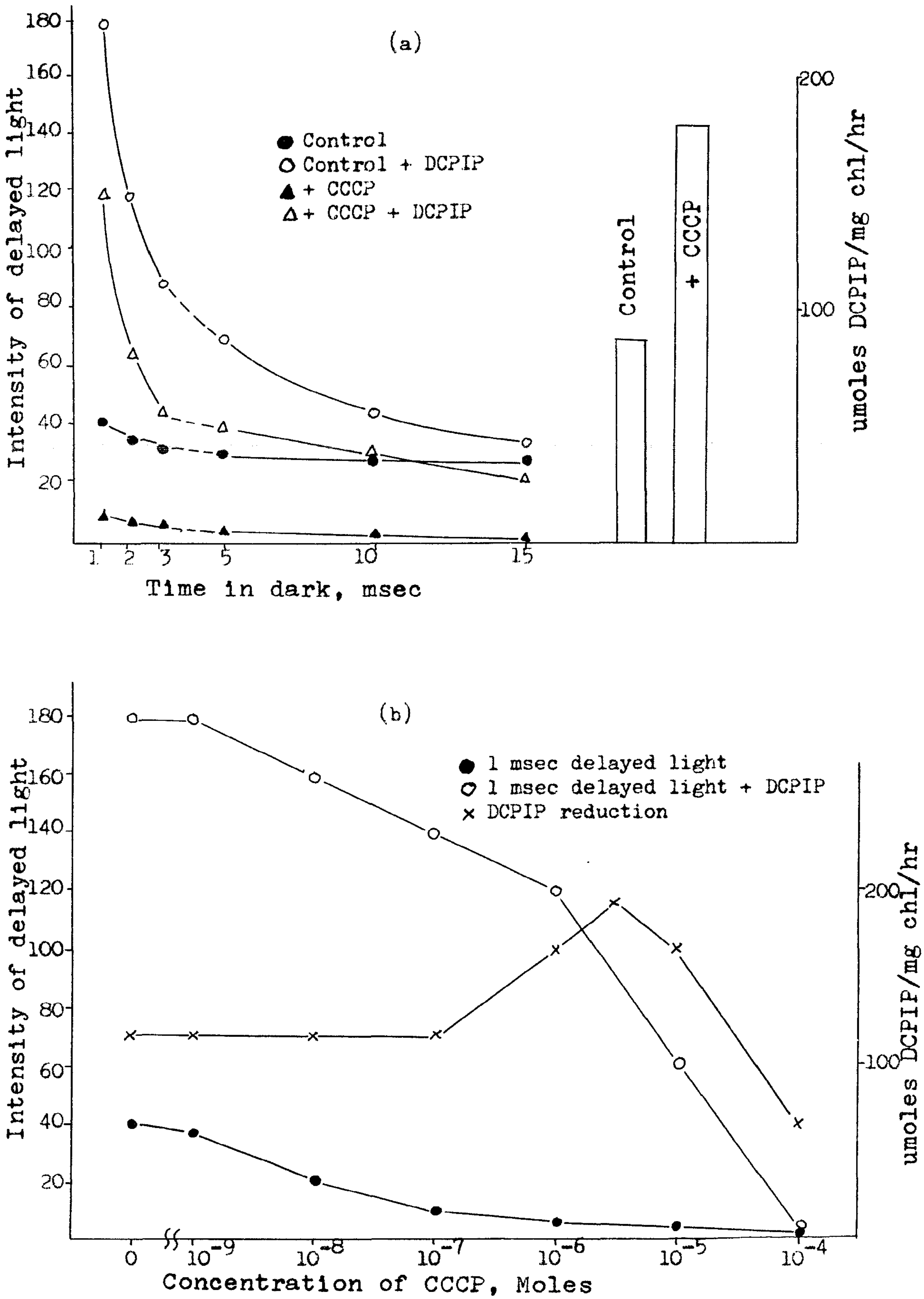

Figure 3-27. a) Delayed light from 1 to 15 msec of chloroplasts with 10^{-6}M CCCP in the absence and presence of DCPIP compared to control chloroplasts. b) The effect of increasing concentrations of CCCP on DCPIP reduction and delayed light at 1 msec in the absence and presence of DCPIP (10^{-5}M).

effective in reducing the absolute emission intensity of delayed light in the absence of an acceptor, but least effective in the presence of an acceptor. However, as can be seen in Figure 3-27, the reduction in delayed light occurs at concentrations where coupled electron flow is not being affected. It appears that CCCP has effects on chloroplasts other than that of an uncoupler, and while they cannot be seen from an examination of photochemistry they are apparent in delayed light.

4. Discussion

Mayne (1968) originally examined the effect of uncouplers on four millisecond delayed light and suggested that the pool of metastable energy used for delayed light emission was located in the coupling mechanism. Clayton (1968), from experiments with photosynthetic bacteria, developed an alternate explanation. He suggested that under phosphorylating conditions or in the presence of uncouplers the flow of electrons and holes away from the reaction centers would be speeded. In the absence of these conditions there would be no means of dissipating high energy phosphorylation intermediates, and electron and hole flow could be slowed or even reversed. The primary electron acceptor could be regenerated in its reduced form by an energy linked reversal of electron transport; delayed light emission could then result from a direct reversal of the primary photoact.

Clayton's theory is consistent with the model of the reaction center of photosystem II proposed by Bertsch (1960). In this scheme delayed light emission would result from the thermal excitation of trapped electrons and holes into conduction and valence bands in the chlorophyll and their subsequent recombination, accompanied by the emission of light. Uncouplers would speed the flow of electrons and holes away from the trapping sites in the dark, and thus lower the population of the conduction band and the probability of recombination.

This hypothesis would explain the decrease in amplitude of four msec delayed light in the presence of electron acceptors. The electron transport chain is turning over faster and trapped charge is being stored for a shorter time. The decrease induced by uncouplers in the absence of an electron acceptor is not adequately explained in this manner. If no electron flow is occuring, high energy intermediates would not accumulate whether or not an uncoupler was present. However, uncouplers have membrane moderating properties even in the absence of electron transport and it may be this action which decreases the intensity of delayed light emission.

Wraight and Croft's experiments (1971) with delayed light induction kinetics showed that the final height reached by delayed light at one msec correlated with the extent of pH gradient and membrane potential generated. Substances which prevented the development of one or both of these phenomena inhibited delayed light. They explain their observation with a model which places the primary donor and primary acceptor on opposite sides of the membrane.

This model follows from two main postulates. First, that the primary donor (Z/Z^+) and acceptor (Q^-/Q) are situated on opposite sides of the thylakoid membrane. Second, that the primary donor and acceptor are in equilibrium with secondary donor (DH/D) and acceptor (A/AH) pools, which are redox couples of the hydrogen carrier type. The pools are in equilibrium with phases on opposite sides of the membrane, and their redox potentials are dependent on the values of the pH in these phases. Interaction with phases on opposite sides of the membrane would alter the probability of reemission of delayed light. Uncouplers, by affecting the flow of ions across the membrane, could alter the environment around the primary donor and acceptor even in the absence of electron flow, and thus affect acceptorless delayed light.

The effect that CCCP at 10^{-8} $\underline{M}$ has on delayed light cannot be explained from its action as an uncoupler, since from figure 3-27 it is apparent that the effect is occuring at a concentration where electron transport is unaffected. Previous researchers have investigated CCCP, but always at concentrations that uncouple phosphorylation (10^{-6} to 10^{-5} M) or at concentrations which inhibit electron transport (greater than 10^{-5} M). Early studies found that

CCCP and FCCP are potent uncouplers of photophosphorylation, exerting at concentrations 10^{-6} to 10^{-5} an inhibitory action on ATP formation with concomitant stimulation of the rate of non-cyclic electron transport (Avron and Shavit, 1963; Gromet-Elhanan and Avron, 1965).

De Kiewiet *et al*. (1965) found that these substances at higher concentrations (above 10^{-5} M) caused an inhibition of the Hill reaction. De Kiewiet *et al*. proposed that the inhibitory site of CCCP in the Hill reaction was at the oxygen evolving process, since 10^{-4} M CCCP did not inhibit, but stimulated to some extent the rate of NADP reduction with reduced DCPIP as the electron donor. Studies on the electron transport system of *Euglena* chloroplasts (Katoh and San Pietro, 1967) confirmed the above observations and further showed that CCCP, as well as DCMU, was effective in inhibiting the action of ascorbate as a donor for NADP reduction in heated *Euglena* chloroplasts. This limits the location of the CCCP inhibitory site on the electron transport chain to between a site where ascorbate, in place of water, donates its electrons to photosystem II, and a site where reduced DCPIP donates its electrons to photosystem I. A similar observation was later reported by Itoh *et al*. (1969) who showed that CCCP suppresses the DCPIP photoreduction in tris inhibited chloroplasts with $MnSO_4$ as the electron donor.

We see effects on delayed light at concentrations of CCCP which are below those which either uncouple electron transport or inhibit it. Delayed light seems to be a more sensitive indicator of the effect CCCP has on photosynthesis than is photochemistry, but the relation between these two effects of CCCP remains unclear.

CHAPTER IV: THERMOLUMINESCENCE*

A. Introduction

We assume that although thermoluminescence and delayed light are different phenomena, they may reflect the same metastable stored energy. Both represent reexcitation of chlorophyll from metastable states of photosynthetic energy conversion. In the case of thermoluminescence the temperature is raised rapidly and the emission is a function of temperature, while in delayed light temperature is kept constant and emission is monitored over time. A correspondence might be expected between the delayed light decay curve and the glow curve. Different thermoluminescence peaks, depending on their trap** depth and frequency factor might contribute to different times of the delayed light decay. Generally, the deeper traps might contribute both to the delayed light in the second time range and to the thermal glow at higher temperatures, and similarly the shallower traps might contribute to the short times of luminescence decay and to glow peaks at low temperatures.

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* Abbreviations: DCMU 3-(3,4-dichlorophenyl)-1,1-dimethylurea, FeCy ferricyanide, DPC 1,5-diphenylcarbohydrazide, tris tris(hydroxymethyl) aminomethane, CCCP carbonyl cyanide 3-chlorophenylhydrazone, CQP chloroquine phosphate

** In this chapter the terms peak, trap and energy storage state, are used synonymously to indicate the position of the stored charge which gives rise to the thermoluminescence. The terms assume no mechanism of storage of this energy.

However, if the frequency factor of a deep storage state is high enough it may have a decay time shorter than that of a shallower trap with a lower frequency factor. This will be discussed in greater detail in Chapter V.

Approximately the same ground is covered in this chapter as in Chapter III, but the analyzing tool is thermoluminescence instead of delayed light. Thermoluminescence of different algae is examined, as well as that of isolated chloroplasts with and without an electron acceptor. Treatments which inactivate photosystem II, and phosphorylation uncouplers are investigated to see how the glow curves are affected. In addition, various physical parameters are measured for the glow curves in order to characterize more fully this phenomenon as it appears in chloroplasts.

B. Glow curves of different organisms

1. Results

Figure 4-1 shows the thermoluminescence of different algae. *Chlorella* and *Scenedesmus* wild type show very similar peak profiles, although *Chlorella's* main peak is shifted 13 degrees higher in temperature. This shift is due to the fact that the heating rate of *Chlorella* in this sample was eight degrees a second while that of *Scenedesmus* was six degrees a second. The shoulders are much closer to each other in peak temperature, 45° in *Chlorella* and 42° in *Scenedesmus.* It is difficult to determine the middle of a shoulder and the temperatures indicated for the shoulders,

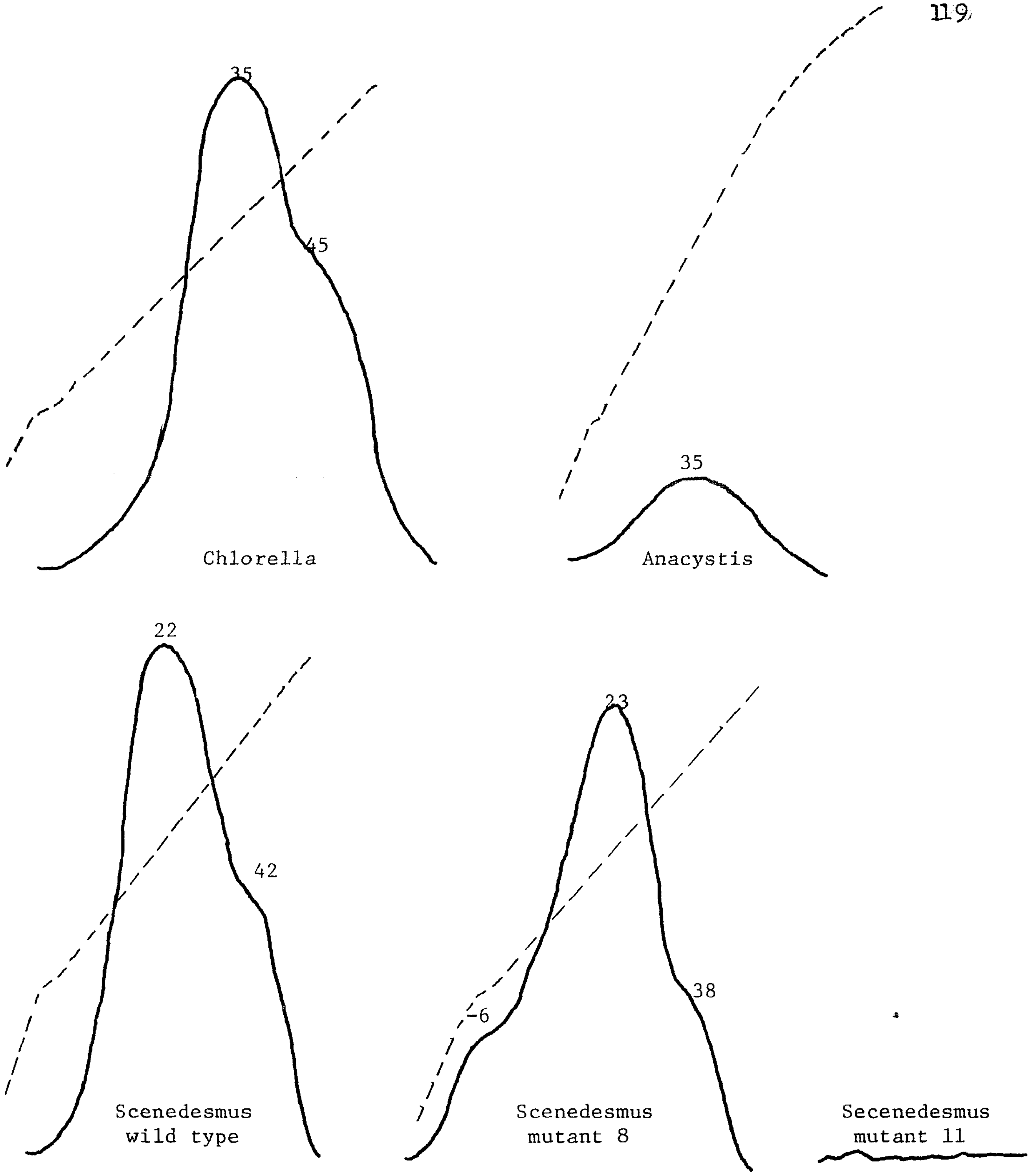

Figure 4-1 Thermoluminescence of whole algae. Cells were spun down at 2000xg for 3 minutes and resuspended in their growth media, to a final concentration of 5 ug chl/ml. Illumination was while cooling from 0° to -196°C, as detailed in the standard procedure in Chapter II. The temperature of each peak is indicated above the peak.

as discussed in Chapter II, are only approximate.

The profiles show one main peak with a distinct shoulder on the high temperature side, similar to the emission profile exhibited by chloroplasts in the presence of DCMU (see figure 4-2). Neither alga has a low temperature peak, which we standardly observe in chloroplasts.

Scenedesmus mutant 8, deficient in photosystem I, has an emission profile like that of isolated chloroplasts (compare figures 4-1 and 4-2) in absence of a Hill acceptor. All three peaks are present in this mutant, although due to difference in heating rate they emerge at somewhat different temperatures from the chloroplasts. In this it differs from *Scenedesmus* wild type which has no peak one. *Scenedesmus* mutant 11, which is inoperative in photosystem II, was also examined. It showed no thermoluminescence in the range from $-20^{\circ}C$ to $+60^{\circ}C$, but it, as did all the other samples, had a peak at $-160^{\circ}C$. This mutant also shows about 300x less D.L.E., as discussed in Chapter III.

The blue green alga *Anacystis* showed very little thermoluminescence, just as it had shown little delayed light. It had some emission in the temperature range where peaks one, two and three appear, but it was impossible to resolve how many of the peaks were present. However, the temperature at which the emission began (figure 4-1) was $0^{\circ}C$ and therefore peak one is probably absent from this alga, as it is from the green algae *Chlorella* and *Scenedesmus*.

The center of the emission is at +35°C.

Figure 4-2 shows isolated spinach chloroplasts in aqueous medium with no additions, and in the presence of the electron acceptor ferricyanide and the photosynthetic poison DCMU. Chloroplasts exhibit three peaks in the temperature range of -30°C to +60°C. The first appears below 0°C and we have called it peak, or storage state, one. The second peak is between +20°C and 35°C. The third peak is a shoulder on peak 2 appearing between 40°C and 50°C. Electron acceptors and electron transport inhibitors affect these peaks differently. Electron acceptors enhance peak 1 and decrease peaks 2 and 3, while DCMU abolishes peak 1 and enhances peaks 2 and 3.

Figure 4-3 shows the effect different concentrations of DCMU have on the emission. Peak one disappears in the presence of DCMU concentrations higher than 10^{-8} M, while peaks 2 and 3 show some enhancement at 10^{-8} M. The maximum enhancement of these two peaks is at the same concentration that photochemistry, measured as ferricyanide reduction, is completely inhibited. At higher concentrations thermoluminescence from all peaks is inhibited, and at 10^{-3} M DCMU no emission is seen.

Two other methods of obtaining glow curves with isolated chloroplasts were tried, both of which had the advantage of giving linear heating rates over most of the temperature range of interest. The methods were suspending

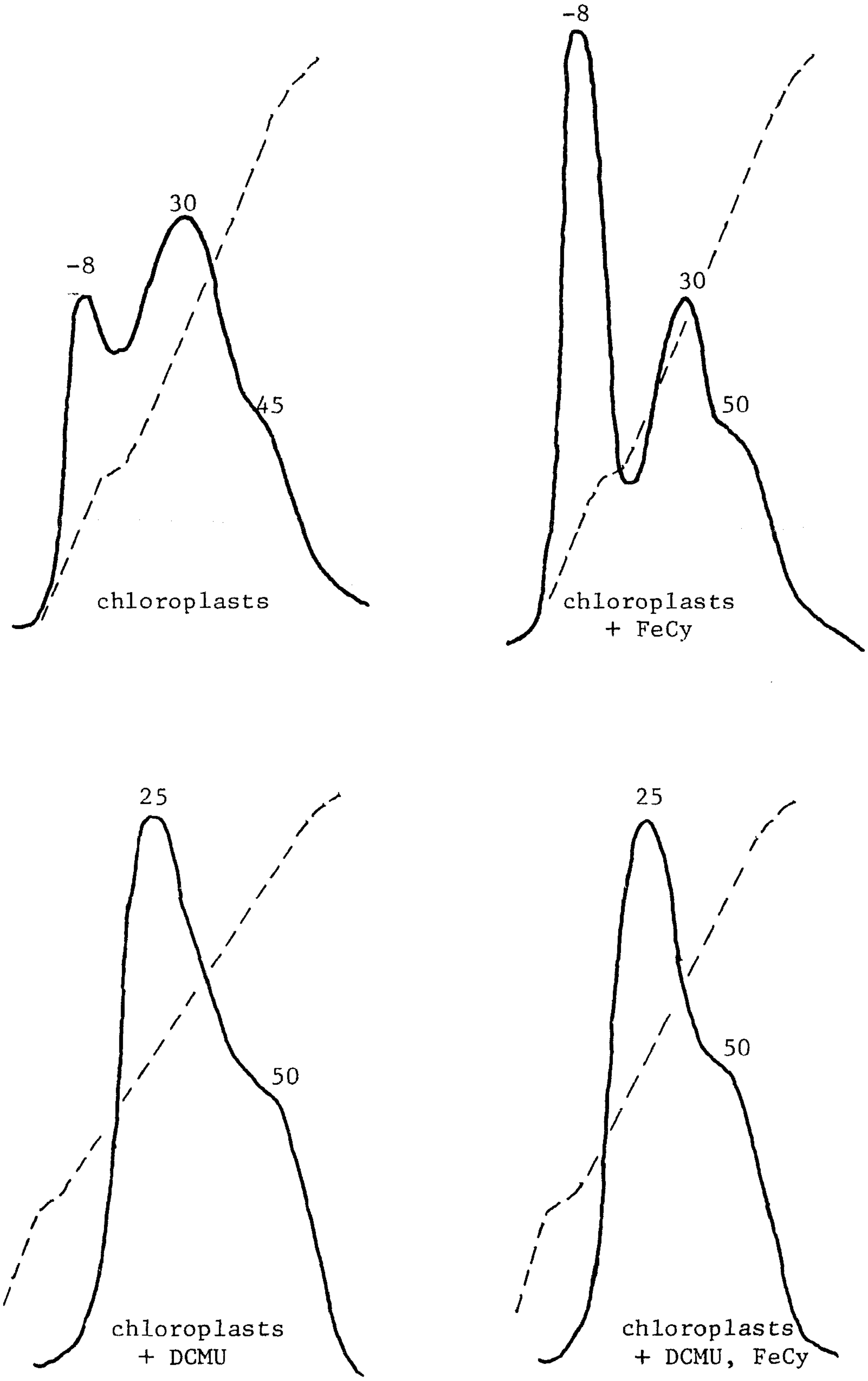

Figure 4-2 Thermoluminescence of isolated chloroplasts in the presence and absence of ferricyanide and DCMU. Reaction mix: 0.025 Tricine (pH 7.8), 0.02 M NaCl, 0.005 M $MgCl_2$, 65 ug chl/ml, and where added $5x10^{-4}$M FeCy, $2x10^{-6}$M DCMU.

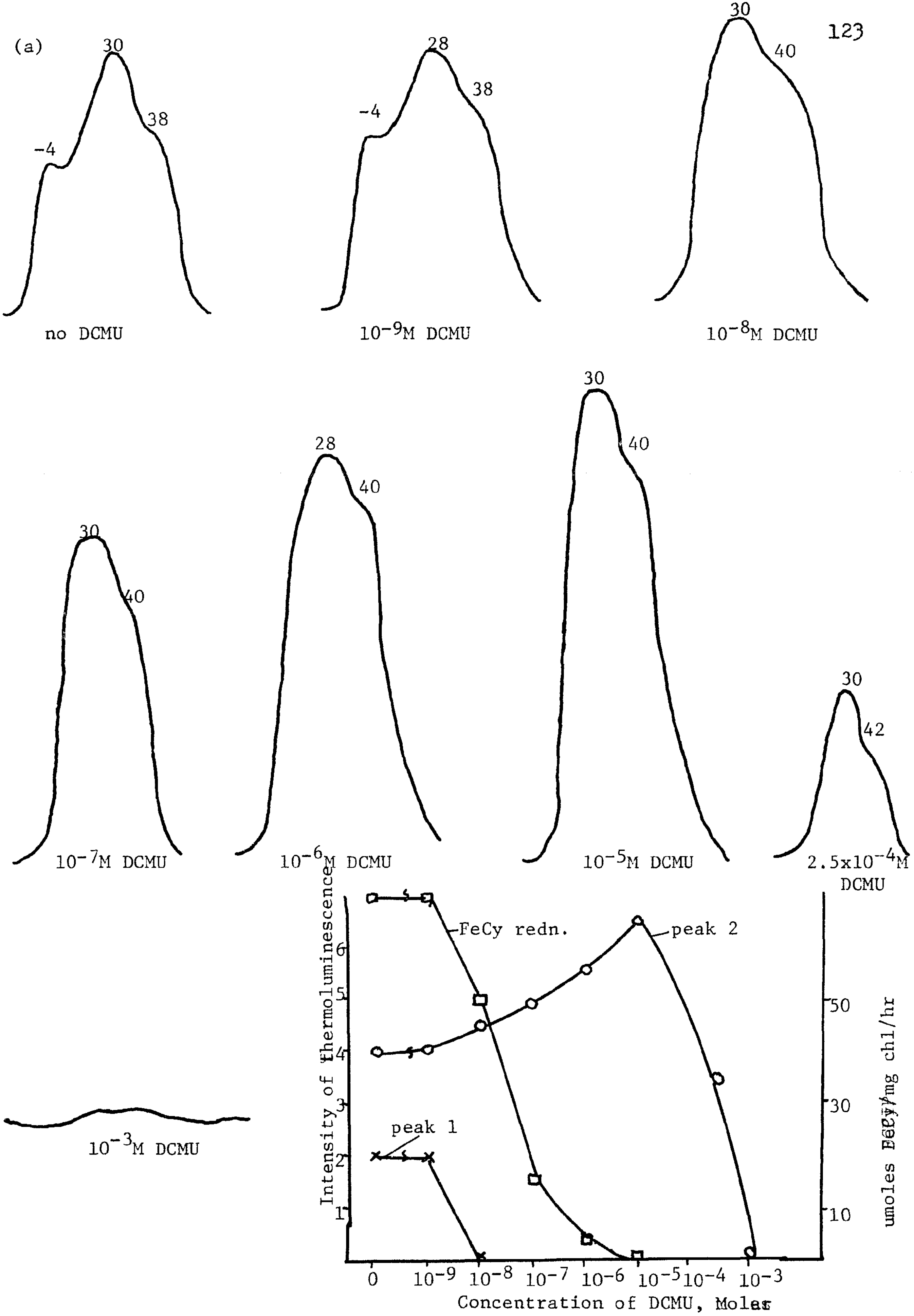

Figure 4-3 Effect of increasing concentrations of DCMU on thermoluminescence of chloroplasts and ferricyanide reduction.

chloroplasts in 10 molal glycerol, and painting a thin layer of chloroplasts on the floor of the sample chamber. The emission profiles are seen in figure 4-4. Chloroplasts suspended in glycerol exhibit thermoluminescence like those in aqueous medium, except that peaks one and three are less distinct. This may be due to the greater linearity of the heating rate. Aqueous medium shows a change in heating rate between -1° and +1°C, the melting temperature of ice, and a slower heating rate above 45° than below it. Ten molal glycerol melts at -20°C and has a linear heating rate until above 50°. The peaks emerge at the same temperatures as those of chloroplasts in aqueous medium.

Chloroplasts painted on the sample holder with a small brush showed emission profiles which differed from run to run. The largest peak appeared over a wide range of temperatures, from -60°C to 0°C, although the heating rate of the sample was always 11 to 12 degrees a second. The profile varied also to a large extent. Figure 4-4 shows a profile with a peak at -40°, a second at 0° and a shoulder at 20°C. Sometimes only two peaks could be distinguished, and, less frequently, only one peak. It was felt that the drying of the chloroplasts caused by this method was creating different artifactual traps, which was reflected by the appearance of different and irreproducible glow peaks.

2. Discussion

The amount of thermoluminescence from the different

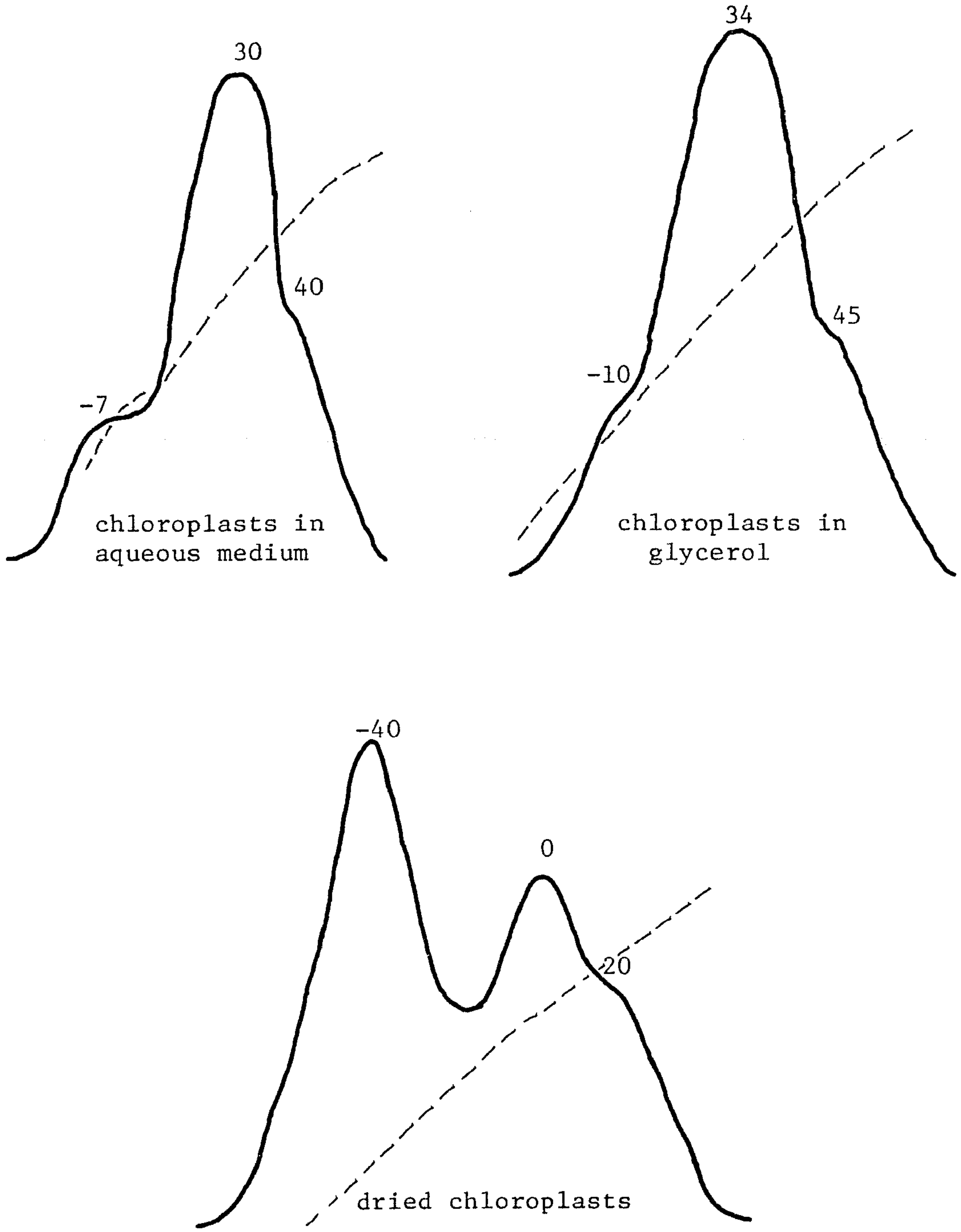

Figure 4-4 Thermoluminescence of chloroplasts obtained with varying reaction mixtures. (a) 50 ug chl suspended in 1 ml of: 0.01 M Tricine (pH 7.8), 0.02 M NaCl, 0.005 M $MgCl_2$. (b) 50 ug chl suspended in 1 ml of 10 molal glycine. (c) undetermined concentration of chloroplasts painted on the bottom of the copper sample holder.

organisms on a per chlorophyll basis agrees well with the amount of millisecond delayed light. Scenedesmus mutant 11 shows neither millisecond delayed light nor thermoluminescence. Anacystis, which shows 5% of delayed light that Chlorella does on a per chlorophyll basis (see figure 3-1), shows 7% as much thermoluminescence (figure 4-1). In addition, Scenedesmus mutant 8 and acceptorless chloroplasts exhibit similar delayed light kinetics (flat decay in the 1 to 15 msec time range), and thermoluminescence emission profiles (three emission peaks). Chlorella and Scenedesmus both show an absence of peak one in their thermoluminescence profiles which look like profiles of isolated chloroplasts in the presence of the inhibitor DCMU. The msec delayed light of the algae show rapid decay kinetics very much like that of isolated chloroplasts in the presence of an electron acceptor. However, the thermoluminescence of chloroplasts with an electron acceptor has an enhanced peak one and smaller peaks two and three. The two algae and spinach chloroplasts with an acceptor show similar millisecond delayed light but very different thermoluminescent properties. One possible explanation of this data is that it reflects a different pool size for storage state 1 in whole algae compared to isolated chloroplasts. This would be due to the relatively slower electron transport rates of extracted chloroplasts compared to chloroplasts *in vivo*.

The purpose of trying two other methods of obtaining glow curves of isolated chloroplasts was to ascertain if having a linear heating rate throughout the temperature range where the three peaks emerged affected the shape of the emission profile. Painting chloroplasts on the bottom of the sample holder did not give reproducible results from one sample to the next. However, suspending the chloroplasts in 10 molar glycerol gave reproducible data. The profile was the same as chloroplasts in aqueous medium except that peak one and peak three were not as clearly distinguished. Ten molal glycerol inhibited photoreduction of ferricyanide 50% under our conditions. Since the thermoluminescence in the two media was similar, and since aqueous media produced more rapid photochemistry, we decided to use aqueous media for our standard conditions.

B. Physical parameters of glow curves

1. Results

The temperature dependence of the trap filling of peaks 1, 2 and 3 is shown in figure 4-5. Samples were illuminated for two minutes at different temperatures, cooled in the dark to liquid nitrogen temperature, and then heated to induce the light emission which was monitored. All three peaks decrease as the temperature at which the sample is illuminated is lowered, but peak 2 and peak 3 are still apparent at -196°C. Peak 1 is not apparent if illumination occurs below -50°C.

The dose-response of the three peaks was also examined (figure 4-6). Illumination was at -35°C so as to

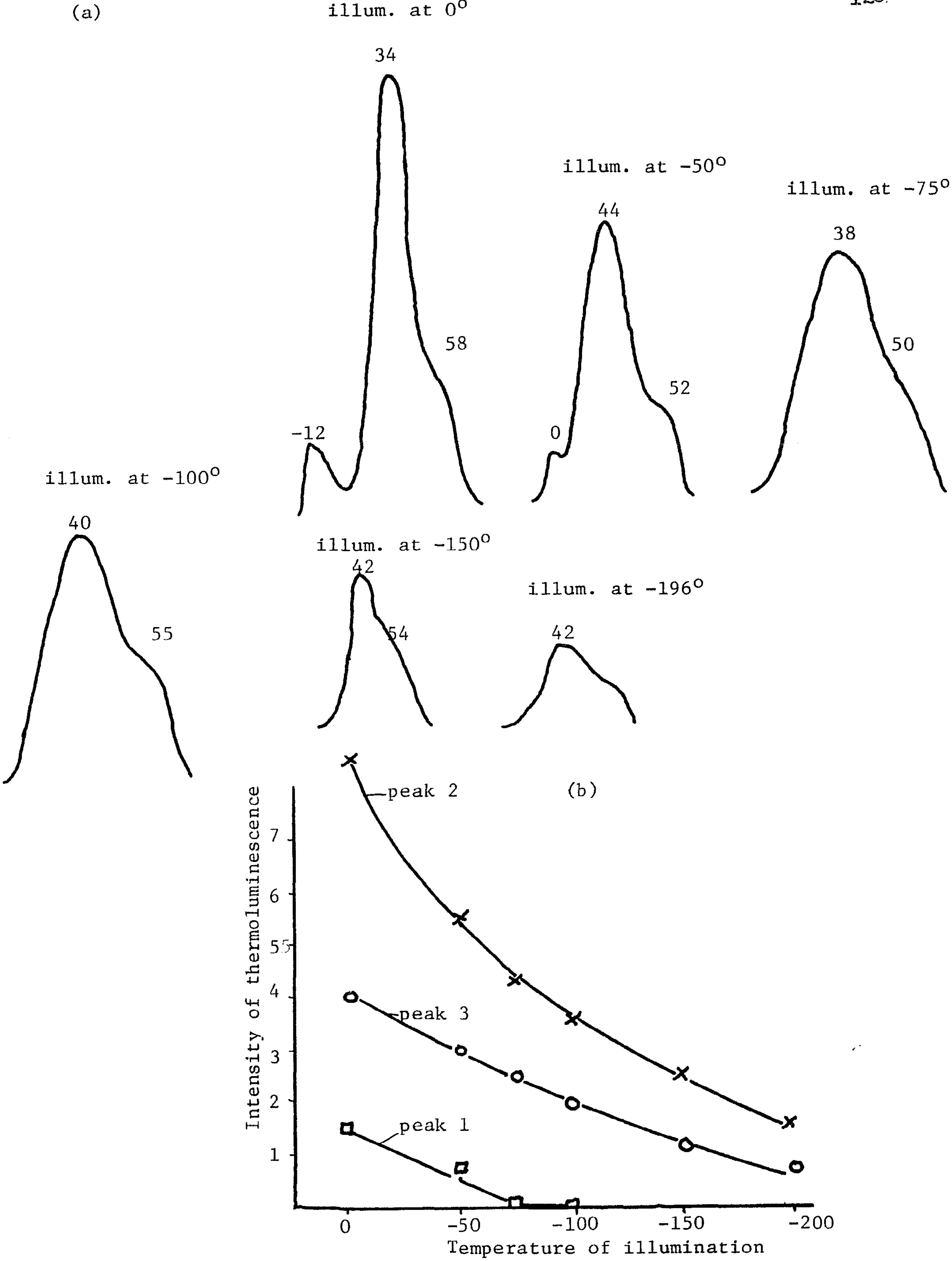

Figure 4-5 The effect of illumination at different temperatures on thermoluminescence. (a) Illumination was for two minutes at the temperature indicated and then the sample was rapidly cooled to liquid nitrogen temperature and heated. (b) Peak heights plotted as a function of temperature of illumination.

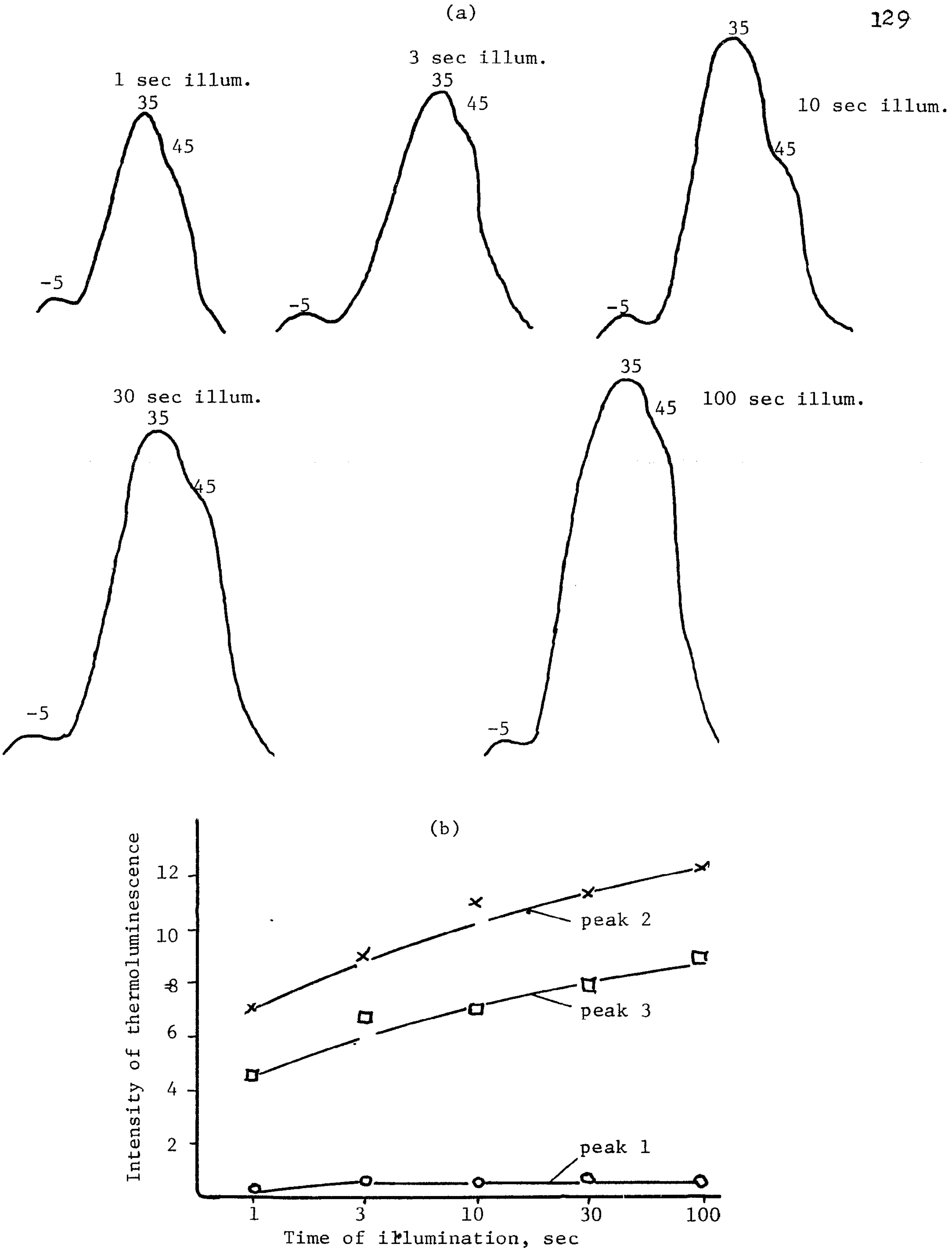

Figure 4-6 Light saturation of glow curves illuminated at -35°C. (a) The glow curves observed after different times of illumination at -35°. (b) Peak height plotted as a function of time of illumination at -35°.

ensure the presence of peak 1, which from the temperature dependence study was seen to disappear below -50°C. Illumination was with white light 2.2 x 10^5 ergs/cm^2/sec. While at -35°C peaks two and three are not saturated at 100 seconds, peak 1 is saturated in three seconds. However, the time required for light saturation of the peaks depends upon the temperature of illumination. At lower temperatures the peaks saturated more quickly (figure 4-7). When illumination is given at -196°C only peak 2 is easily distinguishable. At this temperature peak 2 saturates after three seconds of illumination.

Figure 4-8 shows the results of an examination of whether the reciprocity relationship holds for the glow peaks. The thermoluminescence was examined at -35°C from 0.1 sec to 10 sec at 50% saturation for peak 2. Apparently, untrapping does not occur during the time of illumination, since as seen in figure 4-8 $I \times t$ = constant emission. Peak 1 was too small to measure accurately and peak 3 was also difficult to determine, so figure 4-8b plots storage state 2.

The time course of decay of thermoluminescence in the dark after two minutes illumination at 0°C was examined. At this temperature it took 58 seconds for peak 2 to decay to 37% of its initial value. The decay was exponential (figure 4-9). The expression of the untrapping is $I = dn/dt = -nse^{-E/kt}$ (see Chapter V). At this temperature the energy storage state 1 is not filled well, and energy storage state 3 could not be measured easily as can be seen

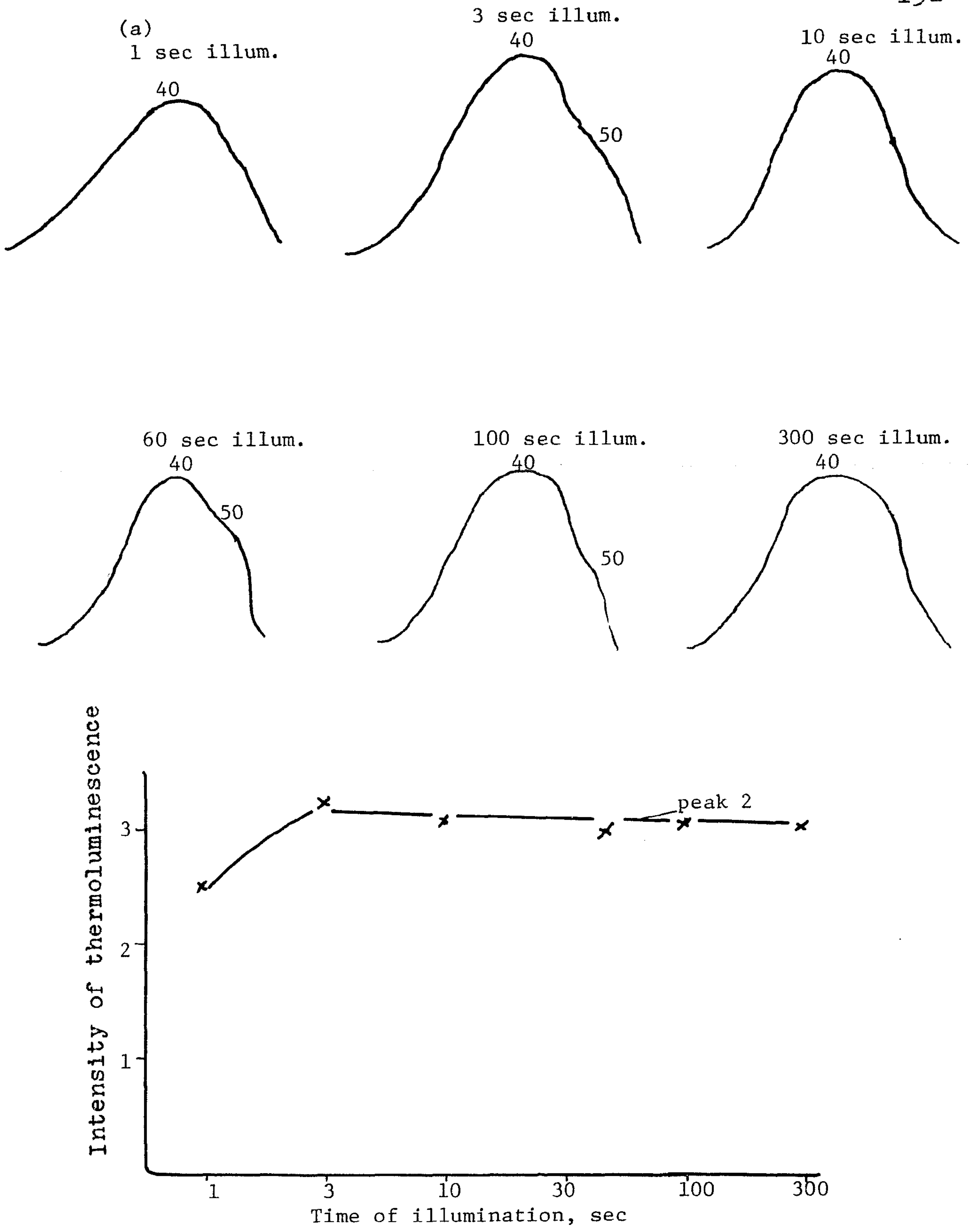

Figure 4-7 Sight saturation of glow curves illuminated at -196°C. (a) The glow curves observed after different times of illumination at -196°. (b) The height of peak 2 plotted as a function of time of illumination at -196°.

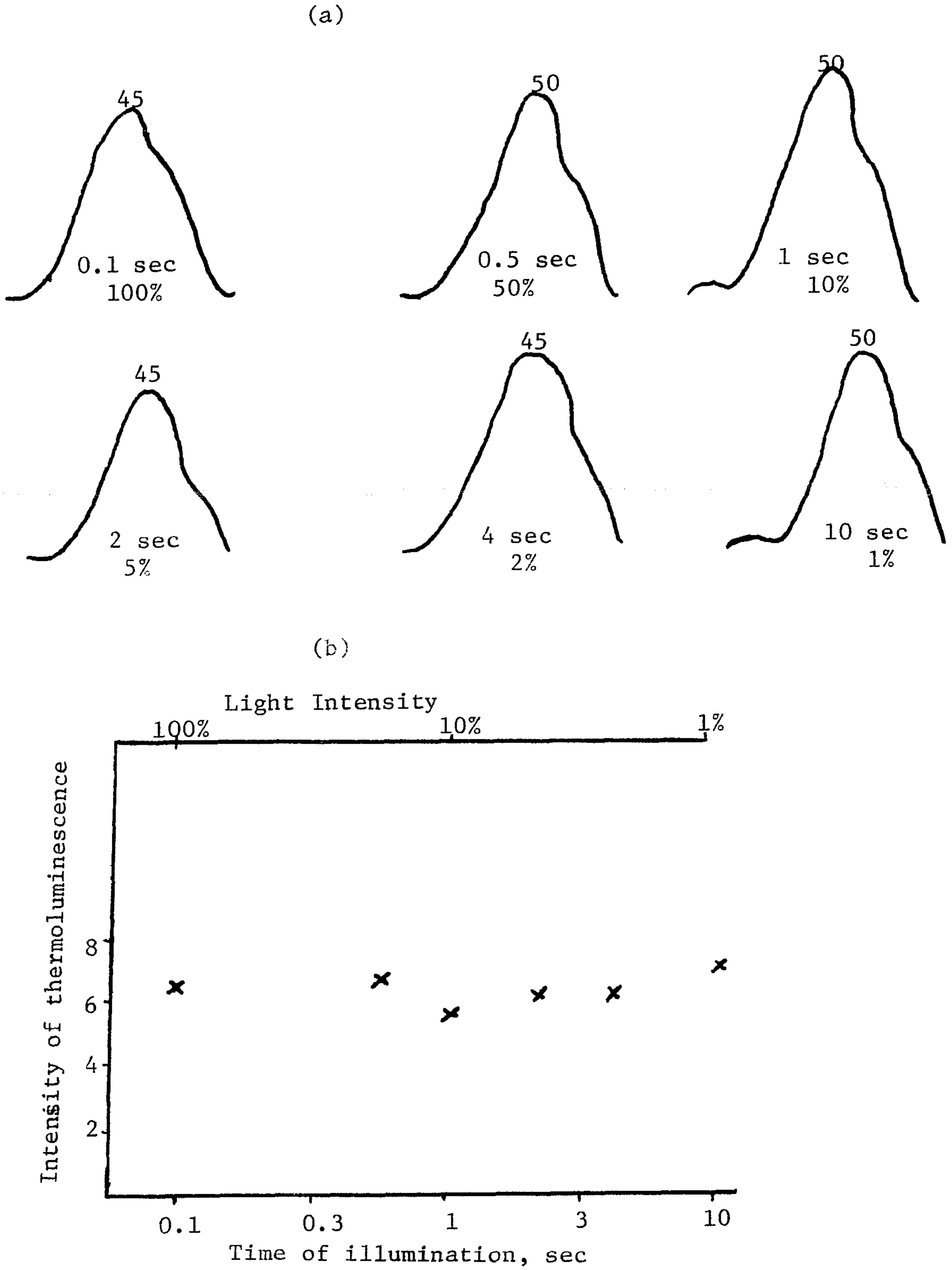

Figure 4-8. Reciprocity in thermoluminescence of intensity of illumination versus time of illumination. (a) The glow curves observed after different times and intensities of illumination (at -35°C). (b) The height of peak 2 plotted as a function of time and intensity of illumination.

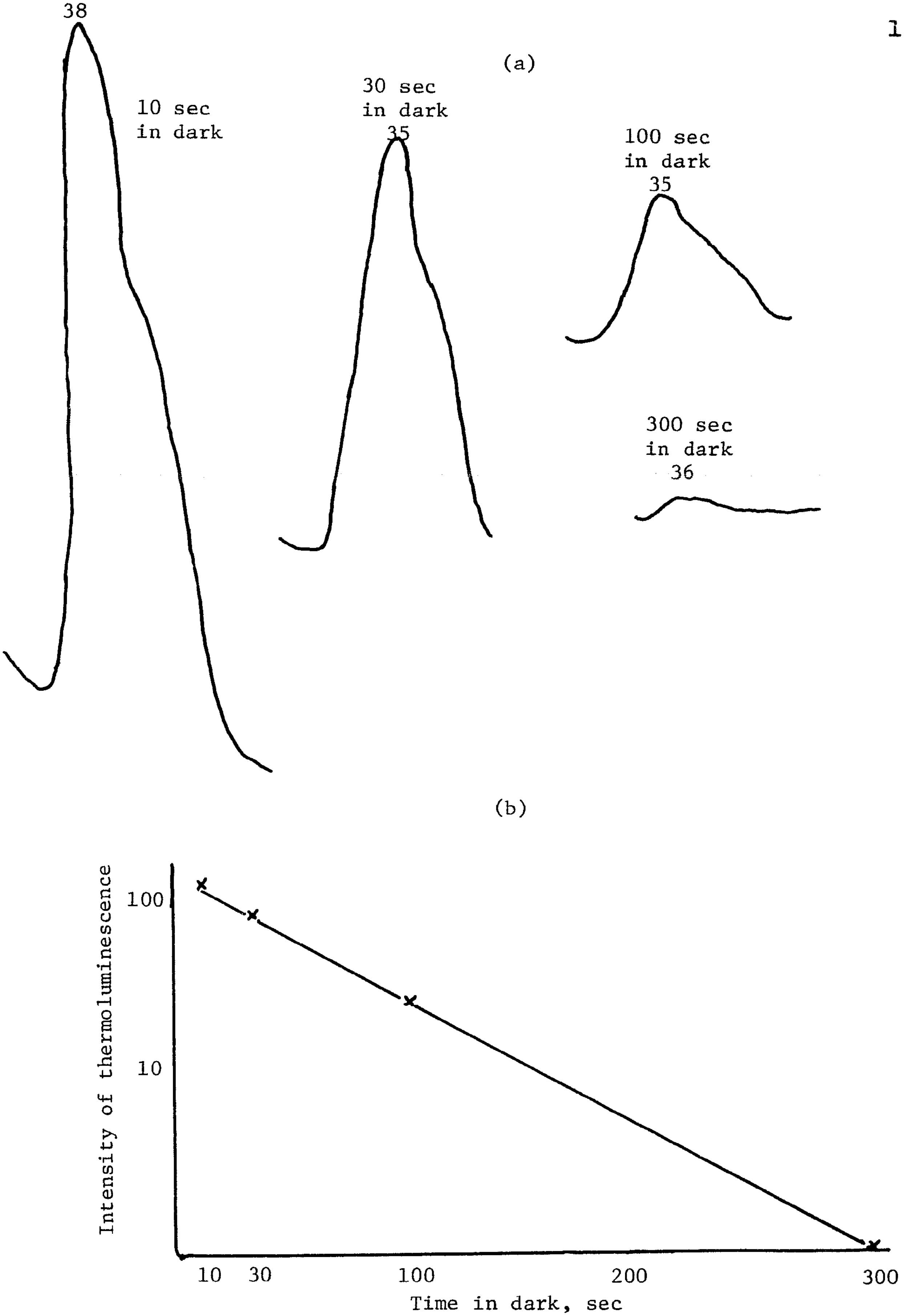

Figure 4-9. Dark decay of thermoluminescence at 0°C. Illumination was for 2 minutes at 0°C. The sample was then kept in the dark at 0°C for the indicated time and then heated. (a) The glow curves after varying times in the dark. (b) The decay of peak 2 plotted on a semilog scale.

from the tracings of the emission profiles. However, the latter state appeared to follow the decay of energy state 2.

2. Discussion

The decrease in the ability of the energy storage states to be charged by illumination at low temperatures as reflected by the decrease in area under the emission envelope has been seen by other investigators. We observed that all three peaks decrease as the sample is illuminated at lower temperatures, but that peak 2 and 3 are still apparent at -196°C while peak 1 is not measurably filled when illumination occurs below -50°C. Arnold (1966) and Rubin and Venediktov (1969) also found that the peak light intensity of Chlorella cells decreased if they were illuminated at lower temperatures. Fleischman (1971) found that the photosynthetic bacterium Rhodopseudomonas viridis emitted no thermoluminescence if the cells were illuminated below -80°C. This phenomenon is also seen in some inorganic semiconductors such as KCl-Tl (Randall and Wilkins, 1945).

The disappearance of peak 1 could be explained on the basis of a thermal step being necessary for charging this energy storage state. Such a large thermal step would not be required for charging the other two energy storage states, since they show less temperature effect. Fleischman (1971) was able to correlate the disappearance of glow peaks in bacteria when illuminated below -80°C with a decrease in light induced P895 oxidation (the bacterial reaction center). However, absorption shifts have not yet been associated with the photosystem II reaction center in the steady state. Witt (1969) observes a shift at 692 nm in flash experiments, which on the basis of decay kinetics can be separated from the shift caused by P700 at that wave length, and which he has identified with the photosystem II reaction center. It is unknown whether at low temperatures this absorption shift would be altered, correlating with a loss of one of the glow peaks.

Light saturation of the energy storage states was found to be dependent upon the temperature at which the sample was illuminated, an effect also reported by Arnold (1966) for Chlorella. Illumination at -196°C gave saturation at 30 seconds while illumination at -77°C took longer than a minute to saturate the emission (Arnold does not distinguish between peaks in this experiment). Our saturation times are somewhat different from the values given above, but the light intensity was not mentioned by Arnold and this may account for the difference.

Reciprocity holds for peak 2 and probably for peak 3, although the data were difficult to measure. State 1 is not charged well by illumination used in the reciprocity experiment. In fact it was found that state 1 was not measurable if the time of full illumination was shorter than one second. Less than full intensity of illumination for one second also did not allow for appreciable filling of the state. A shorter flash of light--the briefest tried was 0.005 sec--caused significant filling of energy states two and three but not of state 1.

The fact that untrapping does not occur during illumination at low temperatures is reflected in the time it takes for the peaks to decay in the dark. The decay of state 2 at 0°C is exponential and takes 58 seconds to reach 37% of its original height. At lower temperatures it takes appreciably longer. At -25°C peak 2 was found to take 27 minutes to reach 50% of its original height. Rubin and Venediktov (1969) also found that the peak emission intensity of *Chlorella* cells did not depend on how long after illumination the sample was held in the dark at -50°C. They were able to wait a number of minutes after illumination before heating with no noticeable effect on the emission intensity.

D. Effect of treatments inhibiting photosystem II

1. Tris aging

Figures 4-10 and 4-11 compare the thermoluminescence of tris aged and untreated chloroplasts in the presence and absence of electron donors and acceptors. Tris aging decreases peak 2 and 3 to 10% of the height of the untreated sample and makes it impossible to distinguish them separately. Peak 1, however, is enhanced threefold. Addition of

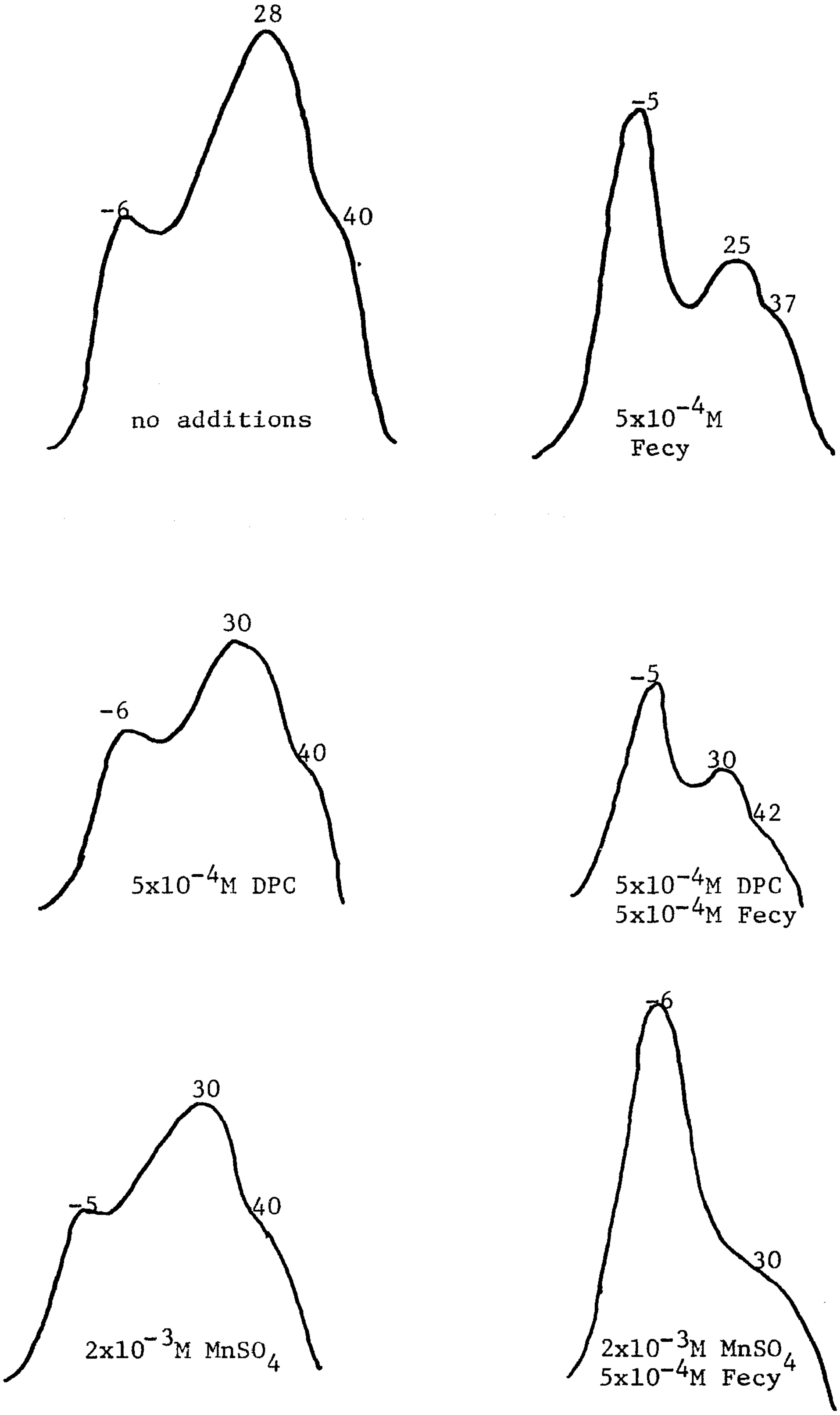

Figure 4-10. Effect of artificial electron donors on thermoluminescence of untreated chloroplasts in the absence and presence of ferricyanide.

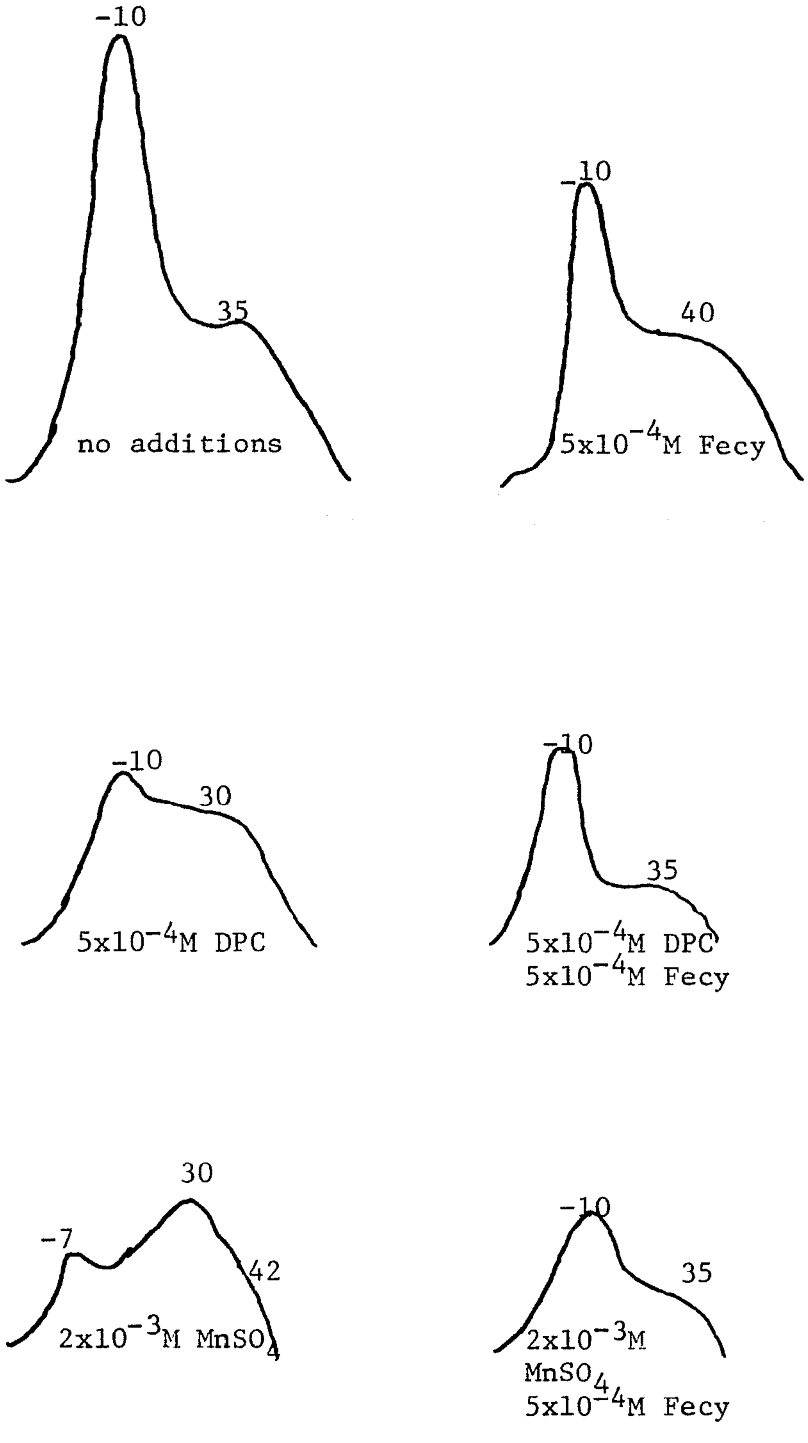

Figure 4-11. Effect of tris aging on thermoluminescence in the presence and absence of ferricyanide and artificial electron donors.

ferricyanide has opposite effects on peak 1 of the two types of chloroplasts: it enhances peak 1 in untreated chloroplasts while diminishing peaks 2 and 3; but decreases peak 1 in tris treated chloroplasts, while not affecting peaks 2 and 3.

Two electron donors were utilized, DPC and $MnSO_4$. Both restored ferricyanide reduction to 45 - 65% of the control after tris aging had inhibited it to 8% of the control. Both donors had some effect on untreated chloroplasts. They decreased the total emission from all three peaks, but did not change the shape of the emission curve. These untreated chloroplasts were also used as controls for heat and ultraviolet irradiated chloroplasts (compare figures 4-13 and 4-16 to 4-10). Peak 1 in tris treated chloroplasts is inhibited four fold by the presence of DPC, while peaks 2 and 3 are unaffected by DPC. The addition of ferricyanide restores a small amount of peak 1, and the two emission profiles of DPC or $MnSO_4$ plus ferricyanide in tris aged or untreated chloroplasts look very similar.

The effect of manganese sulfate is shown in figures 4-10 and 4-11. Its effect on untreated chloroplasts is to decrease all peaks slightly, but not to change the shape of the emission profile. When added to tris treated chloroplasts, $MnSO_4$ decreases peak 1 two fold, and does not measurably change peaks 2 and 3. In the presence of ferricyanide and manganous ion, peak 1 is again increased and peaks 2 and 3 decreased. However, these effects are smaller than in untreated chloroplasts.

We may summarize: inhibition of photosystem II by incubating chloroplasts in high molarity tris-HCl results in reduction of peaks 2 and 3 to 10 - 20% of their untreated value, while peak 1 is enhanced 50 - 100%. Restoring photochemistry by the addition of an electron donor attenuates the height of peak 1, but does not affect the other two peaks. Addition of ferricyanide again increases peak 1 and decreases peaks 2 and 3. The emission curves of the donors plus ferricyanide in the treated chloroplasts are similar to those of untreated chloroplasts, but smaller in magnitude.

2. Heat treatment

Heating chloroplasts to 50°C for increasing lengths of time progressively inhibits photosystem II photochemistry. Thermoluminescence also exhibits a progressive alteration as the time of heating is increased, as demonstrated in figure 4-12. The general effect is the same as that of tris aging; peak 1 is enhanced and peaks 2 and 3 are diminished. The low temperature peak exhibits twofold enhancement compared to the control or untreated chloroplasts. Peaks 2 and 3, on the other hand, decrease with the inhibition of photochemistry. This is shown in figure 4-12b.

The effect of artificial electron donors on heated chloroplasts in the presence or absence of the electron acceptor ferricyanide is similar to their effect on tris treated chloroplasts (compare figure 4-11 and 4-13).

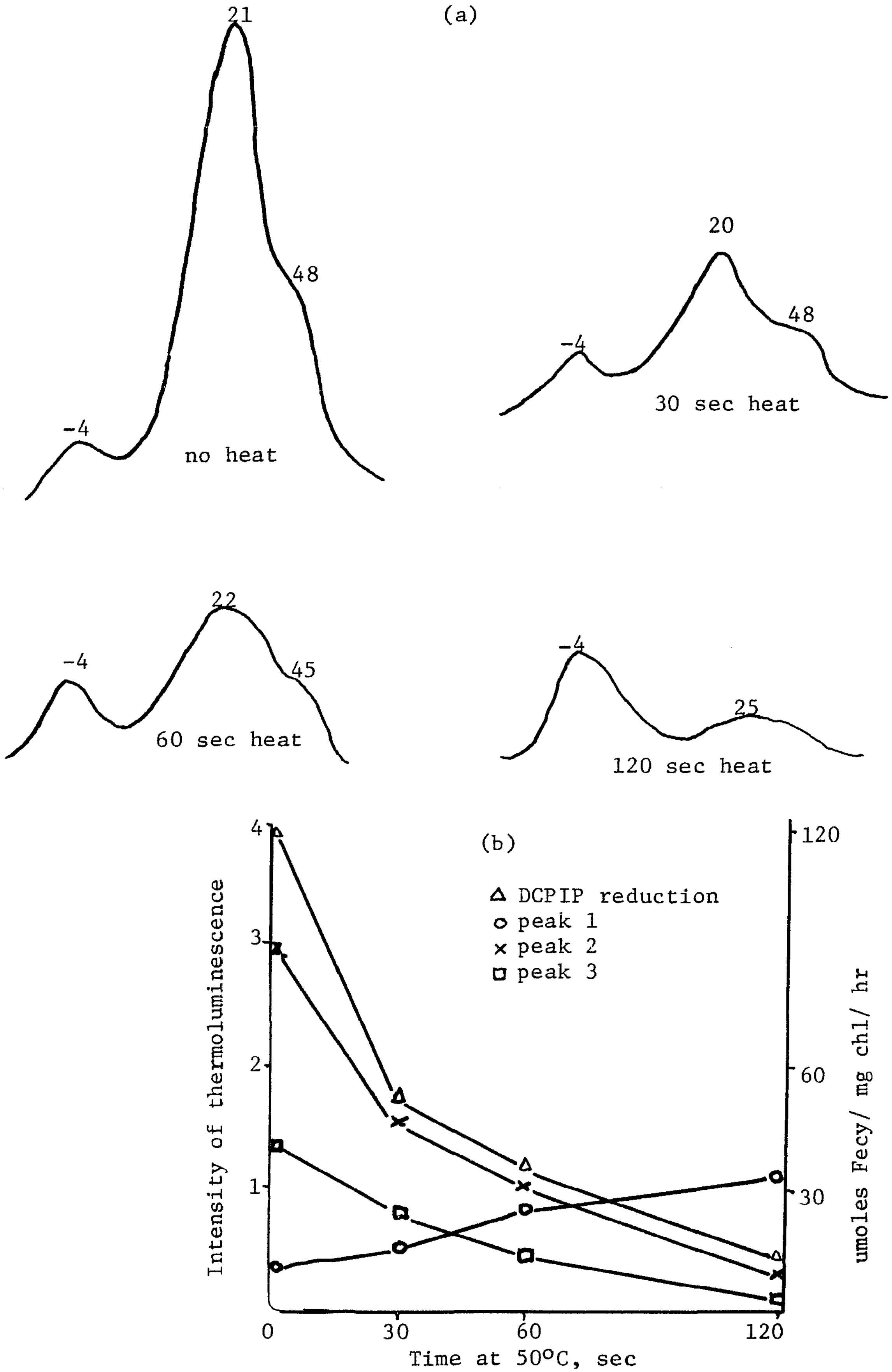

Figure 4-12 Effect of heating chloroplasts to 50°C on thermoluminescence. (a) The glow curves of chloroplasts heated for varying times. (b) The heights of the three peaks and the rate of photochemistry as a function of increasing times of heating.

no additions

$5x10^{-4}$M Fecy

$5x10^{-4}$M DPC

$5x10^{-4}$M DPC
$5x10^{-4}$M Fecy

$2x10^{-3}$M $MnSO_4$

$2x10^{-3}$M $MnSO_4$
$5x10^{-4}$M Fecy

Figure 4-13. Effect of heat on thermoluminescence in the presence and absence of ferricyanide and artificial electron donors. Heating was for 120 sec at 50°C.

Manganous sulfate and DPC both decrease peak 1 two to threefold while not affecting, or slightly enhancing, peaks 2 and 3. The addition of ferricyanide in the absence of an electron donor also decreases peak 1, but not more than 50%. In the presence of an electron donor ferricyanide increases peak 1 and decreases peaks 2 and 3, the same effect it shows in untreated chloroplasts.

3. Ultraviolet irradiation

Thermoluminescence of chloroplasts irradiated with UV light for varying times is seen in figure 4-14. As in tris aged and heated chloroplasts, peak 1 is enhanced by UV irradiation. Peaks 2 and 3 decrease progressively with longer times of exposure and at seven minutes of UV light are 20% of the height of these peaks in untreated chloroplasts. This length of irradiation inhibits photochemistry 95% (figure 4-14b). The inhibition of photochemistry does not follow the decrease in peaks 2 and 3 as well in the case of UV irradiation as it does in heat treatment (compare figure 4-12b and 4-14b). After three minutes irradiation peaks 2 and 3 are diminished 50% while ferricyanide reduction is unaffected.

The effect that artificial electron donors have on the thermoluminescence of UV irradiated chloroplasts is identical to their effects on heated and tris treated chloroplasts (figure 4-15). Peak 1 is decreased threefold while peaks 2 and 3 are unchanged. When ferricyanide is

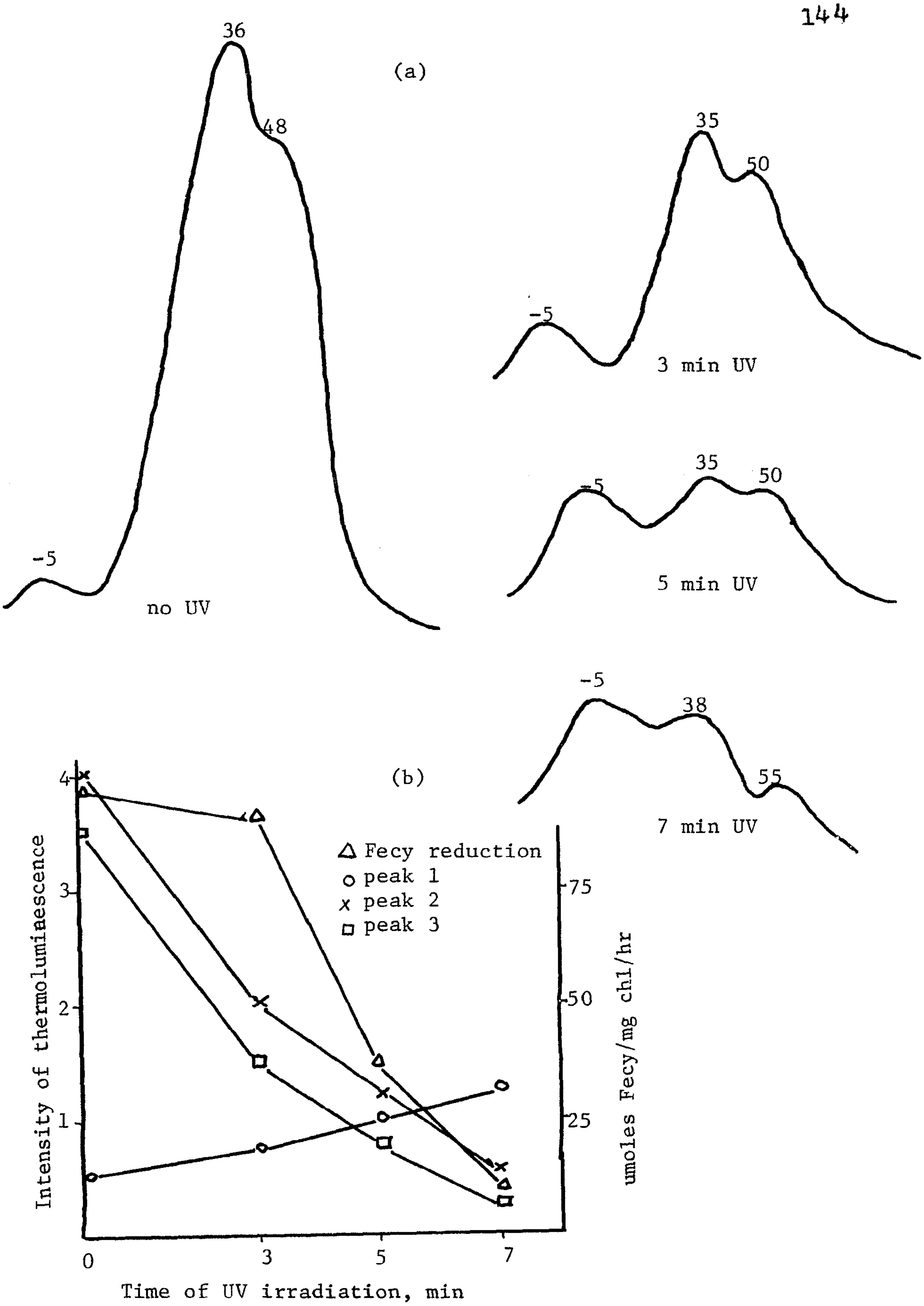

Figure 4-14. Effect of UV irradiation on thermoluminescence of chloroplasts. (a) Thermoluminescence from chloroplasts irradiated for varying times. (b) The effect of increasing time of UV irradiation on the height of the thermoluminescent peaks and on ferricyanide reduction.

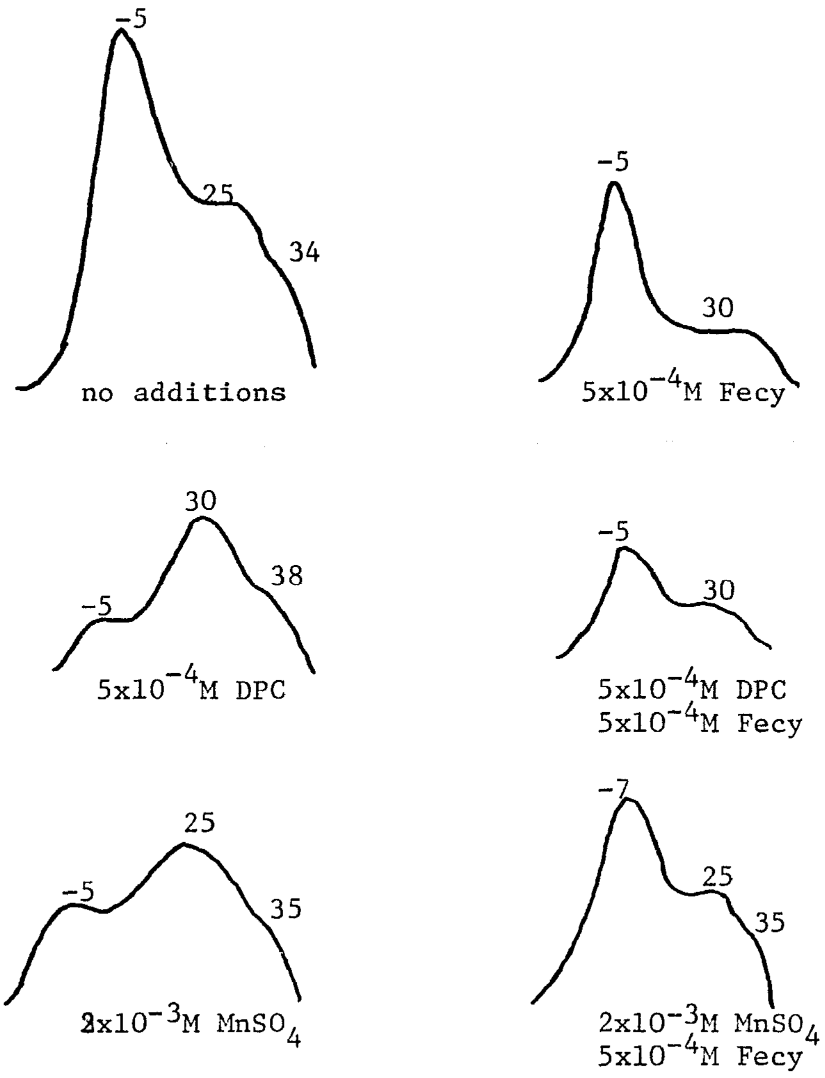

Figure 4-15. The effect of 5 min UV irradiation on thermoluminescence of chloroplasts in the absence and presence of ferricyanide and electron donors.

added to the treated chloroplasts in the presence of the donors peak 1 is enhanced while peaks 2 and 3 are decreased.

4. Chloride deficiency

Chloroplasts which had been depleted of chloride were examined to determine how their thermoluminescence emission profile differed from untreated chloroplasts. The effect on the glow curves of the addition of NaCl, which restores normal electron donation from water, $MnSO_4$ and DPC, which act as an electron donor replacing water, was also examined in both regular and chloride depleted chloroplasts. The results of this examination are detailed in figures 4-16 and 4-17.

The effect of DPC and $MnSO_4$ on untreated chloroplasts has been discussed previously (figure 4-10). The total intensity of the three peaks is decreased but the shape of the emission curve is unchanged. NaCl has no effect on untreated chloroplasts in the absence or presence of ferricyanide.

Chloride depletion (like the other three treatments) enhances peak 1 and decreases peaks 2 and 3 (compare figures 14 -16 and figure 4-17). In this case there was a threefold increase in peak 1 and a 50% decrease in peaks 2 and 3. Ferricyanide decreases this enhancement of peak 1. In chloride depleted chloroplasts NaCl restores the glow profile nearly to that of untreated chloroplasts, and these restored chloroplasts show a profile in the presence of

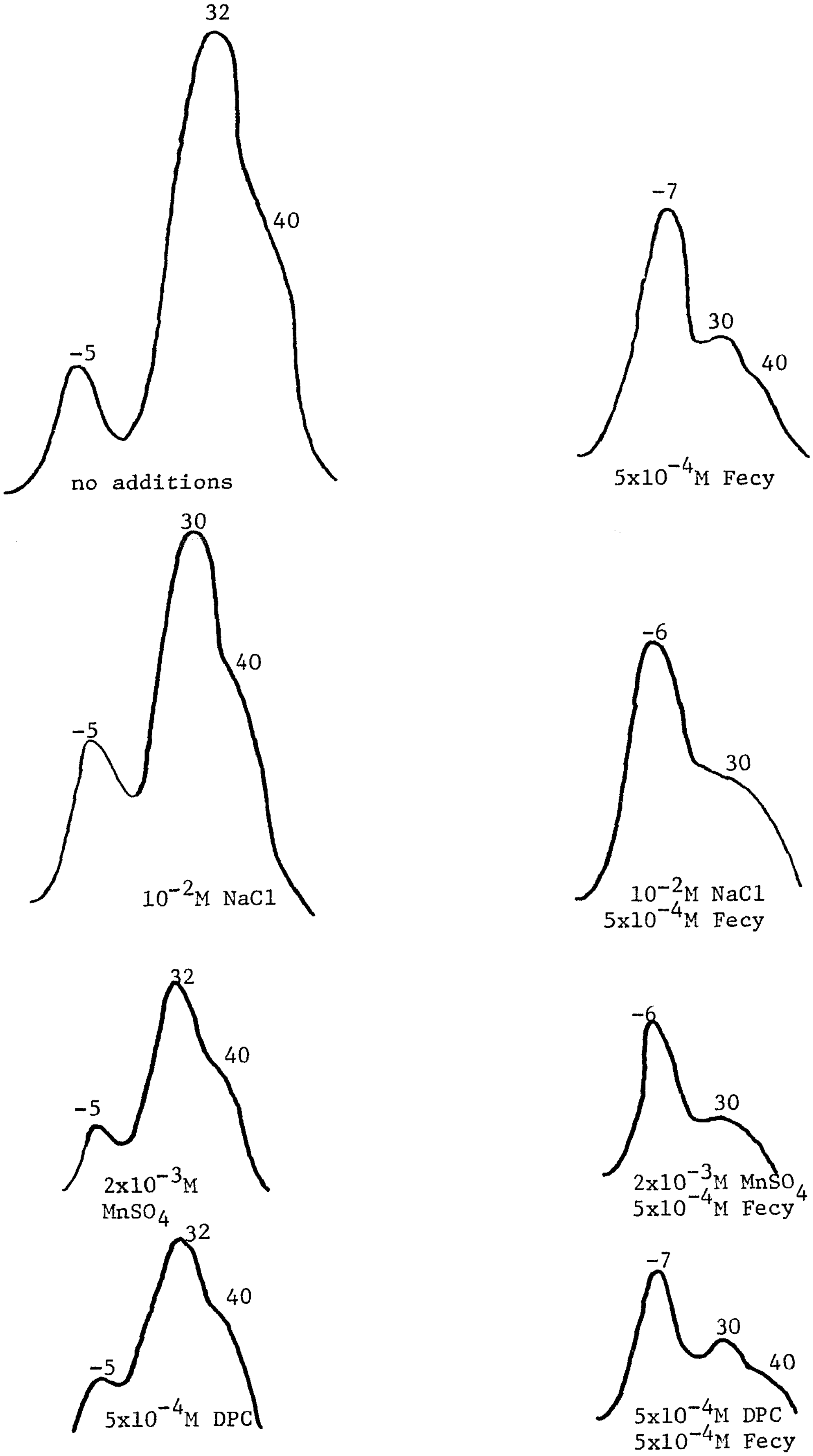

Figure 4-16. The effect of electron donors in the presence and absence of ferricyanide on thermoluminescence of chloroplasts.

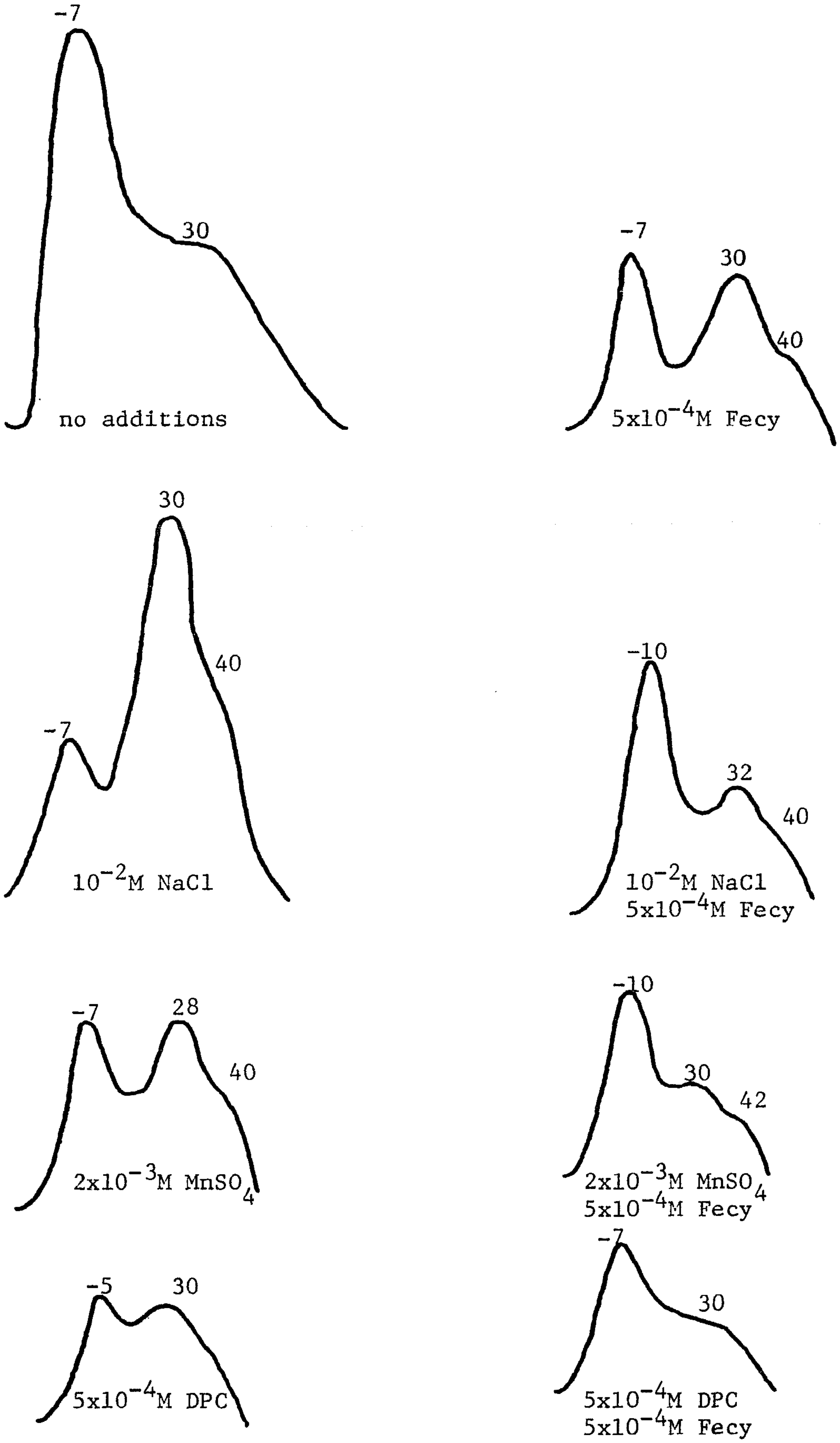

Figure 4-17. The effect of chloride deficiency on thermoluminescence of chloroplasts in the presence and absence of ferricyanide and electron donors.

electron acceptors which is almost like that of untreated chloroplasts. Manganese and DPC have the same effect as on the other treated chloroplasts.

5. Discussion

The four treatments which inactivate photosystem II examined here have similar effects on thermoluminescence. Peak 1 is either increased two to threefold, while peaks 2 and 3 are decreased four to fivefold. In examining the acceptorless delayed light of chloroplasts which had been subjected to these various treatments, it was observed that they all exhibited rapid decay kinetics in the msec time range. This was interpreted as indicating that the treatments induced a wasteful cycle, which dissipated stored energy at a rate which successfully competed with the normal photosynthetic electron transport pathway. The reaction center of photosystem II was functional, in that charge was separated, but this charge was recombining in the cycles induced by the inhibitory treatments.

The thermoluminescence data can be interpreted in a manner which is consistent with this hypothesis of the mode of action of the treatments. An increase in peak 1 and a decrease in peaks 2 and 3 appears empirically to indicate the occurrence of photochemistry, since electron acceptors have similar effects on untreated chloroplasts. The treatments produce a similar emission profile to that observed on activation of a Hill reaction. Therefore, we conclude that photochemistry is occurring in these cases too. Of course, after the treatments the photochemistry does not lead to end products such as oxygen and reduction of a Hill acceptor. Thus, on empirical grounds the thermoluminescent measurement of treated systems implies that treatments induce a useless cyclic recombination which leads to no net photoreduction or photooxidation.

The addition of electron donors does not restore the emission profile of the treated chloroplasts to one resembling untreated chloroplasts. However, chloride (in the case of chloride depleted chloroplasts) does restore thermoluminescence as well as restoring oxygen evolution. Possibly it is necessary to have a fully competent photosystem II to have the normal thermoluminescence profile in the absence of acceptors. Thus the thermoluminescence data indicate that although artificial electron donors may restore photochemistry, they do not completely reverse the effects of the treatments. In this sense thermoluminescence is a more sensitive measurement of the state of photosystem II than photochemistry. Similar conclusions were independently drawn from the msec delayed light data.

E. Effect of uncouplers on thermoluminescence

1. Results

a. Ionophorous uncouplers

Figure 4-18 and Table 4-1 demonstrate the effect

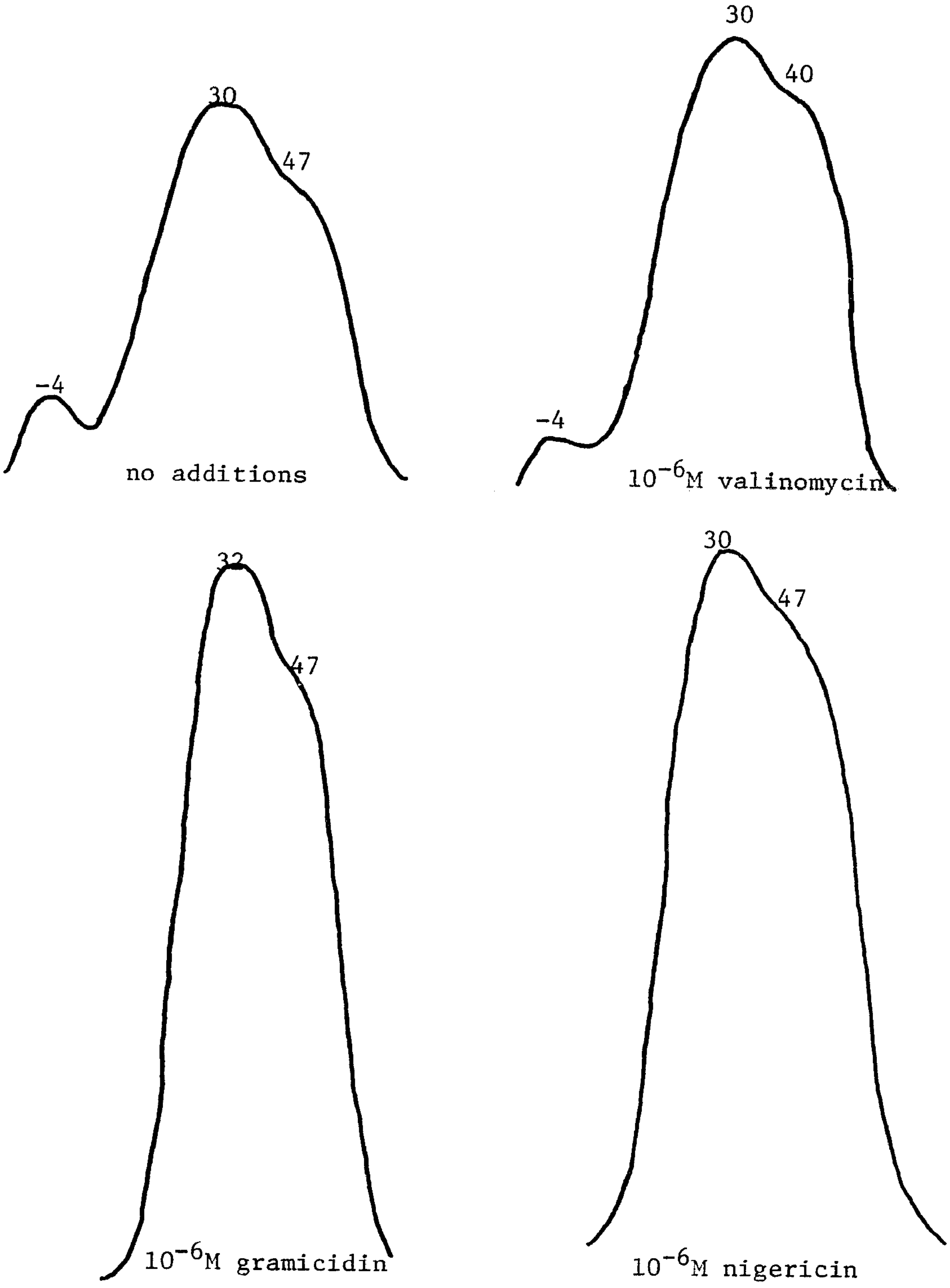

Figure 4-18. The effect of antibiotic uncouplers on thermoluminescence.

Conditions	ueq FeCy/ mg chl/hr	peak height 1	2	3
no addition	84	0.6	3.0	2.7
+ gramicidin (10^{-6}M)	318	0.0	6.5	4.5
+ nigericin (10^{-6}M)	318	0.0	7.0	4.5
+ valinomycin (10^{-6}M)	90	0.2	3.7	3.4
CQP (10^{-4}M)	228	0.0	4.0	3.5
NH_4Cl (10^{-2}M)	276	0.0	5.5	3.5
CCCP (10^{-6}M)	150	0.0	0.0	0.0

Table 4-1. The effect of uncouplers on thermoluminescence emission and on ferricyanide reduction.

that uncouplers have on thermoluminescence of chloroplasts. The antibiotic uncouplers gramicidin and nigericin inhibit the charging of state 1 and increase the height of peaks 2 and 3. Valinomycin, which makes the membrane more permeable to ions but does not act as an uncoupler in the steady state, did not affect peaks 2 and 3 but did inhibit peak 1 60%.

The emission profile of chloroplasts in the presence of these uncouplers is similar to that caused by the photosynthetic poison DCMU. However, addition of an electron acceptor does not alter the emission profile of DCMU treated chloroplasts (figure 4-2), while ferricyanide does alter the glow curve of gramicidin treated chloroplasts (figure 4-19). Peak 1 is increased and peaks 2 and 3 decreased by the addition of ferricyanide to gramicidin uncoupled chloroplasts. As may be seen in Table 4-1 ferricyanide reduction in chloroplasts uncoupled with gramicidin is increased more than threefold compared to control chloroplasts.

b. Amine uncouplers

The effect of this class of uncouplers on thermoluminescence is the same as that of the antibiotic uncouplers; peak 1 is inhibited and peaks 2 and 3 are enhanced. This is shown in figure 4-20 and Table 4-1 for the uncouplers ammonium chloride and chloroquine phosphate. The addition of ferricyanide to ammonium chloride restores peak 1 in a

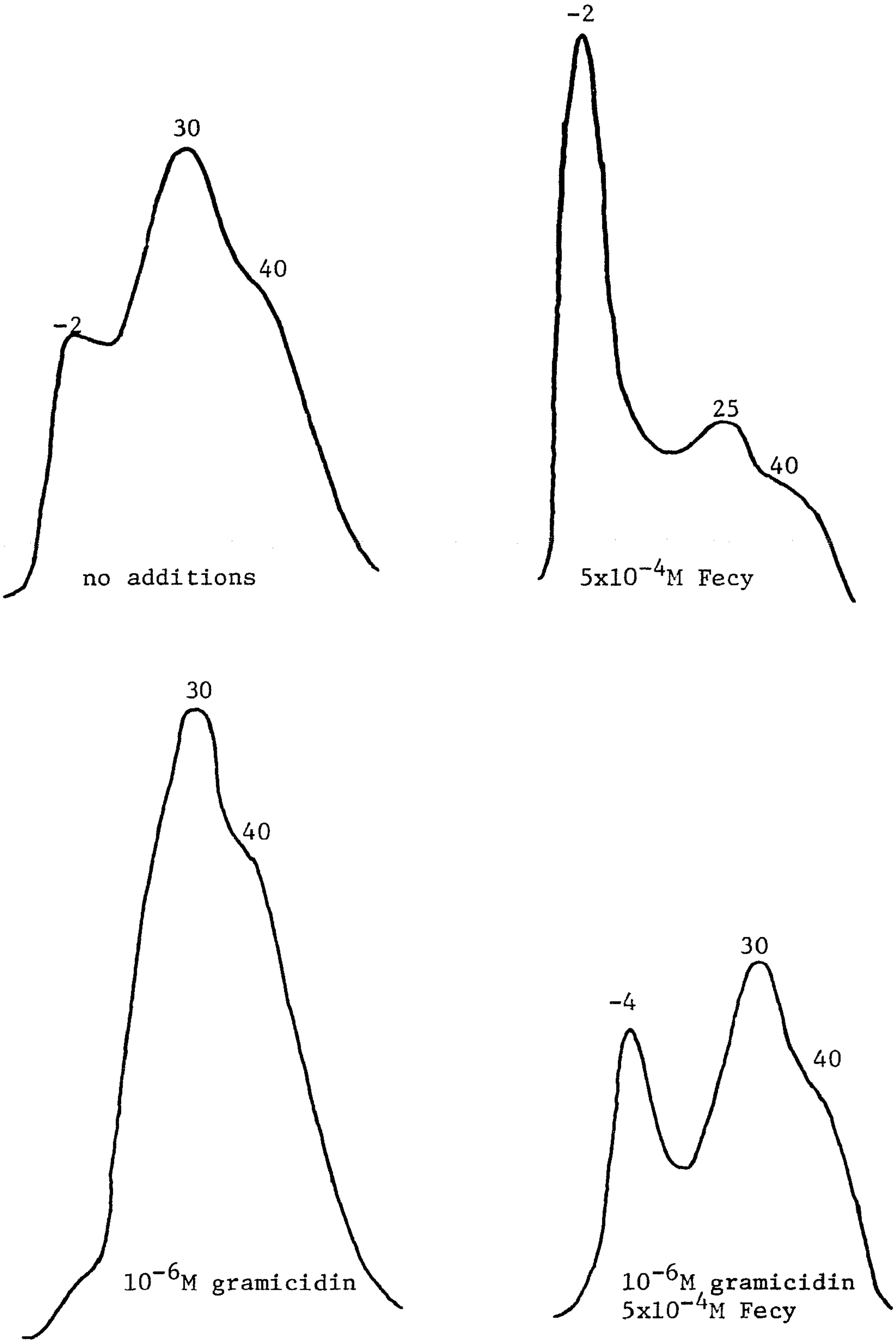

Figure 4-19. The effect of gramicidin on thermoluminescence in the absence and presence of ferricyanide.

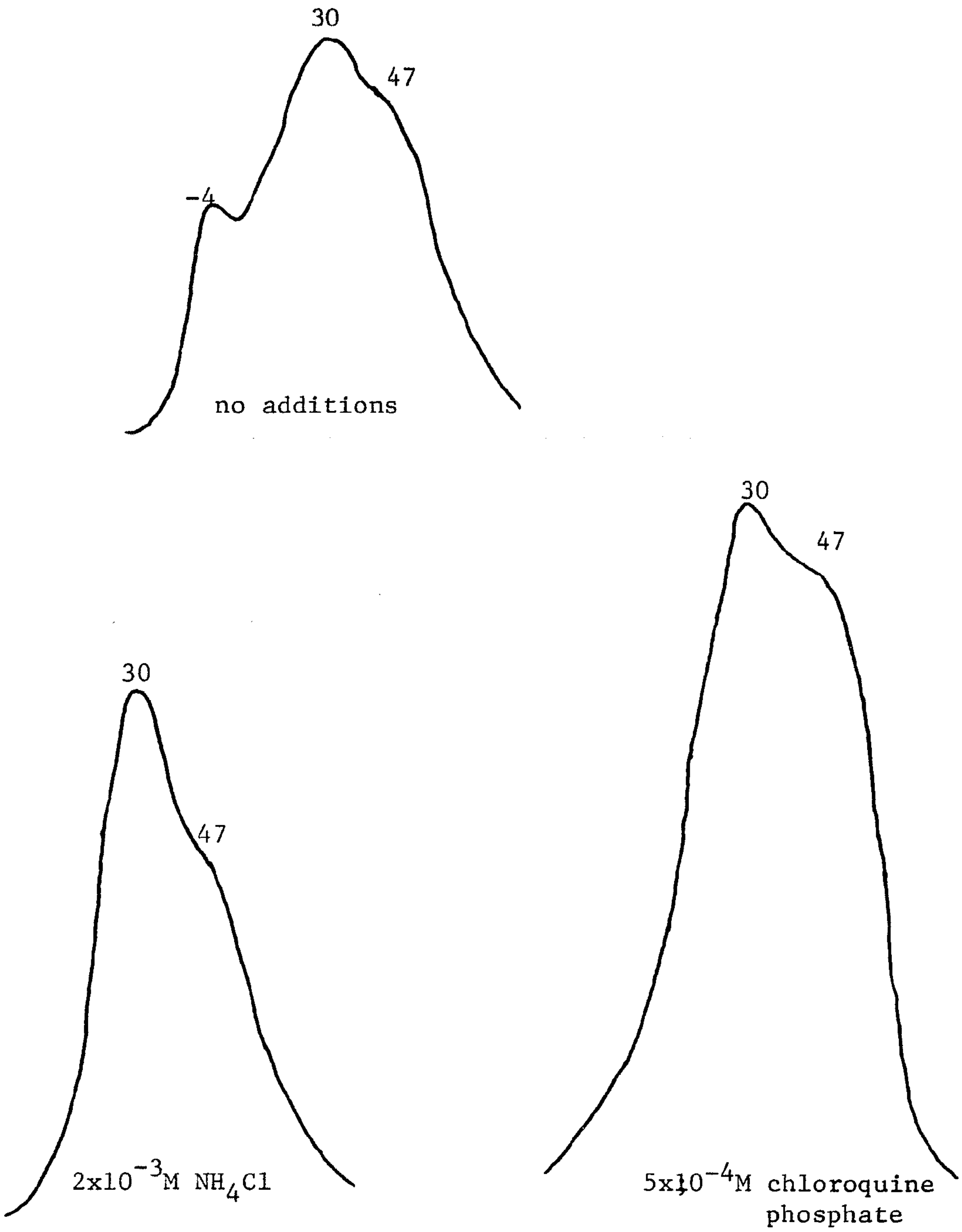

Figure 4-20. Effect of amine uncouplers on thermoluminescence of chloroplasts.

similar manner as it does with gramicidin present (compare figure 4-19 and 4-21).

c. CCCP

This uncoupler exhibits a different effect on thermoluminescence than the other compounds examined thus far. It uncouples over a narrow concentration range from 10^{-6} to 10^{-5} M. In this concentration range all the peaks are diminished and the addition of ferricyanide inhibits thermoluminescence still further (see figure 4-22). Moreover, CCCP has an effect on the glow curve at concentrations below those that affect photochemistry, as is demonstrated in figure 4-22. 10^{-8} M CCCP decreases all three peaks to some extent, and partially inhibits the ferricyanide response, while there is no effect on photochemistry. This was seen in the delayed light data also, where CCCP decreased acceptorless delayed light at a concentration where it did not affect photochemistry.

We feel that CCCP has several actions, and that further work is needed to interpret this data.

2. Discussion

All the uncouplers examined with the exception of CCCP decreased peak 1 and increased peaks 2 and 3 in the absence of Hill acceptors. These results are similar to those obtained in the presence of the photosynthetic poison DCMU. The effect of DCMU on the reaction center is to block exit of charge while uncouplers facilitate

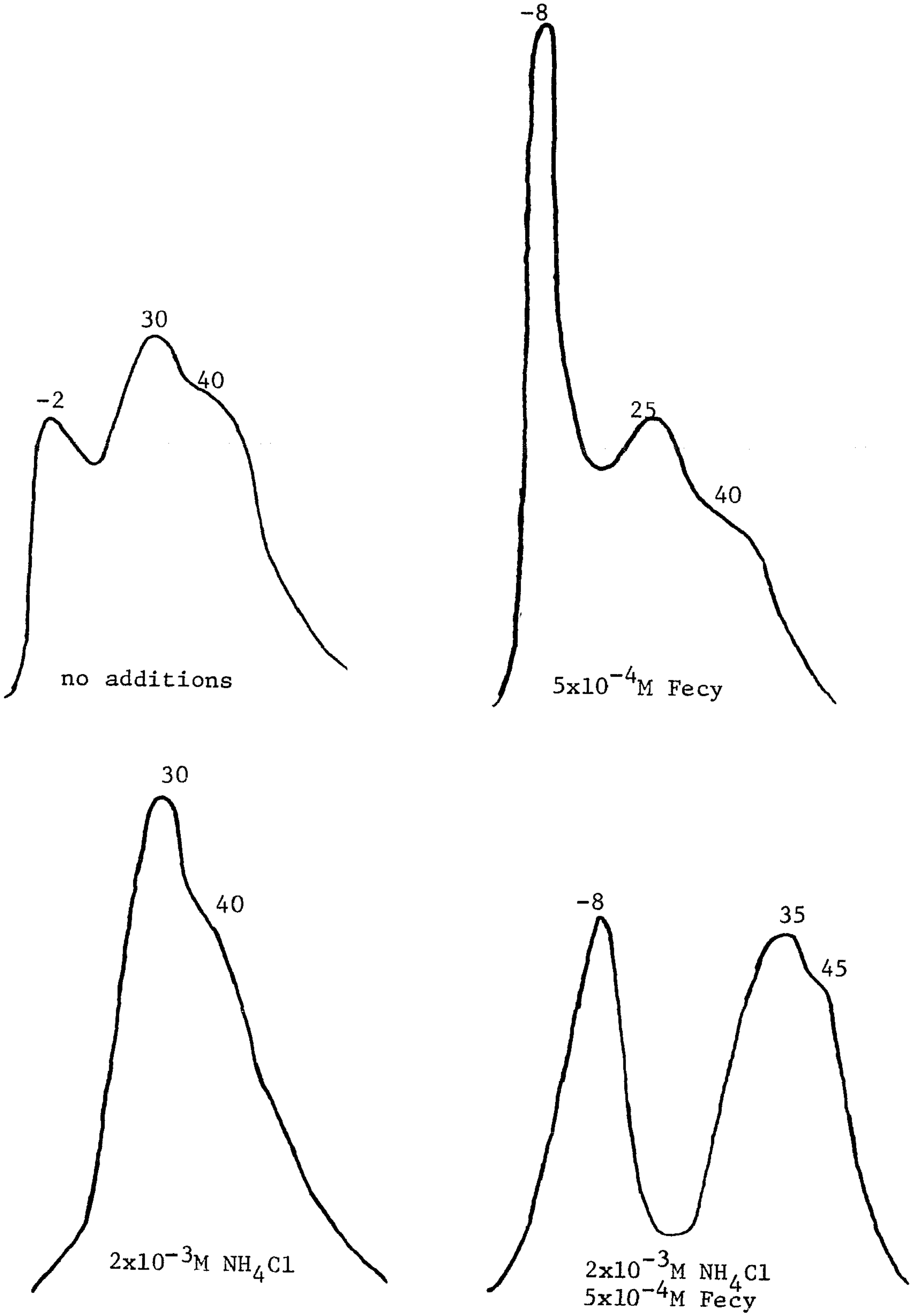

Figure 4-21. Effect of NH_4Cl on thermoluminescence in the absence and presence of ferricyanide.

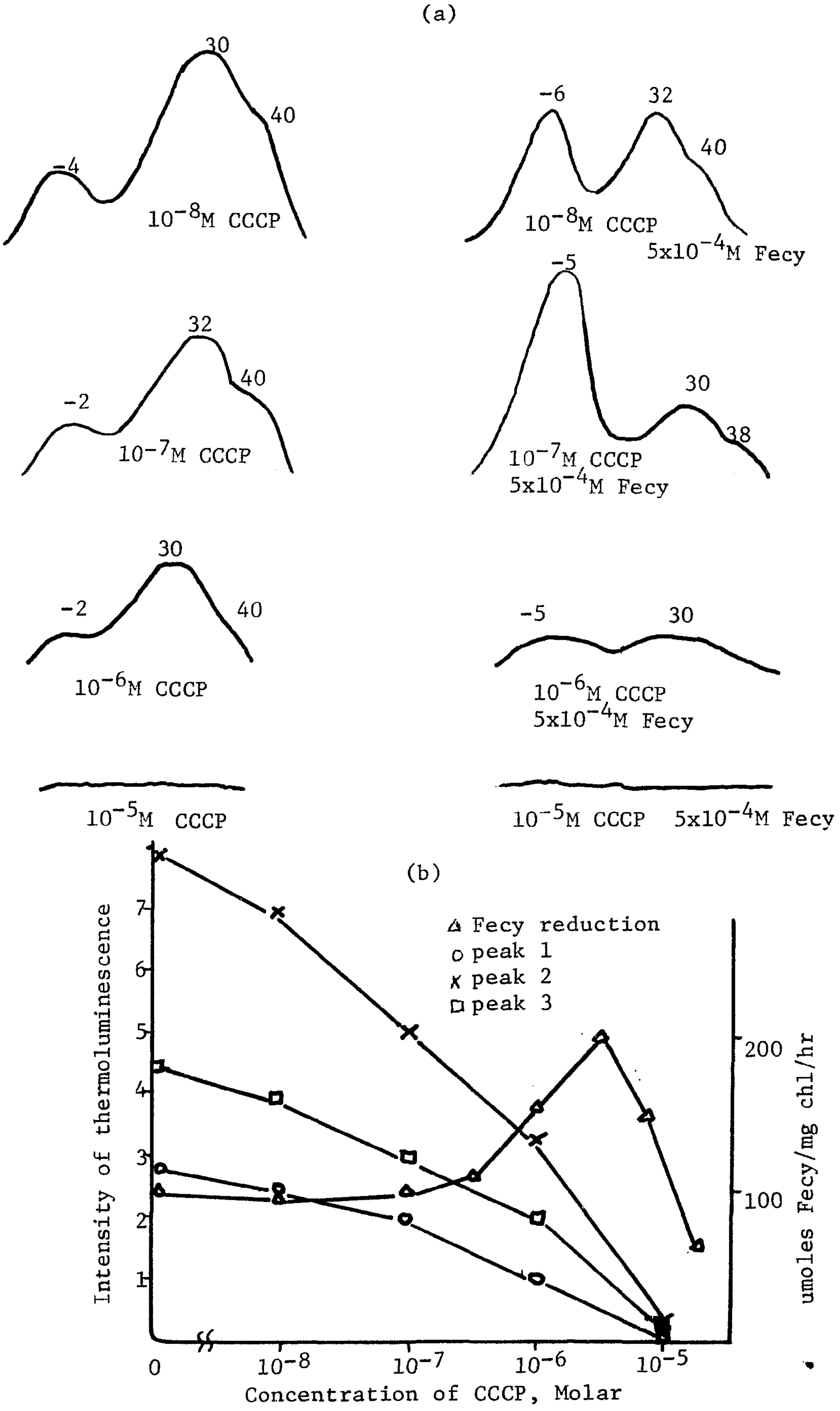

Figure 4-22. The effect of increasing concentrations of CCCP on thermoluminescence in the absence and presence of ferricyanide. (a) The glow curves in the presence of CCCP ± Fecy. (b) The peak heights - Fecy and Fecy reduction plotted as a function of CCCP concentration.

exit of charge, and therefore the similarity in the thermoluminescence emission profiles may be a result of two different modes of action. The effect that Hill acceptors have on thermoluminescence in the presence of uncouplers is to restore peak 1. In the presence of DCMU Hill acceptors have no effect on the emission profile. The percent increase in peak 1 caused by ferricyanide in the presence of uncouplers is much greater than in the absence of uncouplers (0.5 to 3 times increase without uncouplers compared to 10 -20 times increase in height with uncouplers), just as the amount of ferricyanide reduced is greater in the presence of uncouplers.

F. Conclusions

Data from the *Scenedesmus* mutants support the view that it is photosystem II which is the emitter of thermoluminescent glow curves. *Scenedesmus* mutant 8, which is deficient in photosystem I, has all three glow peaks, while mutant 11, which has no functional photosystem II, shows none of these peaks.

Peaks 2 and 3 apparently reflect energy storage sites within the reaction center of photosystem II. They are filled at liquid nitrogen temperature, and they are filled by the shortest flash we were able to give the sample (5 msec), both of which might be taken as indications of a primary photoact. They are enhanced by DCMU (which blocks exit of stored charge into the electron transport chain), and reduced by ferricyanide (which facilitates exit of charge into the electron transport chain). Peaks 2 and 3 are also reduced by treatments which induce an artificial side reaction around photosystem II (tris poisoning, heat, UV irradiation, chloride depletion). Uncouplers enhance these two peaks, with the exception of CCCP which inhibits all three peaks.

Storage state 1 has different characteristics than the other two states. It does not appear to be part of the primary photoact, since it is not measurable if the sample is illuminated for times shorter than one second at full intensity (2.5×10^5 ergs/cm^2/sec). It is also not apparent if illumination occurs below -50°C. It may be a storage state within the reaction center, but one which requires a conformational change brought about by illumination of the chloroplasts to enable it to be charged. This would not occur at low temperatures, as shown by the lack of peak 1 when illumination is below -50°C. Alternatively this storage state may originate in some component of the electron transport chain which would not be filled if the chloroplasts were frozen. At present we are unable to eliminate either of these hypotheses.

Compounds and treatments which alter the functioning ability of photosystem II have a different effect on state 1 than on state 2 and 3. Peak 1 is eliminated by DCMU and enhanced by Hill reagents. It is increased by treatments which establish a cycle around photosystem II, and decreased by artificial electron donors which allow normal photochemistry to compete with this cycle. Uncouplers also inhibit peak 1 in the absence of electron flow.

Certain correlations can be drawn between changes in delayed light and changes in thermoluminescence. In general, treatments of the chloroplasts which cause rapid delayed light decay kinetics also cause greater charging of state 1. The presence of electron acceptors and the four treatments examined are examples of this. Treatments which cause a lowering of msec delayed light without significantly affecting the decay kinetics also cause inhibition of peak 1. The photosynthetic poison DCMU and the uncouplers examined show this effect.

The thermoluminescence data in this study are consistent with the two sided electron-hole model of the reaction center developed by Bertsch (1969), if we accept the interpretation that peak 1 is outside the reaction center. Initially the presence of three peaks was difficult to explain, since Bertsch's model envisions two distinct storage sites, one for electrons and one for holes. However, peak 1 is not the result of a primary photoact, the presence of peaks 2 and 3 in the reaction center of photosystem II is in agreement with the idea that the reaction center may have an oxidizing side spatially separated from a reducing side.

If peak 1 is within the reaction center, modifications of Bertsch's model are necessary. This will be discussed in more detail at the end of the next chapter.

CHAPTER V: THEORETICAL TREATMENT OF THERMOLUMINESCENCE

A. Semiconductors

The energy levels for a simple atom such as an isolated hydrogen atom can be represented as in figure 5-1. The curved lines represent the potential energy of the electron in the electric field produced by the positive nucleus. The various n's are the possible energy levels of the electron which can be obtained exactly by solving the Schrodinger wave equation. A similar potential well can be drawn for an atom with more electrons. Figure 5-2 shows the potential well for a silicon atom, an element often used in semiconductors. Each energy level can contain at most two electrons, of opposite spin. In this case the picture is more complex since each electron is influenced by the repulsion of all the other electrons. Thus, while the innermost electrons see an attractive potential from a nuclear charge of approximately +14, the outer electrons see an attractive potential corresponding more nearly to a charge of +1. However, in all cases the electrons are in distinct energy levels.

These ideas can be extended to describe the energy levels in a silicon crystal. In this case there are a tremendous number of electrons, perhaps more than 10^{20} even in a small crystal. The energy levels are derived in essentially the same manner as before--the wave equation is

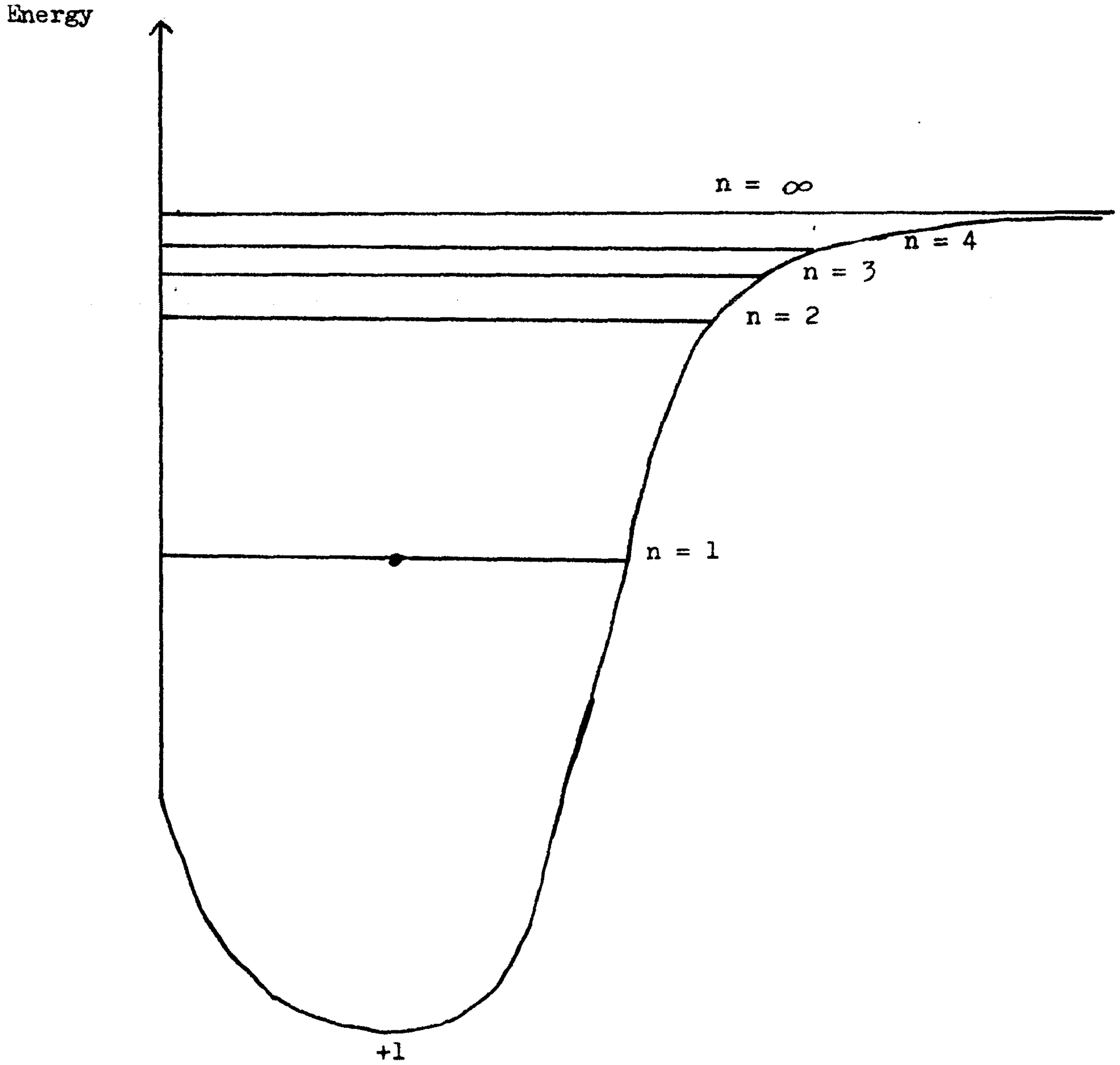

Figure 5-1. Energy levels for a hydrogen atom.

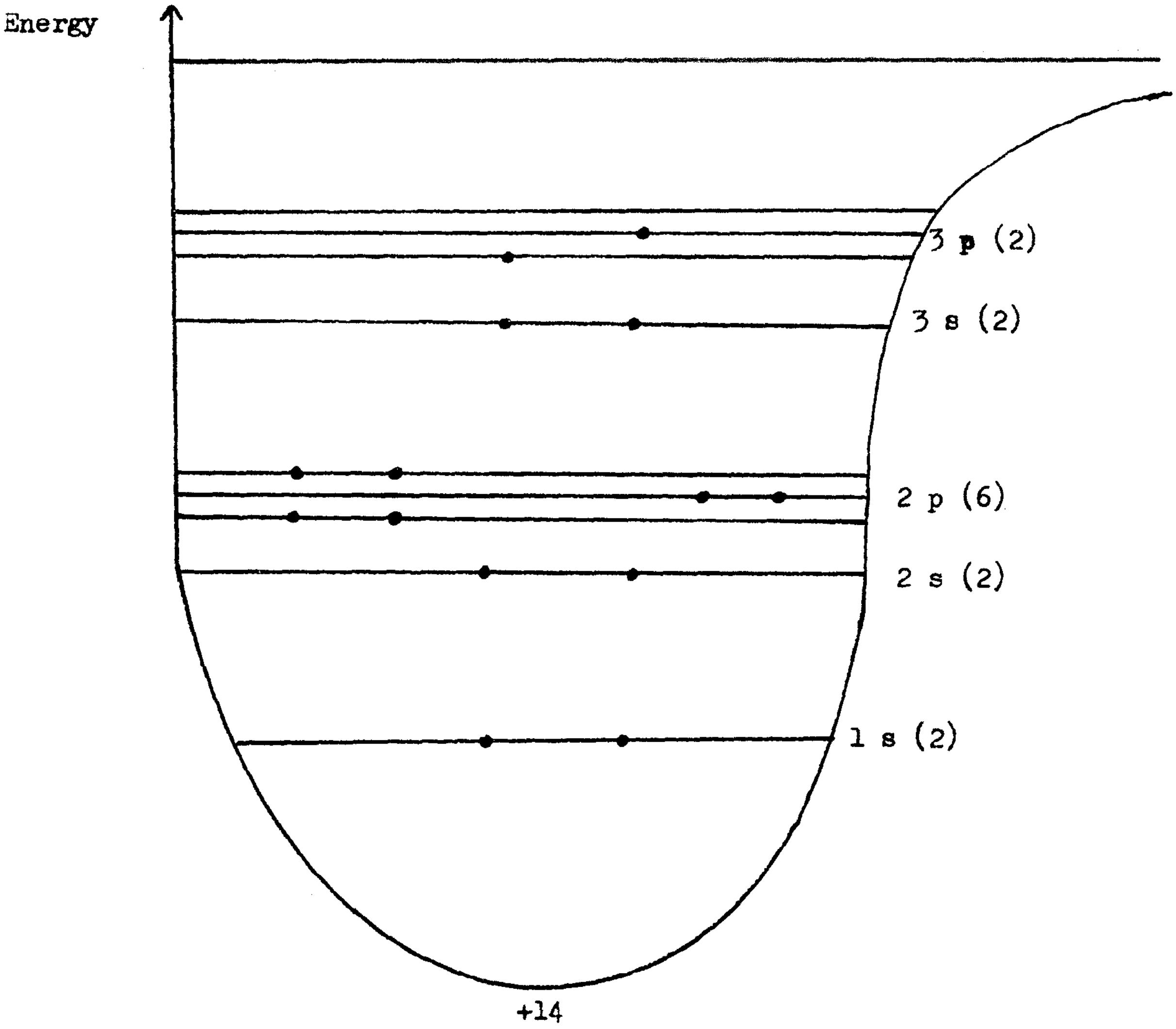

Figure 5-2. Energy levels for a silicon atom.

solved using the appropriate attractive potential. The solution of the wave equation for outer electrons in a periodic potential field results in a series of permissible energy bands, rather than the discrete energy levels of isolated atoms. These energy bands of a silicon crystal are indicated in figure 5-3. The highest energy band which contains electrons is called the valence band; in this case the valence band is completely filled by the four valence electrons of each silicon nucleus. The next highest energy band, which contains no electrons, is referred to as the conduction band. The energy separation between these two bands is called the energy gap. Thus, the electronic properties of a crystal can be represented by energy bands that extend throughout the entire crystal.

The electrical and optical properties of the crystal are determined by the magnitude of the energy gap, in much the same manner as the absorption spectra of simple molecules are determined by the energy differences between the ground state and the excited states. The magnitude of the energy gap depends on the atoms that make up the crystal as well as the crystal structure. If the energy gap is zero, the crystal is an electrical conductor (such as copper). An electrical insulator has a large energy gap (diamond). The intermediate values of energy gap are classified as semiconductors.

Conductivity in an electric field requires a band

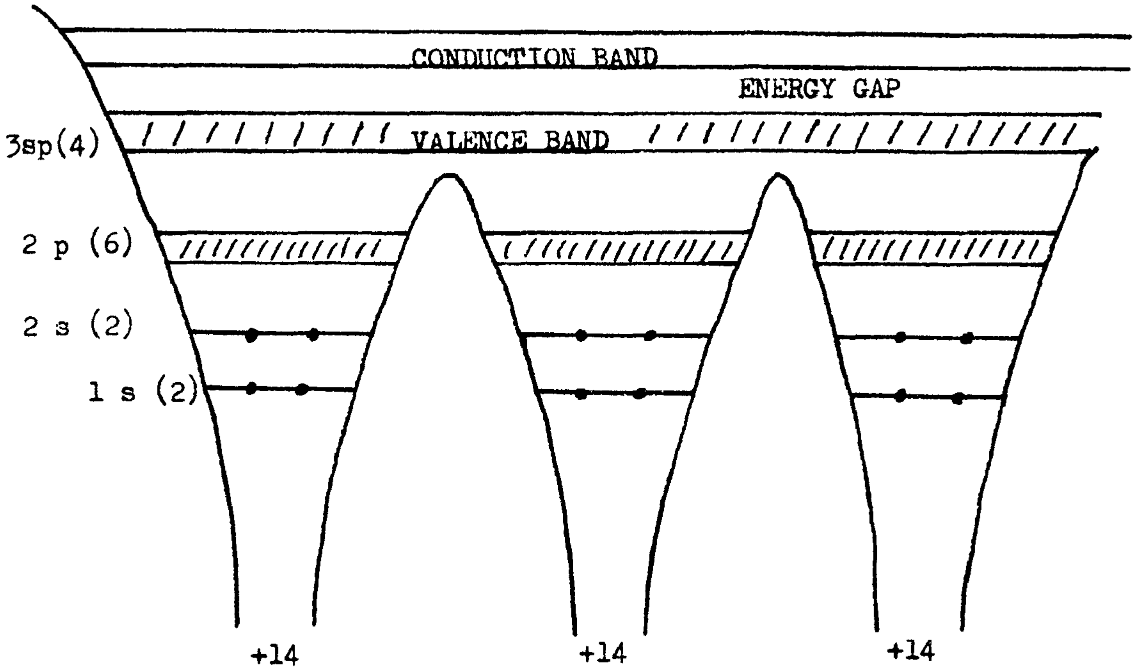

Figure 5-3. Energy bands in a silicon crystal The atomic levels that form the filled bands are indicated at left.

containing some readily available empty electronic levels. A completely filled band cannot conduct because with all levels filled there will be as many electrons moving in one direction as in the other. A completely empty band cannot conduct because it has no charge carriers. Therefore, only partially filled bands permit electronic conduction.

Semiconductors have an energy gap which is sufficiently small so that there is some thermal excitation of electrons from the valence band to the conduction band. Conductivity is then possible because there exists a partially filled band--actually two partially filled bands: the conduction band with some electrons, and the valence band with some holes. Since band energies depend on the periodic bond structure which holds a crystal together, semiconductors also have the property that their conductivity can be considerably modified by the appropriate addition of impurities which cause local alterations in bonding. This leads to the formation of p and n type semiconductors, depending on whether the impurity bonds with an extra electron (n type) or bonds with an electron deficiency (p type), as compared to the rest of the lattice.

It is convenient to represent a silicon crystal by the two dimensional network indicated in figure 5-4. A silicon atom is located at each intersection; each line segment represents two valence electrons, one furnished by each neighboring atom, as shown in more detail in part of

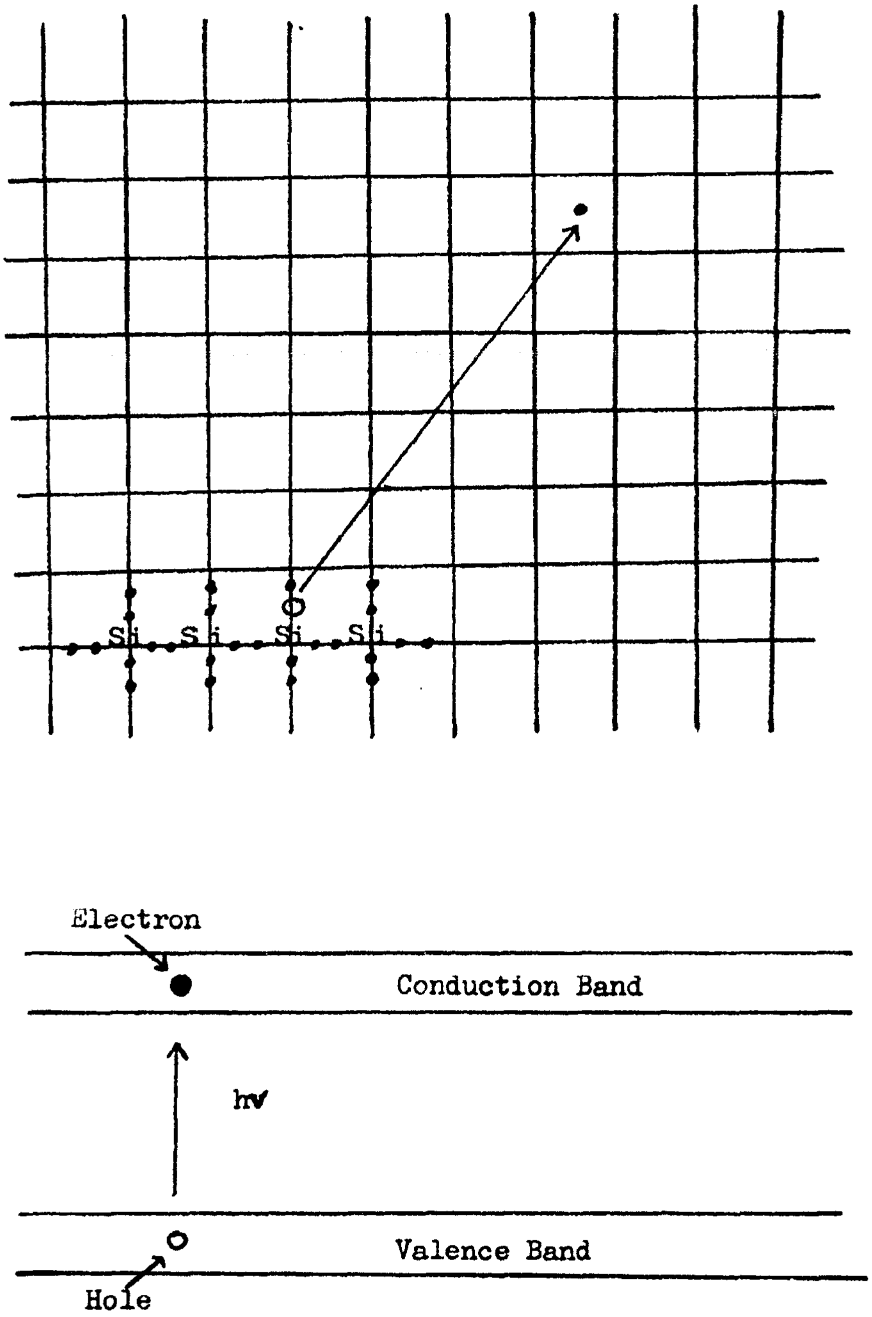

Figure 5-4. A representation of a silicon crystal illustrating the mechanism of charge carrier production by light.

the figure. A photon of light of sufficient energy (1.1 eV) can raise an electron to the conduction band and leave a vacancy (hole) in the valency band. The crystal now contains two partially filled bands, each of which will contribute to electronic conduction. The conduction due to electrons in the almost empty conduction band is called n type conduction. That due to holes in the almost full valence band is referred to as p type conduction, since it is most conveniently described in terms of the motion of the positive holes. The positive charge of a hole is the result of a deficiency of electrons in the vicinity of a silicon nucleus. If an electric field is applied to the crystal, the electron and hole will move in opposite directions; the motion of the hole actually involving the concerted motions of many electrons.

It is possible, as indicated earlier, to modify the electrical properties of silicon crystals by the addition of appropriate impurities into the crystal structure, a process called "doping." For example, an occasional silicon atom may be replaced by a phosphorus atom, as indicated in figure 5-5 . Phosphorus has five valence electrons and only four are needed to fit phosphorus into the silicon crystal. The remaining electron will be associated with the phosphorous at low temperatures, but is readily ionized at room temperature to give a free electron in the crystal and a fixed positive phosphorous ion.

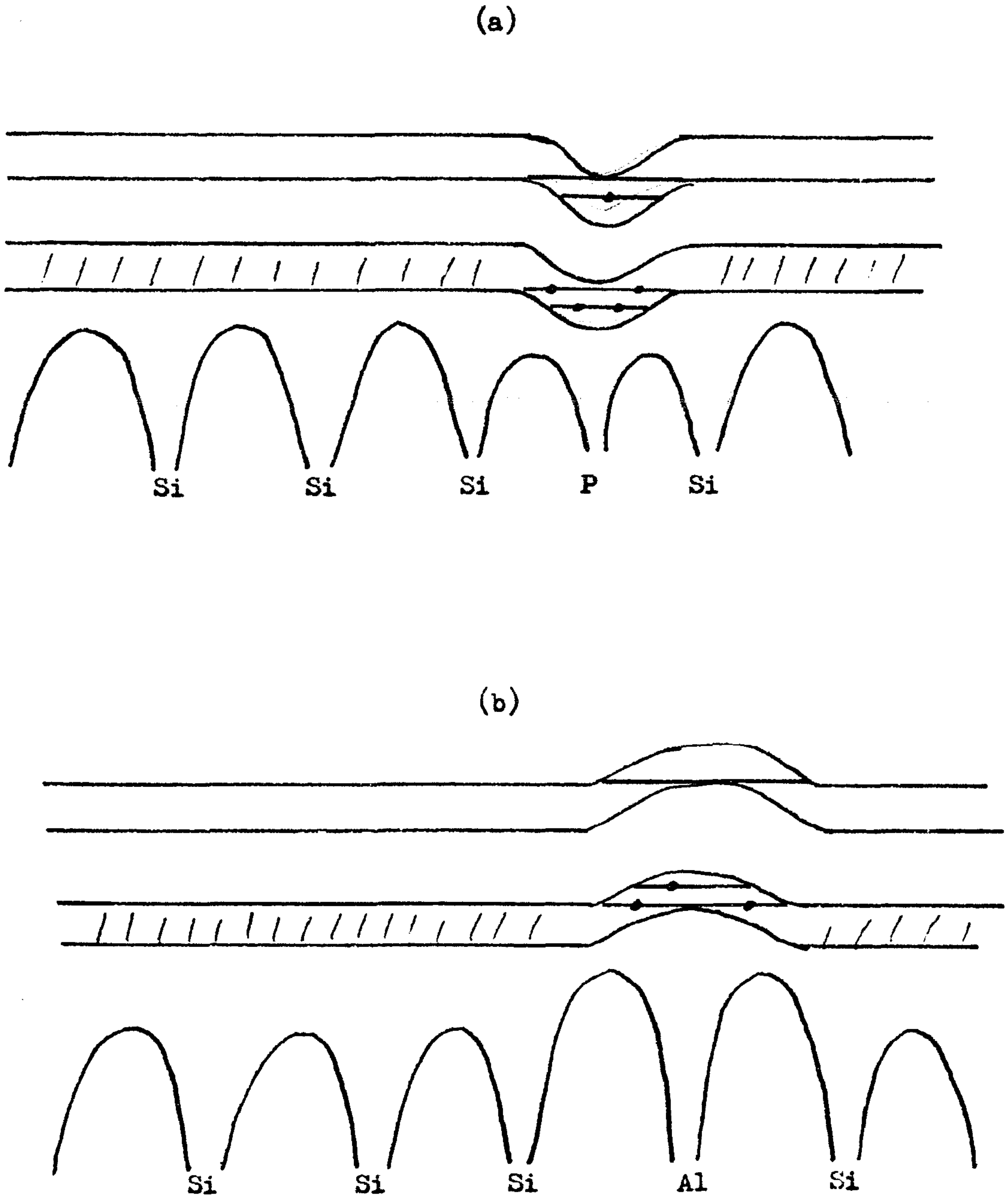

Figure 5-5. (a) Band structure for silicon doped with phosphorous (n type). (b) Band structure for silicon doped with aluminum (p type).

The insertion of the phosphorous into the crystal lattice also perturbs the periodic crystal structure, and consequently the valence band and conduction band are distorted in the vicinity of the impurity atom. This is also shown in figure 5-5. The net result is that localized donor levels are produced that contain the extra electron at low temperatures, but are sufficiently near the conduction band so that thermal ionization is possible at room temperature to give a mobile electron.

Phosphorous-doped silicon will then exhibit electrical conductivity because it has a partially filled conduction band. This is referred to as n type silicon (negative charge carriers). The conductivity will be proportional to the amount of phosphorous added, since each phosphorous atom furnishes one charge carrier. If silicon were doped with a trivalent metal such as aluminum, the result will be as shown in figure 5-5. Instead of an extra electron, as with phosphorous, there will be one electron missing from the valence band for each aluminum atom added. The result is a p type semiconductor whose charge carriers are holes.

Attempts to extend the band model to organic semiconductors have been only partially successful. The origin of the electronic bands is essentially in the periodic structure of the crystal and the interactions between the electrons. In an organic crystal, such as a chlorophyll crystal, the periodic structure is present, but the interactions between electrons on neighboring molecules are relatively weak. The electrons tend to be localized on the individual chlorophyll molecules, and as a result the electronic bands will be very narrow. This results in low mobility of the charge carriers.

Electrical conductivity will be proportional to the number of charge carriers and their mobility. Since the mobility is low for organic crystals they must have a large number of carriers to exhibit appreciable conductivity. In addition to discussing conductivity in terms of band theory, a 'hopping' model has been used for crystals in which the electrons tend to be localized. According to this model a charge on one molecule has a finite probability of transferring to an adjacent molecule. In the presence of an external electric field, this results in an electric current. If the probability for charge transfer to an adjacent molecule is large, the charge is no longer localized and the band model is probably more appropriate.

B. Theoretical treatment

The semiconduction theory of thermoluminescence envisions the originating substance as a crystal containing a small proportion of impurity atoms. These impurity atoms or lattice irregularities give rise to localized electron states with narrow energy levels, either below the level of the conduction band, or above the level of the valence band.

These states serve as traps, and in an n type semiconductor where the impurities form energy levels close to the conduction band, they may capture electrons excited into the conduction band. The release, by thermal agitation, of electrons from these traps into the conduction band and then back to the valence band, gives rise to delayed luminescence, or delayed light. If the crystal is excited by light while cold and is then warmed, light is emitted while heating. This emission of light is thermoluminescence.

A particular temperature of glow corresponds to a particular trap depth. If the energy level of a trapped electron is E electron volts below the conduction band, the electron must absorb at least energy E before it can escape from the trap. It is assumed that the electrons in the traps have a Maxwellian distribution of thermal energies; hence the probability P of an electron escaping from a trap of depth E at temperature T is of the form:

$$P = se^{-E/kT} \tag{1}$$

where k is Boltzman's constant and s is the frequency factor. If n is the number of electrons in the traps at time t, then:

$$dn/dt = -nse^{-E/kT} \tag{2}$$

The intensity of light emission I is proportional to the instantaneous rate of untrapping of electrons.

$$I = Cdn/dt = -Cnse^{-E/kT} \tag{3}$$

Now from equation (2)

$$dn/n = -se^{-E/kT}dt$$

then writing dT = Bdt, where B is the rate of warming, and integrating, we have:

$$\log n/n_o = - \int_o^T 1/B\ se^{-E/kT}dT \qquad (4)$$

and

$$n = n_o e^{-\int_o^T 1/B\ se^{-E/kT}dT}$$

then by substituting equation (4) into equation (3):

$$I = -Cn_o e^{-\int_o^T 1/B\ se^{-E/kT}dT}\ se^{-E/kT} \qquad (5)$$

This expression represents the glow curve for a material containing traps of distinct depths. A sample curve is given in figure 5-6 (from Randall and Wilkins, 1945). Beginning at low temperatures, the curve rises exponentially according to the exponential term in equation (3). When the light emission has continued for some time the number of trapped electrons (n in equation (3)) becomes small and the curve, after reaching a maximum, falls to zero.

An advantage in measuring thermoluminescence rather than delayed light, is that when distinct peaks are observed, the activation energies necessary for the observed peak can be calculated. It is more difficult to use delayed light for this type of measurement, since in each time range more than one process appears to be contributing to the decay

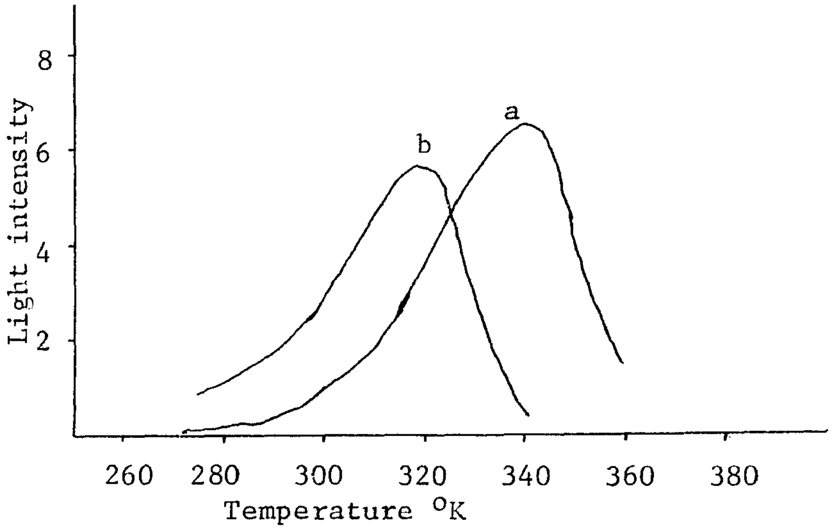

Figure 5-6. Theoretical glow curve for a single trap depth and for two rates of warming. (a) 2.5° per sec, (b) 0.5° per sec. $E = 0.67$ eV; $s = 2.9 \times 10^9$ sec^{-1}. (from Randall and Wilking, 1945)

and it is very difficult to separate the various components. Using thermoluminescence, however, these components are more easily separated, since we assume that each peak corresponds to a single trap depth.

One problem in calculating the trap depth of a particular glow curve is that generally both the frequency factor as well as the trap depth are unknown. All the equations developed thus far include both these unknowns. It is therefore necessary to solve equation (5) for E independent of s. This was done by Grossweiner in 1953.

He found that:

$$E = 1.51\ kT^{*}T^{'}/(T^{*} - T^{'}) \tag{6}$$

and:

$$s = \frac{BEe^{E/kT^{*}}}{kT^{*2}} \tag{7}$$

where k is Boltzmann's constant (8.614×10^{-5} eV), B is the heating rate, T^{*} is the temperature of the glow curve peak, and $T^{'}$ is the temperature of the half height of the glow curve on the low temperature side.

An assumption necessary to Grossweiner's derivation is that E/kT is equal to or greater than 20. This means that for peaks coming between 250 and 300 degrees Kelvin, as do the glow curves from chloroplasts, E must be 0.42 eV or greater for this method of calculating E to be accurate. Equation (6) holds to better than 5% for traps deep enough to satisfy the inequality above, and is seen to be independent of s and of the heating rate B.

In equation (6) it is interesting that the shape of the emission profile will have a profound effect on the solution of E. If the peak is broad, E will be smaller than if the peak emerged at the same temperature and height but was narrow. Grossweiner showed that this relationship held experimentally. Table 5-1 gives his results when glow curves of known trap depth were submitted to his analysis. The two values for E agree quite closely. Intuitively this also seems correct. For instance, consider two glow curves, both with peaks at the same temperature. The first has a half height temperature 10 degrees less than the peak temperature, while the second is broader with a half height twenty degrees below the peak. The shallower trap should correspond to the broader peak--there is less of an energy barrier to be overcome and the probability is greater that random fluctuations will permit electrons to overcome this barrier at temperatures less than the optimum one. A deeper trap will have less chance of overcoming the energy barrier until temperatures much closer to temperatures where the probability of an electron escaping per second is 1.

A second method of calculating the activation energies of the thermoluminescent glow curves: an Arrhenius plot of the initial slope of the glow curve was also done. Since the intensity of emission is proportional to:

$$dn/dt = nse^{-E/kT}$$

then:

$$\ln I = -E/kT + \ln Cns \tag{8}$$

E (eV)	s/B ($^oK^{-1}$)	T^* (^oK)	T' (^oK)	E (calc) (eV)
0.2	10^7	128.8	119.0	0.20
0.4	10^7	249.5	230.2	0.39
0.8	10^7	482.0	446.0	0.78
0.4	10^{11}	171.2	162.5	0.42
0.8	10^{11}	334.5	317.0	0.79
0.4	10^{16}	122.2	117.7	0.42
0.8	10^{16}	240.5	231.2	0.78

Table 5-1. Calculation of trap depths of theoretical glow curves from the temperatures at the maximum and half height (Grossweiner, 1953). Theoretical glow curves were plotted for various values of E and s/B using the equation

$$I = -Cdn/dt = Cn_o se^{-\int_0^T (s/B)\, s^{-E/kT} dT}\, e^{-E/kT} \quad \text{(equation (5)).}$$

Then taking T^* and T' of these curves equation (6) was used to check its accuracy. It was found to be accurate to better than 5 percent.

By plotting log I on the ordinate and 1/T on the abscissa, the slope of the exponential rise in the glow emission is E/2.3k.

Once E and s are found the half life of the traps can also be determined. Assuming the process to be first order, 1/e is:

$$1/e = \frac{\ln 2}{p} = s^{-1}e^{E/kT}\ln 2 \qquad (9)$$

C. Calculations

The activation energy of energy storage states 1 and 2 were calculated by the two methods. The first was the method of Grossweiner, using equation (6). The second was plotting the initial rise in emission on a semilog plot, and determining the slope. Neither of these methods could be applied to state 3 which appears as a shoulder on the high temperature side of peak 2. Neither its initial rise in emission nor its half height on the low temperature side can be measured. Consequently, calculations were performed for only peaks 1 and 2.

The results of the first method, that of Grossweiner, are presented in Table 5-2. Storage state 1 was examined under unenhanced conditions, in the presence of electron acceptors, and after treatments which enhance the height of peak 1. Because of the scatter in the data, Storage state 1

conditions	T^* (°C)	E (eV)	s (sec^{-1})	τ (sec)
no additions	-8	0.878	$5.2x10^{14}$	0.013
no additions	-6	0.823	$2.0x10^{14}$	0.020
ferricyanide	-12	0.850	$2.9x10^{14}$	0.010
ferricyanide	-8	0.726	$5.6x10^{13}$	0.031
UV irradiation	-4	0.820	$2.2x10^{14}$	0.032
UV irradiation	-4	0.778	$5.0x10^{13}$	0.028
chloride deficient	-6	0.727	$5.0x10^{12}$	0.036
chloride deficient	-12	0.724	$3.4x10^{13}$	0.021
Average		0.787	$1.7x10^{14}$	0.024

Storage state 2

no additions	30	0.452	$1.8x10^{7}$	2.045
no additions	36	0.453	$1.3x10^{7}$	2.946
no additions	38	0.531	$1.8x10^{8}$	6.300
no additions	22	0.476	$6.1x10^{7}$	1.552
no additions	22	0.476	$6.1x10^{7}$	1.552
DCMU	28	0.451	$1.5x10^{7}$	2.360
DCMU	22	0.476	$6.1x10^{7}$	1.552
ferricyanide	38	0.443	$5.7x10^{7}$	2.533
ferricyanide	32	0.492	$1.0x10^{8}$	1.700
Average		0.472	$7.0x10^{7}$	2.504

Table 5-2. Calculation of E, s, and τ of storage states 1 and 2 using Grossweiner's equations.

each condition was examined more than once. As can be seen from Table 5-2, the activation energy of this peak calculated by this method falls between 0.724 and 0.878 eV, with an average value of 0.787 eV.

Storage state 2 was also calculated by this method, under varying conditions. The presence of DCMU or ferricyanide does not appear to alter the trap depth, although as seen in the previous chapter, these substances affect the amount of emission from this storage state. As seen in Table 5-2, the activation energy of this state is between 0.443 and 0.531 eV, (with an average of 0.472 eV) regardless of the condition of the chloroplasts.

The results of the second method of calculating the activation energies of the peaks are presented in figure 5-6. Each peak was measured from three different samples. Peak 2 was obtained from samples containing DCMU to eliminate peak 1 and allow the initial rise of peak 2 to be observed. By this method activation energies of 0.723 eV for peak 1 and 0.436 eV for peak 2 were obtained, in good agreement with the values found using Grossweiner's formula.

Since the values for E, the trap depth or activation energy, obtained by these two methods agreed so closely, we felt confident in applying equation (7) to our data to calculate s, the frequency factor of peaks 1 and 2. The results are detailed in Table 5-2. Storage state 1 showed a scatter from between 5×10^{12} tries per second to 5×10^{14} tries

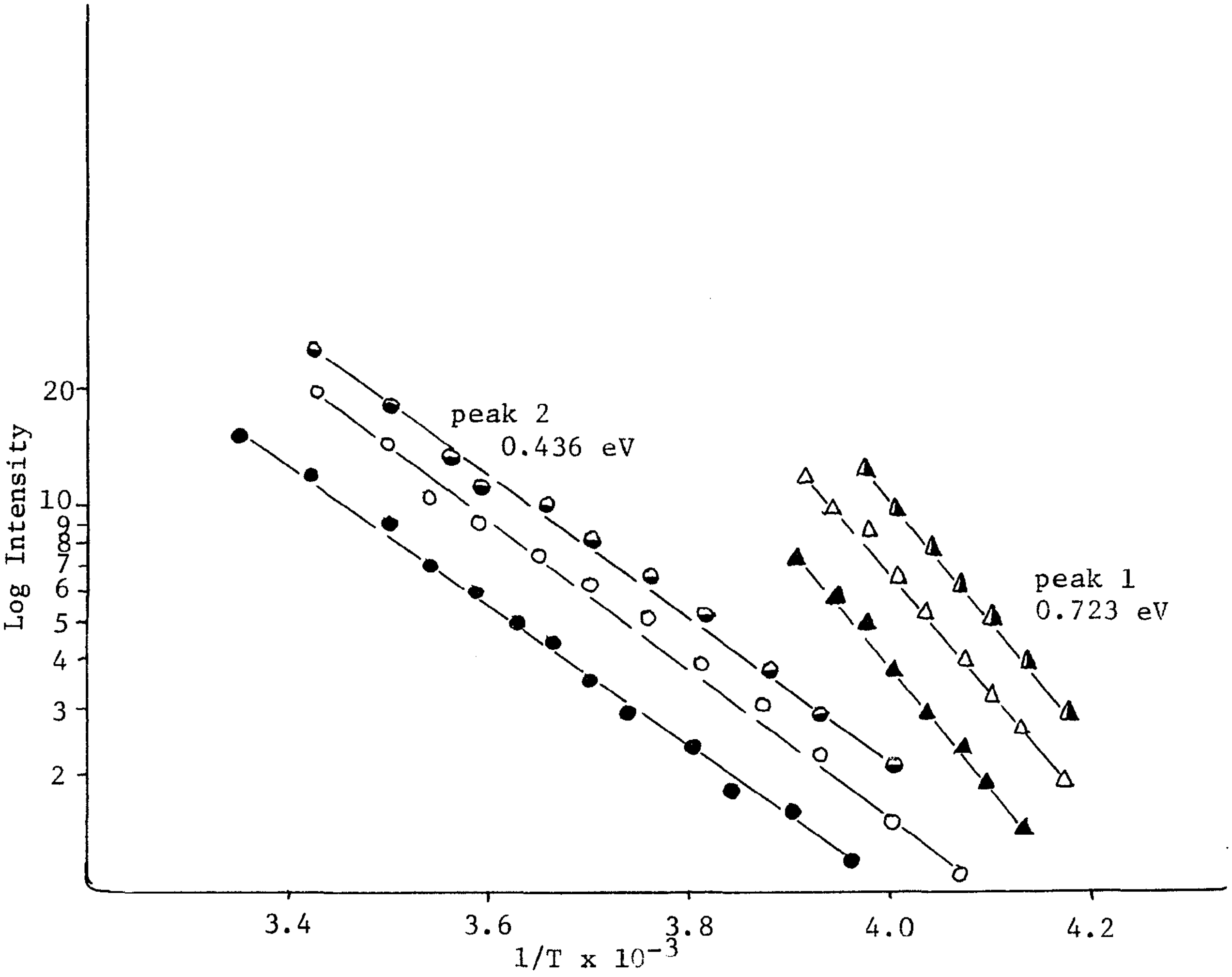

Figure 5-7. Arrhenius plot of the initial slope of peak 1 and peak 2.

per second depending on the sample measured. State 2 varied from 1.5×10^7 to 1.8×10^8 tries per second--less scatter than the date from state 1. Also shown in Table 5-2 is the half life of the two storage states, determined using equation (9). Peak 1 is found to decay in the tens of milliseconds, while peak 2 has a lifetime which extends into seconds.

D. Discussion

The results of these calculations were both interesting and unexpected. The peak emerging at the lowest temperature, storage state 1, was found to have a higher activation energy and frequency factor than the main peak which emerged at a temperature 30 degrees higher. The difference in the frequency factors of the two storage states indicated that they are in some way fundamentally different. This is also supported by the observation that short times of illumination (less than 1 second) does not lead to appreciable filling of state 1, nor does illumination of the sample below -50°C. However, these methods do allow state 2 and 3 to be charged. In addition, state 1 is enhanced by conditions which allow for removal of charge from the reaction center (presence of electron acceptors, treatments such as tris aging) and inhibited by conditions which prevent charge removal (presence of DCMU). One hypothesis might be that some light induced conformational shift is necessary before state 1 can be filled, and that low temperatures and electron transfer inhibitors prevent this while electron acceptors and treatments which set up artificial cycles around photosystem II allow this shift. Alternatively, state 1 may not be in the reaction center, but reflect a pool on the oxidizing side of photosystem II.

Other investigators have attempted to obtain activation energies for energy storage in the photosynthetic apparatus, by utilizing thermoluminescence or delayed light. Arnold first attempted this with thermoluminescence in 1958 in an investigation of dried chloroplast films. The glow curves obtained with these samples were analyzed for E and s and the values were 0.67 eV and 2.45×10^9/sec. However, the peaks were around 80 - 100°C and seemed to have no physiological significance. Further investigation by Arnold (1966, 1968) using whole Chlorella cells, showed an emission profile from -20 to 60°C. Arnold used the frequency factor obtained from dried chloroplasts to analyze these glow curves and got activation energies of 0.53 to 0.62 eV. There is, however, no reason that a frequency factor from dried chloroplasts should be applicable to a different set of thermoluminescent emissions from functional cells.

More recently, Fleischmann (1971) investigated thermoluminescence of photosynthetic bacteria, Rhodopseudomonas viridis. By choosing frequency factors ranging from 10^8, found by Randall and Wilkins (1945) for a number of inorganic thermoluminescent semiconductors, to 2.45×10^9, found by Arnold for dried chloroplast films, he obtained activation energies of 0.45 to 0.55 eV for the thermoluminescence from the bacteria. These values obviously are only accurate if the chosen frequency factors are close to the actual frequency factor.

The Russian researchers, Shuvalov and Litvin (1969) looked at both thermoluminescence and delayed light from the millisecond to the second range of leaves of the water plant, *Trianea bogotensis*. In examining whole *Trianea* leaves, they found a thermoluminescence peak at -15° and a second peak at 20°C. By plotting the initial rise of these two peaks as a function of 1/T they obtained an activation energy for the -15° peak of 0.35 eV and 0.9 eV for the 20° peak. These two energy storage states they were able to correlate with different components of delayed light in the second range. An Arrhenius plot of second delayed light excited by 700 nm light gave an activation energy of 0.9 eV, while the same analysis of second delayed light excited by 640 nm light yielded an activation energy of 0.35 eV. Shuvalov and Litvin conclude that the -15° peak is a component of photosystem II while the 20° peak originates in the reaction center of photosystem I.

It is difficult to compare our data with that of Shuvalov and Litvin, for a number of reasons. Although the glow peaks appear at similar temperatures to the peaks observed by us, the heating rate used by the Russian scientists was 1^{o}/sec, approximately 10 times slower than that used by us. As discussed in Chapter II, a difference in heating rate will change the peak temperature, and the peaks they observe, while appearing to be the same as glow peaks from spinach chloroplasts, may be different. Also they cool their sample to liquid nitrogen temperature and the n illuminate. In our experience this procedure would not allow peak 1 in isolated chloroplasts to be measurably filled, while they do obtain a peak at -15^{o}C from whole leaves. Therefore, it appears that the emission profile from _Triania_ leaves, while superficially resembling that of spinach chloroplasts, is actually quite different.

Their analysis of the delayed light data showed a very good correlation with the thermoluminescence data. However, Bertsch (1965) examined second delayed light of _Chlorella_ and the effect of illumination by different wave lengths and found that the decay kinetics indicated that more than one component was involved in second delayed light decay. At no wave length of illumination was the decay a simple exponential. It seems therefore fortuitous that Shuvalov and Litvin's (1969) two measurements were so closely correlative. In addition, Bertsch (1967) with algal mutants, and Lurie, Cohen and Bertsch (1972) with detergent chloroplast particles, have tried without success to find some part of delayed light originating from the reaction center of photosystem I. Although delayed light can be excited with 700 nm light, it appears nonetheless to originate in photosystem II.

Other researchers have attempted Arrhenius plots of delayed light to determine activation energies for this energy storage process. The first was Bertsch (1969) who found for millisecond delayed light of *Anacystis* in the presence of DCMU, an activation energy of 0.96 eV. Two other groups of investigators have recently examined second delayed light. Roux *et al*. (1971) using isolated chloroplasts in the absence of a Hill acceptor assumed that there were only two pathways for the dissipation of stored energy, a radiative pathway (delayed light production) and a nonradiative pathway (nonradiative deexcitation). By assuming that at any one time one of these pathways predominates, and using conditions where they felt one or the other dominated the kinetics, they were able to find activation energies for the two processes. The E for the nonradiative pathway was 0.45 eV and that of the radiative pathway was 0.9 eV.

Unfortunately, for this elegant analysis it is doubtful whether their initial assumptions are valid for the delayed light in the time range they are investigating. Even in the absence of an electron acceptor there is probably more than one way which stored charge can be removed from the reaction center without producing delayed light. This is supported by the observation that the addition of

DCMU to isolated chloroplasts, alters the decay kinetics of the second delayed light even in the absence of an acceptor. This should not occur if components on the electron transport chain were not contributing to the delayed light decay. Their second assumption, that one pathway always predominates, also appears difficult to justify. If both processes are occurring, it seems that the decay kinetics would reflect this. For example, if three quarters of the stored energy were leaving by a nonradiative pathway and one quarter by delayed light, the decay kinetics would be different from the situation where the nonradiative pathway was dissipating only half the stored energy.

Jursinic and Govindjee (1972) have also recently attempted to determine an activation energy for decayed light in the second time range. They measured delayed light and prompt fluorescence yields in DCMU treated *Chlorella* in the time range of 1 to 10 seconds at temperatures from 0° to 50°C. They used the general expression for the production of delayed light developed by Lavorel (1969)

$$L = \phi J$$

where L is the number of quanta emitted per unit time as delayed light, ϕ is the fluorescence yield and J is the amount of energy made available per unit time for emission as delayed light. Assuming that delayed light is produced by the back reaction of primary photoproducts of photosystem II, a linear relationship should exist between $J^{-\frac{1}{2}}$

and time after illumination. They confirmed this second order relationship at several temperatures (2°, 5°, 10°, 15°). From these analyses, reaction rate decay constants at specific temperatures were calculated. An Arrhenius plot was made for these calculate rate constants. Its slope was 0.36 eV above 18° and 0.43 below $12^{\circ}C$. In the region 12 - $18^{\circ}C$ the slope was 0 indicating no activation energy for delayed light production in this temperature range.

The explanation suggested by Jursinic and Govindjee for this startling result is that the zero slope section of the Arrhenius plot may be a temperature region where a transition between two conformational states exists. Assuming that second delayed light is produced by a back reaction of the primary acceptor and donor of photosystem II (Z^+ and Q^-), this change in slope they suggest may be due to a conformational change implying that a "substrate" upon which the back reaction of Z^+ and Q^- takes place changes its form.

It is apparent that there is a great deal of disagreement regarding the activation energies involved in photosystem II. Depending on the method of calculation values ranging from 0.35 to 0.9 eV have been obtained. Bertsch (1969) in millisecond delayed light and Roux _et_ _al_. (1971) by their method of calculating the radiative pathway of decay of delayed light in the second time range, obtain the same activation energy of 0.9 eV. This agrees well with Shuvalov and Litvin's value for their $20^{\circ}C$

thermoluminescence peak (although they assigned this peak to photosystem I) and with our value calculated by two different methods for peak I. Shuvalov and Litvin (1969) found an activation energy of 0.9 eV for the 20°C peak, while the value for peak 1 of our thermoluminescence emission is 0.75 - 0.85 eV. The other values obtained by the various researchers tend to be approximately half of 0.9 eV. Roux's (1972) activation energy for the nonradiative pathway of decay of stored energy is 0.54 eV, Govindjee's two different slopes in his Arrhenius plot are 0.34 and 0.43 eV, while our activation energy for peak 2 is between 0.45 and 0.55 eV. Arnold (1966) and Fleischmann (1971) in Chlorella and photosynthetic bacteria have obtained activation energies between 0.45 and 0.67 eV. Which of these calculations can be correlated with each other remain to be elucidated. At present the connection remains obscure.

With the activation energy, frequency factor and decay time of peak 1 and peak 2 calculated, we may make some further speculation on the origin of these glow peaks. Peak 2 from all the data appears to be charged by the primary photoact of photosystem II. We have two interpretations of where peak 1 originates, and from the thermoluminescence data we are unable to eliminate either as possibilities. The first interpretation is that peak 1 is not a primary photoact, but reflects the filling of a pool outside the photosystem II reaction center (on the water side of photosystem II). Data supporting this theory are that peak 1 is not filled by illumination shorter than approximately one second (indicating that it is not part of a primary photoact) and is not charged by illumination below -50°C (indicating that electron transport may be necessary for its charging). It is enhanced by conditions which lead to removal of charge from the reaction center and presumably increase the filling of pools (electron acceptors, treatments which induce a cycle). It is inhibited by electron transport inhibitors which prevent electron flow to intermediates on the electron transport chain (DCMU). It is a deeper trap which has a different frequency factor than peak 2. This reflects the fact that it may be a component of the enzyme system between photosystem II and water, which is being reexcited to the first excited singlet of chlorophyll a.

The second interpretation is that peak 1 is in the reaction center of photosystem II, but comes into existence through a light induced conformational shift, and becomes the predominant storage site when photochemistry (normal or aberrant) is occurring. Illumination at low temperatures prevents this conformational shift, and too short a time of illumination does not induce it. The absence of electron transport (DCMU) inhibits its appearance, while cycle inducing treatments and electron acceptors enhance the charging of this storage site. The membrane-modifying properties of uncouplers also affect this trap, but uncouplers in the presence of an electron acceptor enhance the filling of state 1. The fact that it is a deeper trap (approximately 0.8 eV) allows it to be a more efficient energy storage mechanism for energy which will be utilized for photosynthesis than peak 2 (0.47 eV).

Peak 1 has an inherent half decay time of 0.01 - 0.03 sec while peak 2 is 1 - 3 seconds half decay. The actual decay of these peaks in a functioning system may be quite different, but if nothing is drawing stored charge out of these storage states the decays would be in the time range indicated above. As a result, peak 1 may have a greater contribution to msec delayed light than peaks 2 and 3. This is supported by the finding that conditions which inhibit peak 1 lower the overall emission of msec delayed light, while these same conditions in general enhance peaks 2 and 3.

CHAPTER VI: CONCLUSIONS

A. Relationship between Delayed Light and Thermoluminescence

Both delayed light and thermoluminescence are properties of semiconductor systems. Previous research indicated that delayed light in photosynthesis originated in the reaction center of photosystem II (Bertsch, 1967). We have added to the evidence leading to this conclusion by a study of detergent subchloroplast particles enriched in either photosystem II or photosystem I. We found that the only delayed light emitted by photosystem I particles was due to photosystem II contamination. The thermoluminescence peaks observed by us also originate in photosystem II. An examination of algal mutants which lacked a functional reaction center for either photosystem I or photosystem II, showed that thermoluminescence peaks were absent in the mutant deficient in photosystem II, but present in algae which lacked a functional photosystem I.

The question then arose as to how these glow peaks, which represented some metastable storage state in the reaction center of photosystem II, contribute to msec delayed light emission. It was possible to make correlations between delayed light emission at 1 msec and peak 1 in the thermoluminescence curves. Conditions which enhance delayed light emission at 1 msec, also enhance charging of peak 1 (addition of ferricyanide and inhibitory treatments). Conditions which decrease delayed light emission at 1 msec, decrease peak 1 (DCMU and uncouplers). The half life of peak 1 is around 10 msec. Therefore the charge stored in the metastable state reflected by peak 1 might make a major contribution to delayed light at 1 msec.

Peaks 2 and 3 do not correlate well with delayed light emission from 1 to 20 msec. DCMU and uncouplers enhance peaks 2 and 3 while decreasing msec delayed light emission. Ferricyanide and inhibitory treatments enhance 1 msec delayed light emission and decrease delayed light emission from 5 to 20 msec. These conditions decrease peaks 2 and 3. Peak 2 has a half life of about 1 second. It therefore is possible that this peak contributes more to delayed light emission at times longer than 20 msec after illumination. Further investigation is needed to check this possibility.

B. Activation energies of thermoluminescence peaks

Calculations were done for peaks 1 and 2. Two means of calculation were used which are described in Chapter V. The results from these two methods were similar. The two peaks showed very different activation energies, frequency factors and half lives. Peak 1 has an activation energy of 0.78 eV, a frequency factor of 10^{14} and a half life of 20 msec. Peak 2 has an activation energy of 0.47 eV, a frequency factor of 10^7 and a half life of a second. Calculations were not done on peak 3. However, since it was always affected in the same manner as peak 2, we have assumed that it has a similar activation energy.

These activation energy calculations are in the same range as calculations from delayed light or thermoluminescence done by other researchers. Their work has been discussed in Chapter V. However, since methods of data collection and analysis have been so different it is impossible to correlate our results with those of other investigators.

Interestingly, the deeper trap is the one enhanced by activation of photochemistry, either normal or cyclic in nature. This trap, being deeper, is more efficient in storing trapped charge, and appears to be enhanced at the expense of traps 2 and 3. It is possible that the removal of charge from the reaction center into photochemical pathways in some way potentiates this trap to become the major one in charge storage.

C. Mechanism of quantum conversion

The finding that thermoluminescence and delayed light are properties of the reaction center of photosystem II is consistent with the idea that the reaction center functions as a semiconductor. Our view, based on the data presented in this thesis, is that the reaction center contains three traps which can store energy absorbed by the antenna chlorophyll and transferred to the reaction center. Two of these traps have an activation energy of about 0.47 eV and are the major traps when photochemistry is not occurring. The third has an activation energy of about 0.78 eV, and becomes the major trap when photochemistry is occurring.

The first two traps probably do not make a major contribution to delayed light in the 1 to 20 msec time range, but may contribute to delayed light at longer times. The deeper trap does make a major contribution to msec delayed light emission. The increase in delayed light emission at 1 msec upon activation of electron transport is due to the fact that the ability of charge to leave the reaction center and enter the electron transport seems to trigger a change which makes trap 1 the dominant storage state of the excited charge.

This explanation of the msec delayed light and its increase in emission intensity at short times upon activation of electron transport is in conflict with the explanation from Bertsch's semiconductor model. The electron hole model explains the increase in delayed light at 1 msec as due to an increase in nonthermal delayed light originating from the increased population of unpaired electrons and holes in the bands once electron transport is activated. Our explanation is that electron transport activates a deeper electron trap which contributes in a major way to msec delayed light.

Our explanation of quantum conversion is that the reaction center functions as a semiconductor in its charge storage, but we feel that the changes in delayed light decay kinetics in the msec region can be explained by the different filled states of the traps and the relative contribution they make to delayed light in the different time periods.

There is a second explanation of the thermoluminescent data, namely, that peak 1 may originate from some component outside the reaction center. This is supported by the fact that storage state 1 has a very different activation energy and frequency factor from peaks 2 and 3. Also it appears to need either a light induced conformation change or electron transport to fill it. DCMU would prevent its charging by preventing electron transport, and ferricyanide would increase filled pools in the electron transport chain by facilitating electron transport.

In terms of the relationship of delayed light to thermoluminescence this explanation necessitates one of two choices. Either peak 1 contributes to msec delayed light in which case msec delayed light is not just a reflection of conditions within the reaction center but also of electron transport chain components, or peak 1 does not contribute at all to msec delayed light, and delayed light from 1 to 20 msec does not reflect the conditions of any of the storage states we have examined.

We prefer the explanation that peak 1 is within the reaction center. This has the attractiveness of being a simpler formulation. However, at the present time we have no conclusive evidence which would enable us either to choose between the two explanations or to discard both in favor of another theory.

D. Mechanism of Action of Inhibitory Treatments

Four treatments were examined which inhibited photosystem II activity without affecting photosystem I. These were: tris treatment, heat, UV irradiation and chloride depletion. All were found to act by inducing a cycle which led to useless recombination of the charge separated by the reaction center of photosystem II. The effects of the treatments on both delayed light and thermoluminescence led us to this conclusion.

Treated chloroplasts exhibited rapid delayed light decay kinetics from 1 to 20 msec. Thermoluminescence from the treated chloroplasts showed an enhancement of peak 1 and a decrease in peaks 2 and 3. Both these phenomena occur normally when photochemistry is initiated by the addition of electron acceptors. In the case of the treatments the two phenomena were exhibited in the absence of electron acceptors, indicating that the treatments themselves were leading to removal of charge stored in the reaction center.

From the effect that electron donors, and the photosynthetic poison DCMU have on delayed light (our work) and fluorescence (from papers by other researchers) in treated chloroplasts, we were able to present a detailed model of where the cycles instigated by the different treatments act (see fig. 3-22 and discussion).

We are continuing to investigate conditions which affect photosystem II on the water splitting side. Thus far all the treatments we have examined except one appear to induce cycles. (The exception is inhibition with high concentrations of ammonia, which appears to block electron flow in a similar manner to DCMU, but functions on the water splitting side of photosystem II.)

The finding that many varied treatments have the same effect on photosystem II, may say something fundamental about the makeup of the components on the oxidizing side of photosystem II. It appears that all these treatments both prevent water from acting as the normal electron donor, and induce an abnormal pathway for useless recombination of separated charge. Although we do not know what component or components is affected by the treatments, it seems possible that they may effect small conformational changes in the thylakoid membranes.

E. Uncouplers

Uncouplers have two actions which affect energy storage in photosystem II--as reflected by delayed light emission. These are their effect on membrane permeability, and their effect on the rate of electron transport. The first effect, that of modifying membrane permeability, alters the ion balance on either side of the thylakoid membrane, affecting either or both the pH gradient and the membrane potential. CCCP allows for proton exchange across the membrane, while gramicidin makes the membrane permeable to monovalent cations. Both destroy the pH gradient and the membrane potential. Valinomycin alters the membrane to allow for free passage of potassium ions. This does not affect the pH gradient but does destroy the membrane potential. Nigericin exchanges potassium ions for hydrogen ions, thus preventing the pH gradient but not affecting the membrane potential. Amines move across the membrane in an uncharged form, becoming protonated on the inside and dissipating the pH gradient.

This selective increase in the permeability of the membrane caused by uncouplers occurs in the presence or absence of electron transport. It may explain the decrease in delayed light emission from 1 to 20 msec caused by uncouplers even without an electron acceptor present. We presented a modification of Wraight and Crofts (1971) model of the reaction center to explain these results. We have placed the electron trap on the inside of the membrane and the hole trap on the outside. Both are affected by the microenvironments around the trapping sites, and changing the microenvironments (by the addition of uncouplers) would affect the probability of reemission of delayed light and lead to less delayed light emission in the msec time range. As yet we have been unable to separate the effects that the membrane potential or pH gradient have on energy storage at photosystem II, but this is under continuing investigation.

The second effect of uncouplers, that of stimulating electron transport occurs in the presence of a Hill acceptor. In the presence of an uncoupler and a Hill acceptor delayed light emission at 1 msec is not stimulated as much as it is in the absence of an uncoupler. Bertsch's model suggests that delayed light should be enhanced at 1 msec when electrons are being more rapidly removed from the reaction center into the electron transport chain. This rapid emptying and refilling of the traps would create more unpaired charge in the bands and thus lead to an increase in delayed light at short times. This is not what is observed.

One explanation may be that the steady state turnover time of photosynthesis in the presence of uncouplers is faster than the 20 - 40 msec measured by Arnold in Chlorella. In terms of Bertsch's model this would shift his nonthermal delayed light into the usec time range (which we do not monitor) and make msec delayed light almost purely the result of back reactions of the stored electrons and holes. With faster electron transport there would be a smaller population of stored charge and therefore lower delayed light.

In terms of our explanation of thermoluminescence, uncouplers reduce the level of storage state 1, and the addition of ferricyanide increases storage in this state, but not to the extent seen in the absence of an uncoupler. If peak 1 is the major contributor to delayed light from 1 to 20 msec, then the effect that uncouplers have on it correlates with their effect on delayed light in this time range.

APPENDIX

The following is a solution for the equation

$$I = -C(dn/dt) = Cnse^{-E/kT} \qquad (1)$$

for E and s independently. This equation is an expression of the decay process of a first order glow curve, where n is the number of electrons in traps of depth E at time t, s is a frequency factor and C is a geometry constant.

$$dn/dt = -nse^{-E/kT} \qquad -dn/dt = nse^{-E/kT} \qquad (2)$$

$$dn/n = -se^{-E/kT}dt$$

Now let $T = \alpha + Bt$, where α is the temperature at t_o and B is the heating rate. Then dT = Bdt so:

$$dn/n = -s/B\ e^{-E/kT}dT$$

$$(d\ \ln n/dn = 1/n \quad \text{so}\ d\ \ln n = dn/n)$$

then,

$$d\ \ln n = -s/B\ e^{-E/kT}dT$$

and integrating,

$$\ln n/n_o = -\int_o^T s/B\ e^{-E/kT}dT$$

$$n/n_o = \exp\left(-\int_o^T s/B\ e^{-E/kT}dT\right)$$

$$n = n_o \exp\left(-\int_o^T s/B\ e^{-E/kT}dT\right) \qquad (3)$$

The expression for n in equation (3) is substituted in equation (2)

$$-dn/dt = n_o s \exp\left(-\int_o^T s/B\ e^{-E/kT}dT\right) e^{-E/kT}$$

Now,

$$I = -Cdn/dt = Cn_0 s \exp(-\int_0^T s/B\ e^{-E/kT} dT)\ e^{-E/kT}$$

By taking two different intensities we can set up a ratio. Let I' equal the maximum intensity of the glow peak. Then T' is the temperature the intensity is maximum. I* will equal the half maximum intensity on the low temperature side, and T* will be the temperature at which half maximum emission is seen. Then,

$$\frac{I'(T')}{I^*(T^*)} = \frac{1}{2} = \frac{Cn_0 s \exp(-\int_0^{T'} s/B\ e^{-E/kT} dT)\ e^{-E/kT}}{Cn_0 s \exp(-\int_0^{T^*} s/B\ e^{-E/kT} dT)\ e^{-E/kT}}$$

$$\tfrac{1}{2} = \exp(-E/k(1/T' - 1/T^*))\exp(-\int_0^{T'} s/B\ e^{-E/kT} dT + \int_0^{T^*} s/B\ e^{-E/kT} dT)$$

By subtracting one integral from the other, we get,

$$\tfrac{1}{2} = \exp(-E/k(1/T' - 1/T^*))\exp(\int_{T'}^{T^*} s/B\ e^{-E/kT} dT) \qquad (4)$$

It is possible to integrate $\exp(\int_{T'}^{T^*} s/B\ e^{-E/kT} dT)$ by parts

$\int u dv = uv - \int v du$

Let $u = e^{-E/kT}$; $dv = dT$

Then $du = E/kT^2\ e^{-E/kT} dT$ and $v = T$

so: $s/B \int_{T'}^{T^*} e^{-E/kT} dT = Te^{-E/kT} \Big|_{T'}^{T^*} - \int_{T'}^{T^*} T\ E/kT^2\ e^{-E/kT} dT$

$$= (T^*\ e^{-E/kT^*} - T' e^{-E/kT'}) - E/k \int_{T'}^{T^*} 1/T\ e^{-E/kT} dT \qquad (5)$$

We will define f(x) as $-E_1(-x)$, which equals the expression $\int_x^{\infty} e^{-u}/u\ du$. Let $u = E/kT$. Then $du = E/kT^2\ dT$. When $T = T'$ $x = E/kT'$. Now we will use these definitions to work with the expression $\int_{T'}^{T^*} 1/T\ e^{-E/kT}\ dT$

$$-\int_{T'}^{T'} 1/T\ e^{-E/kT}\ dT = -\int_{T'}^{\infty} 1/T\ e^{-E/kT}\ dT + \int_{T^*}^{\infty} 1/T\ e^{-E/kT}\ dT$$

$$-E_1(-E/kT') = \int_{x}^{\infty} e^{-u}/u\ du = -\int_{T'}^{\infty} e^{-E/kT}/E/kT \cdot E/kT^2\ dT = -\int_{T'}^{\infty} 1/T\ e^{-E/kT}\ dT$$

and,

$$-E_1(-E/kT^* = -\int_{T^*}^{\infty} 1/T\ e^{-E/kT}\ dT$$

Therefore,

$$-\int_{T'}^{T^*} 1/T\ e^{-E/kT}\ dT = \int_{T^*}^{\infty} 1/T\ e^{-E/kT} dT - \int_{T'}^{\infty} 1/T\ e^{-E/kT}\ dT$$
$$= E_1(-E/kT^*) - E_1(-E/kT')$$

and equation (5) is,

$$s/B \int_{T'}^{T^*} e^{-E/kT} dT = (T^* e^{-E/kT^*} - T' e^{-E/kT'}) + E/k \left[E_1(-E/kT^*) - E_1(-E/kT')\right]$$

Since $-E_1(-x) = \int_{x}^{\infty} e^{-u}/u\ du$ this integral has the form of the series $-E_1(-x) = e^{-x}(1/x - 1/x^2 + 2!/x^2 - 3!/x^3 + \dots)$. $x = E/kT$. When x is larger than 20 less than 10% error is made by dropping everything after the second term in the above expansion.

Therefore,

$$e^{-E/kT} = T^* e^{-E/kT^*}/(E/kT^*) - T' e^{-E/kT'}/(E/kT') \quad (6)$$

Substituting equation (6) into equation (4) gives

$$\tfrac{1}{2} = \exp -E/k(1/T'-1/T^*) \exp s/B\ (T^* e^{-E/kT^*}/(E/kT^*)) - (T' e^{-E/kT'}/(E/kT'))$$

If we take the log of both sides and rearrange things a bit we get,

$$(E/k)(1/T'-1/T^*) - 0.693 = skT^{*2}e^{-E/kT^*}/(EB$$

$$1-(T'/T^*)^2 \exp(-E/k)(1/T'-1/T^*) \qquad (7)$$

Now if equation (1) is differentiated with respect to T, at the glow peak maximum we have,

$$E/k = (s/B)e^{-E/kT^*}T^{*2} \qquad (8)$$

Therefore, at the glow peak maximum the coefficient $skT^{*2}e^{-E/kT^*}/EB$ on the right side of equation (7) is unity. Also the exponential term in the right side of equation (7) is approximately $e^{-1.693}$. The factor $(T'/T^*)^2$ may be neglected as it affects the value for E by less than two percent where $s/B \; 10^6$. With these approximations we have as the solution to equation (7),

$$E = 1.51kT^*T'/(T^*-T')$$

Once E is evaluated, s can be found from equation (8).

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