# THE EFFECTS OF UNCOUPLERS ON THE INTENSITY AND DURATION OF DELAYED FLUORESCENCE AND ON THE RATE OF ELECTRON TRANSPORT IN ISOLATED CHLOROPLASTS

By

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ABSTRACT

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Information is presented in this thesis about the effects of electron transport uncouplers, electron transport inhibitors, and rates of electron transport on the delayed fluorescence from spinach chloroplast lamellae. A laser phosphoroscope was combined with a spectrophotometric device to permit simultaneous determinations of microsecond delayed fluorescence and noncyclic electron transport under nearly steady-state conditions of most of the electron transport-generated transmembrane electrochemical gradients.

Uncouplers of photophosphorylation and the presence of phosphorylation conditions increased the intensity of that part of the delayed fluorescence observed less than 100 μs after light extinction but inhibited that part of the delayed fluorescence observed after longer delays. This initial increase in delayed fluorescence implies that electrochemical gradients (which are decreased or abolished

under such conditions) are not important factors contributing to the back-reactions responsible for the delayed fluorescence.

The initial intensities of the delayed fluorescence at pH's above neutrality were well correlated with the rates of electron transport when the rate-limiting steps in transport between the two photosystems were increased by uncouplers or decreased by the inhibitors diuron or cyanide. On the other hand, inhibitors of electron transport on the oxidizing side of photosystem II did not inhibit delayed fluorescence nearly as severely as they inhibited prompt fluorescence and, therefore, it seems probable that the chemical back-reactions responsible for delayed fluorescence actually increased. Presumably, different rates of electron transport alter the amount of oxidized reaction center chlorophyll P680 and the amount of reduced electron acceptor (Q) formed in the light and, in so doing, alter their initial rate of back-reaction and enhance the intensity of the delayed fluorescence.

The decay of delayed fluorescence was not a simple exponential function but consisted of two or more phases. The rate of decay of the delayed fluorescence was unaltered by the presence of uncouplers during the first 100 μs after light extinction. Thereafter the decay was much faster in the presence of uncouplers than in their absence. Thus the residual delayed fluorescence after 100 μs had a half-life of about 800 μs in the absence of uncouplers but a half-life of

300-500 μs in the presence of uncouplers. These observations probably can be explained in terms of the known increase in the rate of oxidation of plastoquinone in the presence of uncouplers, since an increase in the rate of this oxidation might begin to affect the level of $Q^-$ within a few hundred microseconds.

In this thesis are also described the effects of a wide range of uncouplers on phosphorylation and on electron transport at pH values ranging from 6.5 to 9.0. Large increases in the rates of electron transport with little if any inhibition of phosphorylation were found near neutrality when the pK's of the uncoupling amines were also near 7.0.

To my wife, Sally

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| | |
|---|---|
| BCD | Binary-coded decimal |
| CCCP | carbonylcyanide 3-chlorophenyl hydrazone |
| Chl | chlorophyll |
| DCMU | 3-(3,4-dichlorophenyl)-1,1-dimethylurea |
| DF | uncorrected counts of delayed fluorescence |
| DF* | corrected counts of delayed fluorescence |
| DMAP | 3-dimethylaminopropionitrile |
| GD | gramicidin |
| HEM | N-β-hydroxyethylmorpholine |
| HEPES | N-hydroxyethylpiperazine-N'-ethanesulfonic acid |
| I | intensity of the light absorbed |
| J | flux of excitons injected into the antenna chlorophyll as a result of the back-reactions that give rise to delayed fluorescence |
| LED | light-emitting diode |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| $P_i$ | orthophosphate ($PO_4^{-3}$) |
| $P/e_2$ | phosphorylation efficiency as moles of ATP formed per pair of electrons |
| P680 | reaction center chlorophyll associated with photosystem II |
| PF | counts of prompt fluorescence |
| PS I | photosystem I |
| PS II | photosystem II |
| Q | primary acceptor of photosystem II |
| TAPS | 3-tris(hydroxymethyl)-methyl-3-aminopropane sulfonic acid |
| Z | first secondary electron donor of photosystem II |

# SECTION I

# THE EFFECTS OF UNCOUPLERS AND ELECTRON TRANSPORT INHIBITORS ON MICROSECOND-DELAYED FLUORESCENCE

# INTRODUCTION

Delayed fluorescence was discovered by Strehler and Arnold (1951) in chloroplasts. They were performing a firefly extract assay for the determination of ATP in these chloroplasts when they noticed that even the controls which omitted the firefly extract exhibited a delayed fluorescence that persisted for several minutes. Early experiments were quick to establish that the phenomenon is related to the process of photosynthesis. It was found that the delayed fluorescence (also called luminescence or delayed light emission) occurs in photosynthetic systems - be they leaves, algae, chloroplasts, or photosynthetic bacteria - and is lacking in non-photosynthetic systems. The action spectrum for delayed fluorescence was found to parallel closely that for photosynthesis in green (Strehler and Arnold, 1951), bluegreen, and red algae as well as photosynthetic bacteria (Arnold and Thompson, 1956). Heat treatment of chloroplasts (Strehler and Arnold, 1951) and experiments with Mn-deficient mutants of algae (Kessler et al., 1957) also suggested that at least part of the photosynthetic apparatus must be functionally intact to permit delayed fluorescence.

Emission spectra for both delayed fluorescence and prompt fluorescence are very similar in many organisms (Strehler and Arnold, 1951; Arnold and Thompson, 1956; Clayton, 1965), suggesting that the light emitted as delayed

fluorescence is due to the return to the ground state of chlorophyll molecules in the lowest excited singlet state and is not the result of the phosphorescence of some long-lived triplet state which would emit quanta at longer wavelengths than those observed for delayed fluorescence. (But see below.)

## Recombination Hypothesis

To explain this phenomenon, Arthur and Strehler (1957) proposed what has come to be called the recombination hypothesis (see Lavorel, 1975a). Its major tenets are these: a) Delayed fluorescence results from a back-reaction (recombination) of the primary photoproducts of photosynthesis. In fact, such an occurrence can be induced chemically in the dark by first oxidizing and then reducing chlorophyll a dissolved in organic solvents (Goedheer and Vegt, 1962). b) The emissions at times longer than the times required for immediate back-reactions are made possible by the reversal of the slower electron transfer steps associated with some dark reduction of Q by the electron carriers on the reducing side of photosystem II and some dark oxidation of the reaction center chlorophyll P680 by some electron carriers on the oxidizing side of photosystem II. c) The electron from the reduced acceptor is injected into the oxidized chlorophyll molecule forming an excited singlet state exciton. This exciton migrates into the antenna

chlorophylls where it may decay with the production of a quantum of light. d) Fluorescence and delayed fluorescent-excitons are not distinguishable. e) The excitons are subject to radiative or non-radiative decays, migration, and trapping by "open" centers (That is, by doing the photochemical act of charge separation to give $Q^-$ and $P680^+$).

## Triplet State Hypothesis

Investigations with organic molecules in fluids and crystals (Priestley and Haug, 1968), and with chlorophyll in vitro (Parker and Joyce, 1966) have suggested that delayed fluorescence might involve the triplet state indirectly through intersystem crossing back to the singlet state (see Malkin, 1977). The likeliest mechanisms are triplet-triplet annihilation (where two triplet excitons combine to form one singlet exciton), absorption of a quantum of light by a chlorophyll molecule in the triplet state, or thermochemical excitation of the triplet chlorophyll to the singlet (E-type emission as demonstrated with eosin. Parker, 1968). The second and third approaches raise the triplet chlorophyll to a higher triplet state which may then be converted to a singlet state and then fluoresce. It should be pointed out, however, that detection of the triplet state in chloroplasts or whole organisms is by no means a simple task (especially at room temperatures) and although there are a few reports of its existence

(Uphaus et al., 1974; Leigh and Dutton, 1974; Haberkorn and Michel-Beyerle, 1977), theories invoking its involvement in the delayed fluorescence of photosynthetic organisms (Stacy et al., 1971) seem less tenable than the recombination theory.

## Semiconductor Hypothesis

Several lines of evidence have led to a semiconductor explanation for the phenomena of delayed fluorescence. Tollin et al. (1975) observed delayed fluorescence at an extremely low temperature (77°K) which forced them to rule out the participation of any enzymatic process. Also, experiments by several laboratories (Arnold and Sherwood, 1975; Arnold and Azzi, 1968; Ichikawa et al., 1975 Lurie and Bertsch, 1974) determined that algae, leaves, and chloroplasts could give rise to thermoluminescence. This is light (fluorescence) emitted in the dark at specific temperatures by material that is slowly warmed after having been frozen to 77°K during illumination. Bertsch and his coworkers (Bertsch and Lurie, 1971; Bertsch et al., 1971) proposed a two-quantum electron-hole model to account for thermoluminescence and for the stimulation by artificial electron donors of the delayed fluorescence associated with Tris-treated chloroplasts. In his model, delayed fluorescence arises within an aggregate of chlorophyll molecules. The absorption of two quanta create two electron-hole pairs.

In one of the pairs, the hole oxidizes a primary donor and in the other pair, the electron reduces a primary acceptor. This leaves an electron and a hole that are separated and are free to migrate. There is a significant probability that these mobile species will recombine before being trapped again, and such an event could give a quantum of delayed fluorescence.

Such a model should operate even at 77°K (Type 1). To explain the two other phenomena (thermoluminescence and tris-donor effects) two variations were proposed. One (Type 2) requires thermal activation of the resulting hole and electron before they can migrate and the other (Type 3) requires reverse electron flow to create the electron and hole.

## Relation to Photosystem II

In higher plants, the majority of the delayed fluorescence detected seems to be emitted by the chlorophylls associated with photosystem II. The action spectrum for delayed fluorescence corresponds to the action spectrum for photosystem II whereas light that stimulates PS I exclusively (> 700nm) depresses the intensity of delayed fluorescence (Goedheer, 1962). Mutants lacking photosystem II show very little delayed fluorescence while mutants lacking only photosystem I have normal delayed fluorescence (Bertsch and Azzi, 1965; Haug et al., 1972). Subchloroplast particles enriched in photosystem II produce more delayed fluorescence

than those enriched in photosystem I (Lurie et al., 1972).

It should be mentioned that there have been some reports of delayed fluorescence arising from photosystem I (Shuvalov, 1976) but its intensity is much less than that associated with photosystem II.

## Lavorel Model

Lavorel proposed in 1968 that the delayed fluorescence intensity could be expressed in a manner analogous to the fluorescence intensity. In the latter case, the relationship is:

$$F = \Phi I.$$

Where F is the fluorescence intensity, $\Phi$ is the quantum yield for fluorescence, and I is the intensity of the light absorbed. In the case of delayed fluorescence, the relationship is:

$$L = \Phi J.$$

Where L is the delayed fluorescence intensity and J is the flux of excitons injected into the antenna chlorophyll complex as a result of back-reactions in the photosystem II reaction center. These two formulae have the quantum yield term in common, reflecting the supposition that once an exciton is formed - either by the light excitation of the reaction center pigment via the antenna chlorophyll or by the reduction of the oxidized reaction center pigment by $Q^-$ - it will disappear in a manner that is independent of its method

of creation. In other words, conditions which affect the yield of prompt fluorescence (such as cations, pH gradients, etc.) should affect the delayed fluorescence yield in a similar manner.

As Clayton (1969) has pointed out, it is important to consider which component of the fluorescence yield to use. The yield of fluorescence is normally considered to have two components. The so call "dead" fluorescence yield is an invariable portion of the fluorescence and is thought to be due to those chlorophyll molecules which are not in contact with reaction centers. There is also a variable component of the fluorescence yield, however, which responds to the condition of the reaction center. It is this potentially variable portion of the fluorescence which reflects the fate of the singlet excitons responsible for both delayed fluorescence and prompt fluorescence.

Certain difficulties with the Lavorel model arise however if the excitation energy can migrate freely between the reaction centers. If this is the case, the yield per unit is not only dependent upon the reaction center being open or closed, but also on the state of neighboring reaction centers (the so called *ilot* or "cluster" effect of Lavorel and Joliot, 1972). Under these circumstances the value of $\Phi$ for delayed fluorescence and for prompt fluorescence may not be the same.

It should be remembered that J will depend upon the competing reactions that lower the amounts of the reactant species, $Q^-$ and $P680^+$ available to back-react. Such reactions

are the oxidation of $Q^-$ by a secondary acceptor and the reduction of the oxidized chlorophyll by a secondary donor, Z.

In some models (Lavorel) the ZChlQ reaction center complex is considered to be one unit. In this case, the active species is $Z^+ChlQ^-$, a photoelectric dipole. Lavorel's notation however seems to minimize the importance of the state of the reaction center chlorophyll. True, the reduction of the pigment by Z (forming $Z^+$) is one of the fastest electron transfers under consideration (see discussion) but it may become significant when one considers the delayed fluorescence arising very soon after the light extinction.

## The Importance of Delayed Fluorescence

It is not clear what function if any delayed fluorescence plays in the plant or in the chloroplast. The quantum yield for delayed fluorescence is only about $10^{-4}$ (Zankel, 1971) and its role in photosynthesis must necessarily be minimal. However, its role in photosynthesis research may be considerable since it is a useful tool with which to investigate the properties of photosystem II. It is important as a non-intrusive indicator of a variety of conditions related to electron transport, ion fluxes, and ATP synthesis.

The intensity of microsecond delayed fluorescence varies with the same periodicity (with saturating flashes) as the oxygen yield in chloroplasts and algae. This suggests

use of delayed fluorescence as another experimental tool to probe the mechanism of oxygen liberation and the condition of the so called "S states" (Kok et al., 1970). Also, as will be shown, the millisecond delayed fluorescence seems to reflect the energized state of thylakoid membranes and as such may be employed to corroborate the values of membrane potentials obtained by electrochromic shift determinations (Barber, 1972).

As shown in this thesis, the microsecond delayed fluorescence reflects the steady-state rates of electron transport through photosystem II.

Finally, as considered in the discussion, the kinetics of the decay of delayed fluorescence may provide information about the rate constants of the separate electron transfer reactions associated with photosystem II (Vierke, 1979).

## Techniques for Measuring Delayed Fluorescence

Since much of the information about delayed fluorescence can only be interpreted when it is clear how the information was obtained, it is important to review briefly the experimental techniques at an early point in this thesis.

There are only two components common to all delayed fluorescence experiments - a light source and a light detector. When one starts to add additional components (oscilloscopes, rotating and triggered shutters, stopped-flow devices, signal averagers) in addition to alterations in the

light sources (xenon lamps, xenon lasers, pulsed lasers) and light detectors (photocells, photomultipliers, photon counting) the explosion in different techniques (and hardware) is astonishing. Moreover, all this is compounded by a wide range of photosynthetic organisms studied (red, blue-green, and green algae, bacterial chromatophores, frozen and non-frozen chloroplasts, intact plants). Unfortunately, the profusion of techniques has created a very diverse body of phenomena - all of which are in some way related to delayed fluorescence - but conclusions reached with one approach often are not applicable to a different approach.

Since the discovery of delayed fluorescence, there has been one dominant theme in the development of new approaches. This theme can be identified by a single word, "faster". In the twenty-eight years since delayed fluorescence was discovered, the minimum time for light measurement has decreased from hundreds of milliseconds to a few microseconds. This has been possible in large part because of equally impressive reductions in the cut-off times of the actinic light. Since the prompt fluorescence intensity is three orders of magnitude greater than the delayed fluorescence intensity, any "tail" of the actinic light swamps the delayed light with prompt fluorescent light and makes relaible determinations of delayed light impossible.

Nearly all delayed fluorescence experiments can be divided into three groups; phosphoroscope, single flashes, environment-triggered. These divisions are by no means

clear-cut and combinations of all of the methods have been reported.

The phosphoroscope method was first employed in the study of the phosphorescence of organic molecules. With the "Becquerel" type of phosphoroscope, the sample is placed between two rotating sectors with openings at a fixed angular distance. The decay of delayed fluorescence after the actinic light pulse is determined by altering this angular distance or by varying the speed of rotation of the sectors. The minimum time after light extinction is about 0.1 ms with this technique, the limitations being the width of the beams being chopped, the width of the chopping windows, and the speed of the rotating sector.

Lavorel (1971) and Haug et al. (1972) have modified the phosphoroscope approach by using a laser as the light source. Due to the parallel nature of the light beam produced, a converging lense can focus the beam almost to a point, the chopping of which by a high-speed rotating sector gives extinction times of less than 250 ns (Haug et al., 1972).

The phosphoroscope technique however has the possible disadvantage that, since the delay of delayed fluorescence lasts for times longer than the occultation period, some of the consequences of successive illumination periods may be summed. To avoid this difficulty, Zankel (1971) and others have resorted to single flash techniques. In this case, the sample is given a brief (3 μs) saturating flash of actinic light. In some cases it has been necessary to synchronize

the flash with a shutter to cut off the tail of the flash (Zankel, 1971) but in recent years, lasers have been used to give nanosecond-long pulses (Jursinic and Govindjee, 1977; Van Best and Duysens, 1975). To increase the signal-to-noise ratios, flow systems have been used with the flash technique so that repetitive measurements can be made but the same biological material is never subjected to more than one light flash.

Delayed fluorescence can also be triggered by perturbations of the system other than a flash of light. Salt injections, acid injections, and base injections can all be done by stopped-flow - a technique in which the two liquid volumes to be mixed are expelled simultaneously by syringes into a common tube, where mixing rapidly occurs ($\simeq$ 50 ms). The biological material can also be perturbed by physical methods - either electric fields or temperature jumps - with a consequent emission of light.

All of the above techniques have required a laboratory setting with bulky power supplies, oscilloscopes etc. Recently, Melcarek et al. (1977) have described the construction of a portable solid state device that can simultaneously monitor prompt and delayed fluorescence. The light source is a light emitting diode and the light detector is a HAV4000A large area sensor. With this apparatus they have measured the effects of temperature on the delayed fluorescence and prompt fluorescence of the leaves of chilling sensitive and chilling resistant plants (Melcarek and

Brown, 1977).

## Effects of Uncouplers and the Importance of the High Energy State

Uncouplers have been known for many years to affect delayed fluorescence (Mayne, 1968). The bulk of the data accumulated about these effects have been acquired by workers using the phosphoroscope technique and have measured the effect of uncouplers on millisecond-delayed fluorescence. Almost all uncouplers inhibit such delayed fluorescence. These data and data to be discussed below have led many investigators to believe that the so called "high energy state" is important in the generation of delayed fluorescence from photosynthetic organisms.

The term "high energy state" is a phrase that was first used to describe the conditions necessary for photophosphorylation by chloroplasts. The chemiosmotic theory, as formulated by Peter Mitchell (1961, 1966), states that the endergonic phosphorylation of ADP by inorganic phosphate involves a membrane-bound ATPase that is driven to make ATP by a pH gradient established across the membrane. This pH gradient is thought to be generated in plants by the light-driven oxidation of water and plastohydroquinone on the inside of the membrane (each reaction producing internal $H^+$). Recently it has become clear that a membrane potential due to the unequal distribution of cations and anions can also contribute

much of the energy required for ATP synthesis (Schuldiner et al., 1973; Graan, 1979).

Uncouplers of photophosphorylation are agents which abolish the phosphorylation capacity of chloroplasts without inhibiting electron transport. This is thought to occur by the abolishment of the pH gradient by making the membrane "leaky" to protons (Good, 1977). The ways in which uncouplers do this need not concern us here but will be dealt with in the next part of this thesis. Suffice to say that the hydrogen ion concentrations on the two sides of a thylakoid membrane are equal in the presence of uncouplers. Agents exist however which partition into membranes and can act as carriers of specific small cations. In the presence of such ionphores, membrane potentials may be abolished (Pressman, 1976).

## Crofts-Fleischman Hypothesis

In the presence of either uncouplers or ionphores, the millisecond-delayed fluorescence is diminished (Mayne, 1967). This observation led to the suggestion that the millisecond-delayed fluorescence is somehow stimulated by the high energy state, a suggestion which was supported by the further observations that chloroplasts, after being incubated in an acidic medium, emitted delayed fluorescence when the pH of the surrounding medium was rapidly raised and a transmembrane pH gradient was generated (Mayne and Clayton, 1965; Mayne, 1968). This delayed fluorescence induced by a pH increase

was abolished by uncouplers. Very soon thereafter, Miles and Jagendorf (1969) found that chloroplasts emitted delayed fluorescence when the external medium was exposed to a rapid increase in salt concentration. Such an event causes a membrane potential whenever the less permable anion lags behind the cation in the diffusion of the salt from outside to inside.

The above considerations induced Crofts to postulate (as quoted in Fleischman 1971; see also Crofts et al., 1971) that the pH and other ion gradients can stimulate delayed fluorescence. His proposal begins as a modification of Lavorel's equation (1968) and takes into account the observations of Arnold and Azzi (1971) on the glow peaks observed upon the warming in the dark of frozen, preilluminated chloroplasts:

$$L = \Phi J = -\Phi \frac{dN}{dt} = \Phi NFe^{-E_a/kT} \qquad (1)$$

where: L is the intensity of the light emission (in quanta)

$\Phi$ is the efficiency with which excited PS II chlorophyll fluoresces

J is the rate of the dark generation of excitons

N is the number of trapped electron or holes

F is a frequency factor containing rate constants and entropy terms

The exponential factor ($e^{-E_a/kT}$) is the fraction of the electrons that have enough energy to return to the chlorophyll singlet.

$E_a$ is the activation energy

k is the Boltzman constant

T is the absolute temperature

Since the electron transfer from the singlet to the acceptor occurs rapidly and at very low temperatures, there is probably no activation energy and $E_a$ may be the difference in energy between an electron in the acceptor and one in the chlorophyll singlet level (Fleischman, 1971).

The initial photochemical charge separation of photosynthesis probably occurs across the membrane, reducing Q to $Q^-$ toward the outside and oxidizing P680 to $P680^+$ toward the inside (Kraan et al., 1970). A membrane potential (positive toward the inside) might then facilitate the return of an electron from $Q^-$ to $P680^+$ and lower the activation energy required for this process. In considering this effect, Crofts modified equation (1) to

$$L = \Phi NFe^{-(E_a - \Psi)/kT} \qquad (2)$$

where $\Psi$ is the membrane potential.

To explain the stimulation of delayed fluorescence by a pH gradient, Crofts et al. (1971) suggested that the electron-carrying redox couples $Q/Q^-$ and $Z/Z^+$ are in equilibrium with pools of secondary donors and acceptors which are hydrogen-carrying redox couples. These pools are supposed to be themselves in equilibrium with the aqueous phases on the opposite sides of the membrane and their redox potentials dependent on the pH of these phases. Thus the

pH difference across the membrane could increase the availability of $Q^-$ or $Z^+$ (or $P680^+$) or both.

For thermodynamic reasons, Crofts et al. suggested that the effects of pH and $\Psi$ should be combined into a single equation using the concept of protonmotive force. As defined by Mitchell (1966), the protonmotive force (pmf) is the energetic term which drives phosphorylation and is given by:

$$pmf = \Psi - 2.303 \frac{RT}{F} \Delta pH.$$

Thus:

$$L = \Phi NFe^{-(E_a - pmf)/kT} \qquad (3)$$

Kraan et al (1970) have proposed a similar model for the effects of pH gradients on delayed fluorescence. They assumed however that Q and Z are themselves able to take up a proton when reduced.

Delayed fluorescence can also be induced by a pH-mediated reverse electron flow (Shahak et al., 1977). Abrupt changes in the pH of the medium are thought to alter the redox potential of the plastoquinone pool. Reduced molecules of plastoquinone may then reduce Q to $Q^-$ (with a corresponding increase in the fluorescence yield) in an electron transfer that is sensitive to DCMU and insensitive to DBMIB.

The Crofts-Fleischman hypothesis has been used extensively in the past decade to explain the alleged effects of the high energy state on delayed fluorescence. Since its promulgation however, there have been several hints that the hypothesis might be wrong. Neumann et al. (1973) and Felker et al. (1974)

showed that under certain conditions, uncoupling by ammonium salts enhanced millisecond delayed fluorescence instead of inhibiting it. However, such conditions might be merely substituting an ammonia gradient for a pH gradient because it was also shown (Felker et al, 1974) that under such conditions there is an uptake of $NH_4^+$. Furthermore, this perplexing stimulation of the delayed fluorescence was observed only with ammonia whereas methylamine, which seems to uncouple (and be taken up) in the same manner as ammonia, was inhibitory to millisecond delayed fluorescence.

In this thesis, a laser phosphoroscope capable of measuring the delayed fluorescence emitted within microseconds has been used to study the effects of uncouplers and phosphorylating conditions under steady-state conditions. This thesis also reports the first simultaneous determinations of the rate of electron transport (ferricyanide reduction) and the intensity of microsecond-delayed fluorescence.

In the course of the studies reported here it was observed that uncouplers and phosphorylating conditions increase rather than decrease the intensity of the delayed fluorescence when the fluorescence is measured less than 100 microseconds after light extinction. The stimulation of microsecond delayed fluorescence is correlated with the rate of electron transport. Nevertheless the often-observed inhibition of millisecond delayed fluorescence by uncouplers was confirmed. Thus uncouplers actually increase the fluorescence which is observed in the first 100 μs but speed

the decay in the capacity for delayed fluorescence during the next 900 μs.

Such results will be discussed in the light of the Crofts-Fleischman model and in the light of other models that deal with the decay kinetics of delayed fluorescence.

## METHODS

### Isolation of Chloroplasts

Chloroplasts were isolated from commerical spinach leaves by the methods of Ort and Izawa (1973) or of Hall et al. (1971). All procedures were carried out in a cold room at about 4°C and the isolated chloroplast preparations were stored on ice until used.

In the first method, approximately 30 g of fresh turgid spinach leaves (Spinacea oleracea L.) were washed with cold distilled water and the midribs removed. These leaf sections were then ground in a blender for 5 s in approximately 90 ml of a solution containing 0.3 M NaCl, 0.03 M Tricine-NaOH (pH 7.8), 3 mM $MgCl_2$, and 0.5 mM $Na_2EDTA$. After being filtered through thirty thicknesses of well-rinsed cheese-cloth, the homogenate was centrifuged for 2 min at 1600 x g. The chloroplast pellet was resuspended with an artist's brush in approximately 100 ml of a solution containing 0.2 M sucrose, 5 mM HEPES-NaOH (pH 7.4), 2mM $MgCl_2$, and centrifuged for 45 s at low speed (<1000 x g). This step was intended to remove chloroplasts that had clumped together as well as cellular debris. The green supernatant was then filtered through two layers of tissue (Kimwipes) and the filtrate centrifuged at 1600 x g for three minutes. The pellet was washed once and resuspended in a few ml of the

suspension medium with a final concentration of 1-2 mg chlorophyll/ml.

In the second isolation method (Hall et al., 1971), approximately 50 g of washed spinach leaves were cut into strips and placed in a blender. Before blending, 100 ml of a freshly prepared solution of 0.4 M sorbitol, 0.5 M MES-NaOH (adjusted to pH 6.5), 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1% crystalline bovine serum albumin, and 2 mM ascorbic acid was added. The mixture was then ground for 3 s and filtered through thirty thicknesses of rinsed cheesecloth. The filtrate was centrifuged for 90 s at 2900 x g and rapidly slowed. The chloroplast pellet was resuspended in a few ml of a solution containing 0.4 mM sorbitol, 50 mM HEPS (adjusted with NaOH to pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$ at a concentration of between 2 and 3 mg chlorophyll/ml.

This second method of chloroplast isolation was used because other experiments in our lab indicated that chloroplasts thus prepared had somewhat greater efficiencies of photophosphorylation. In the experiments reported here however, no noticeable differences were observed between the chloroplasts isolated by the two techniques. Therefore most of the experiments reported here were performed with chloroplasts isolated by the first, simple method.

To determine the chloroplyll concentration of the chloroplast suspension, a 0.1 ml aliquot of the suspension

was diluted with 10 ml of 80% acetone. This was mixed well and centrifuged at 3000 x g for five minutes. The absorbance of the resulting supernatant was measured at 645, 663, and 710 nm, with the 710 reading subtracted from the former values to correct for any "absorbance" not due to chlorophyll but rather due to some relatively wavelength-independent scattering. The concentration of the chlorophyll solution was calculated with the following formula:

$$(A_{663} \times 8.02) + (A_{645} \times 20.2) = \text{concentration of chlorophyll in } \mu g/ml$$

## Hydroxylamine Treatment

In one experiment, the electron transport-mediated oxidation of water was inhibited by pretreating the chloroplasts with hydroxylamine and EDTA. Such a treatment is thought to remove the Mn from the poorly-defined "water-splitting apparatus" and thus block $O_2$ production (Ort and Izawa, 1973).

For the treatment, chloroplasts were isolated by the first method and their concentration adjusted to around 100 µg/ml. A few ml of this suspension were centrifuged at 1600 x g for three minutes and the supernatant removed. The pellet was resuspended in the $NH_2OH$ treatment solution at a concentration of approximately 100 µg/ml. The $NH_2OH$ treatment solution contained: 0.2 M sucrose, 5 mM HEPES-NaOH (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA, and 3 mM $NH_2OH$. The

chloroplasts were incubated in this mixture for twelve minutes in the dark at room temperature. At the end of this time, the chloroplast suspension was diluted with the same cold suspension medium described for the first isolation procedure, pelleted, and washed twice with the same solution.

## Cyanide Treatment

In some experiments it was necessary to vary the rates of electron transport by pretreating the chloroplasts for varying lengths of time with a solution containing KCN (Ort et al., 1973). This pretreatment was done in an ice bath in the dark with 0.1 M sucrose, 0.1 M Tricine-NaOH (pH 8.0), 1 mM $MgCl_2$, 50 mM KCN, and 50 μM potassium ferricyanide. These treated chloroplasts were then added directly into the reaction mixture without removing the KCN, except by dilution.

Nigericin and gramicidin were added in ethanol. The final concentration in the reaction mixture was always less than 3%. This concentration of ethanol alone had no effect on the phenomena being studied.

## Laser Phosphoroscope

The laser phosphoroscope employed was a modification of the one described by Beall and Haug. Briefly, the light produced by a continuous output argon laser (principal lines at 488 and 515 nm) was focused by a microscope objective and chopped at the focal point by a notched disk rotating at 250 revolutions per s. This gave a cycle of 1 ms light and 1 ms dark with a light-dark transition time of less than 250 ns. The chopped beam was then collimated and directed through the bottom of a 1 x 1 x 4.5 cm quartz cuvette. Prompt and delayed fluorescence were measured at a right angle to the actinic beam by a single photomultiplier tube cooled with a solid $CO_2$. A 690 nm interference filter was placed between the cuvette and the photomultiplier to shield it from stray actinic light. The output of the photomultiplier, working into a 50Ω load, gave discrete pulses 1-3 ns in length, each pulse associated with individual photons striking the cathode surface. To measure the delayed fluorescence, only those pulses generated during a specified period after light extinction were counted. To do this, a timing circuit was activated at the instant of light extinction. This timing circuit created a counting "window" by opening a gate from the photon detector to the counter for a prescribed time. Under many circumstances this window opened 5 μs after light extinction and closed 10 μs after

light extinction. The pulses arriving during 10,000 of these gated counting periods were summed (one counting period every 2 ms for 20 s). The duration of the counting period and time of opening of the counting window could be varied independently so that the rate of decay of delayed fluorescence could be determined. When longer delays occured before the fluorescence was measured, the rate of photon counting was so much slower that the window was extended considerably, to 25 μs when the delay was 500 μs and to 100 μs when the delay was 1 ms. However, in the figures, the data presented have been corrected for this change in counting time. Prompt fluorescence was measured with the same photomultiplier by allowing the gate to open to the counter while the actinic light was still on.

Delayed fluorescence is reported as the ratio of counts during 5 μs divided by the prompt fluorescence in 5 μs for the following reasons: We are interested not so much in the actual fluorescence as in the thermochemical reactions responsible for the excitation of the chlorophyll. Since delayed fluorescence and prompt fluorescence seem to come from the same chlorophyll (see Introduction) it is reasonable to assume that the chemically-excited chlorophyll responsible for delayed fluorescence fluoresces with the same efficiency as the light-excited chlorophyll responsible for prompt fluorescence. Thus, dividing by the prompt fluorescence should help to correct for variations in the fluorescence yield of excited chlorophyll although, to be sure, the prompt

fluorescence yield was not measured and could not be measured at the same instant as the delayed fluorescence. Nevertheless these corrected values for delayed fluorescence should reflect the recombination reactions we wish to measure more accurately than the delayed fluorescence itself. (However, several assumptions are invoked in these corrections which are only probable and not certain.) The "corrected" values have the added advantage of normalizing the results of the various experiments since each data point represents a separate experiment and there are inevitable small variations in the actinic light intensity, chloroplasts density, and chloroplast condition.

In the same experiments electron transport was measured as the reduction of ferricyanide. This was done by observing the changes in the ferricyanide absorbance at 420 nm. A weak light was passed through a 420 nm interference filter before it passed through the cuvette. This beam was then detected with a photomultiplier which was protected by another 420 nm interference filter to screen out the 488 and 515 nm actinic light. The signal from the photomultiplier was processed by a logarithmic amplifier before it was fed into a strip chart recorder so that the rates of change in the concentration of ferricyanide could be directly determined and recorded.

In all cases the values given for delayed fluorescence intensity were the mean of 5 determinations. Prompt fluorescence was measured after 20 s of preillumination and

immediately before and after the delayed fluorescence determinations. All experiments were conducted at room temperature, 23°C. The average intensity of the light from the laser impinging of the reaction cuvette was approximately 80 $mW/cm^2$ (measured with a Yellow Springs Radiometer). The reaction mixture occupied a cubic volume 1 cm on each side.

The constrast ratio of the measurements of the apparent delayed fluorescence and actual prompt fluorescence observed when there was really no delayed fluorescence at all was determined by replacing the chloroplast-containing reaction mixture with a solution of chloroplyll in acetone. With this chlorophyll solution, the counts falsely attributed to the 5-10 μs delayed fluorescence were always less than one per 10,000 counts of prompt delayed fluorescence measured over the same length of time. The intensity of the delayed fluorescence from chloroplasts was routinely ten times or more higher than this noise level.

# RESULTS

Uncouplers of phosphorylation and phosphorylating conditions (the presence of ADP + $P_i$) increase the initial intensity of delayed fluorescence (Figures 1 and 2). This stimulation is largest with ammonia (over three-fold at 5 μs) and with ammonia persists up to a millisecond after light extinction. With the other uncouplers and with ADP + $P_i$ however, the increases in intensity seen at 5 μs are smaller and the rate of decay of the delayed fluorescene is larger. Thus at some time between 100 and 500 μs after light extinction the uncouplers and phosphorylating conditions begin to decrease the remaining delayed fluorescence intensity. It should be noted that these stimulations of delayed fluorescence are real and not artifacts of the method of correcting for the fluorescence efficiency of excited chlorophyll (see Methods) since they are of a similar magnitude whether or not such corrections are made. The results are the same when methylviologen is used as the electron acceptor for the Hill reaction instead of ferricyanide. The decay of the delayed fluorescence does not follow simple exponential kinetics, as other investigators have already reported (see Lavorel, 1975a). The log of the intensity of delayed fluorescence was not a linear function of the time after light extinction (see Appendix I, Figures 1 and 2). It is important to note however that

Figure 1 Delayed fluorescence in spinach chloroplasts in the presence and absence of the uncouplers, $NH_4Cl$, nigericin, methylamine-HCl, and gramicidin. Reaction mixtures contained in 1.0 ml: chloroplasts containing 5-10 µg chlorophyll; $MgCl_2$, 2.0 µmol; Tricine-NaOH (pH 7.8-8.1), 50 µmol; potassium ferricyanide, 0.5 µmol; sucrose, 100 µmol; gramicidin, 5µg; nigericin, 5µg. DF* is 1000 times the ratio of the number of delayed fluorescence photons counted in 5 µs in the dark to the number of prompt fluorescence photons counted in 5 µs in the light. At 500 and 1000 µs after the light was turned off, the periods of counting of delayed fluorescence photons were increased to 25 and 100 µs respectively with appropriate corrections made to express the data on the basis of 5 µs. Solid lines represent delayed fluorescence in the presence of the uncoupler and dotted lines represent delayed fluorescence in the absence of the uncoupler.

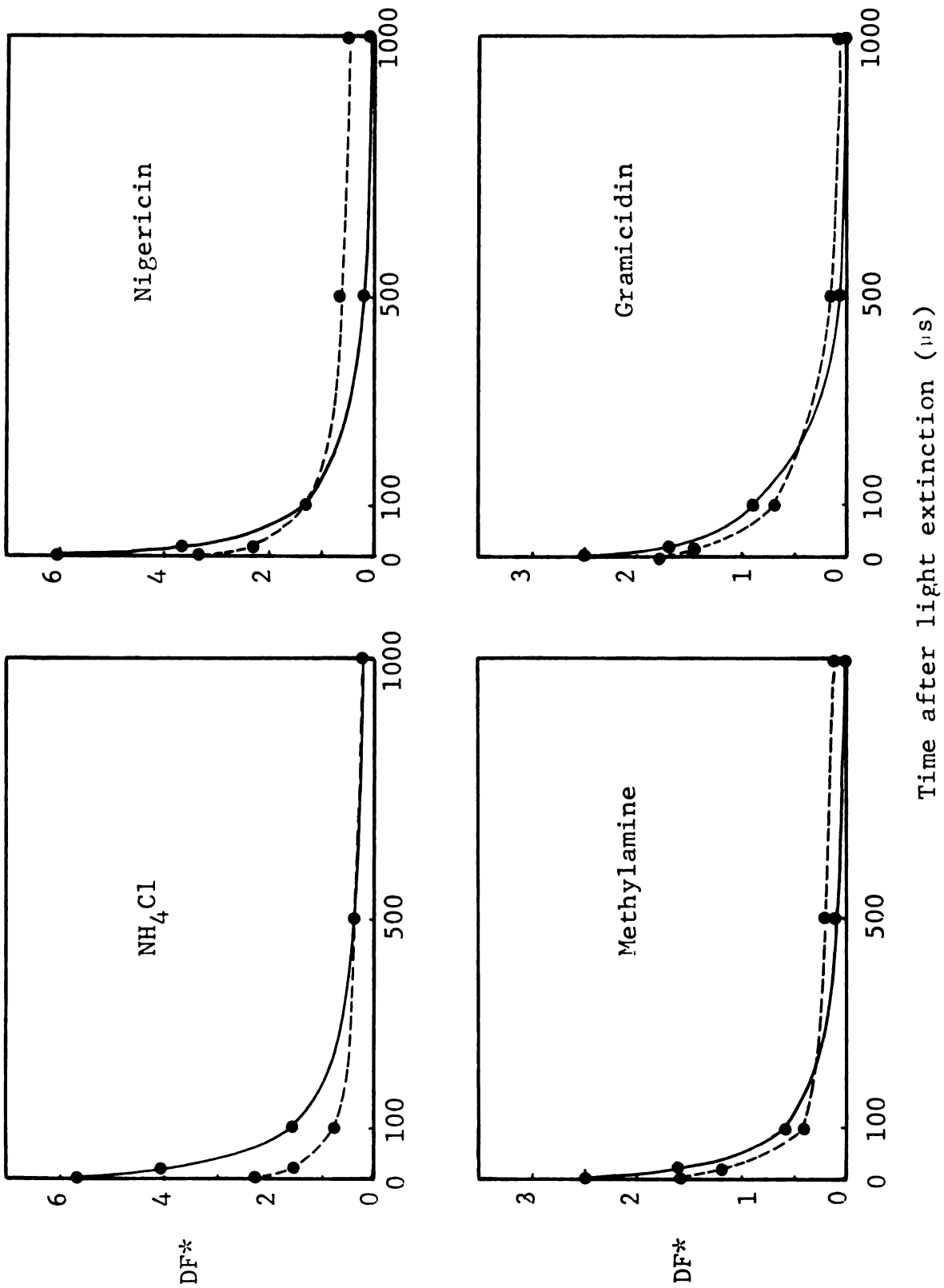
Nigericin
Gramicidin
$NH_4Cl$
Methylamine
DF*
Time after light extinction (μs)
0
100
500
1000
2
4
6
1
3

Figure 1.

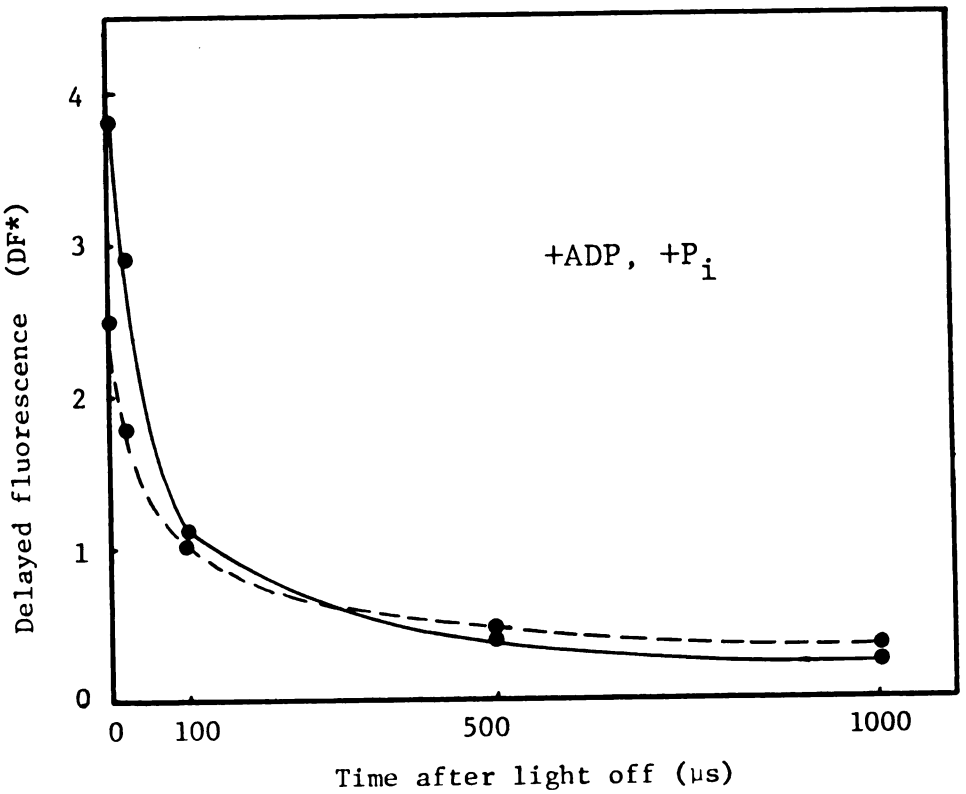


Figure 2 Delayed fluorescence in spinach chloroplasts in the presence and absence of ADP and orthophosphate. Reaction conditions and data presentations as in Figure 1. When added, ADP was 1.5 μmol, $K_2HPO_4$, 5 μmol.

the initial downward slopes of the logarithmic decays curves were nearly equal in the presence or absence of the uncouplers. It was only at longer times (>100 μs) that the rates of decay in the uncoupled chloroplasts were noticeably greater than in the controls. Unfortunately the method of measurement did not allow for the collection of enough data points to detect separate, distinct phases in the decay.

It is clear from these observations that the delayed fluorescence cannot be dependent on any uncoupler-inhibited state of the membrane system. On the contrary, some processes which are enhanced by uncouplers or by ADP plus $P_i$ must be responsible for delayed fluorescence. Since all uncouplers share with ADP + $P_i$ both the ability to increase the electron transport and the ability to increase 5 μs delayed fluorescence, a correlation between electron transport rates and the initial intensity of delayed fluorescence was sought. Table I of Appendix I confirms this correlation between electron transport rate and the intensity of 5 μs delayed fluorescence. In these experiments, electron transport was varied in three ways. (1) It was greatly diminished by omitting an exogenous electron acceptor. (2) It was almost abolished with the inhibitor DCMU. (3) Electron transport was greatly increased by the use of uncouplers in which case, as already noted, the increase in microsecond-delayed fluorescence was marked. In the first two cases with lowered electron transport rate, the intensity of μs delayed fluorescence was much lowered. In the case of DCMU, delayed

fluorescence was almost absent. It is particularly important to note that the increase in delayed fluorescence caused by uncouplers in the presence of an exogenous electron acceptor, did not occur when the electron trasport rate was limited by the absence of an exogenous electron acceptor. That is to say uncouplers do not increase the intensity of the μs-delayed fluorescence when the uncouplers fail to increase the electron transport rate.

Increases in the concentration of DCMU decreased both the electron transport rate and the intensity of the delayed fluorescence (see Figure 3). The insert of the figure illustrates the good correlation that exists between these two parameters. However the disparity between the slopes of the lines with and without the uncoupler gramicidin suggest that the uncoupler has some inhibitory effect which is in addition to its effect on electron transport. A linear relationship was also found between the rate of electron transport and the initial delayed fluorescence when the rate of electron transport was inhibited by cyanide (Figure 3, Appendix I).

This correlation between the electron transport rate and the intensity of the initial delayed fluorescence was not found when the electron transport was varied in other ways (i.e. by varying the light intensity, by inhibiting water oxidation, or by lowering the pH of the medium). Increases in light intensity gave increases in delayed fluorescence when the electron transport was already light-saturated

Figure 3 The effect of the electron transport inhibitor DCMU on the 5-10 μs intensity of delayed fluorescence in the presence and absence of gramicidin. Reaction conditions were the same as in Figure 1. Gramicidin when added was 5 μg. Closed circles indicate the presence of gramicidin and open circles indicate its absence.

Insert shows the correlation between DF* and the rate of electron transport under these conditions. The difference in slopes of the lines with and without gramicidin shows that gramicidin has some inhibitory effect on delayed fluorescence in addition to the effect arising from differences in electron transport rates.

Rates of electron transport (E.T.) determined simultaneously as ferricyanide reduction and are expressed as μmol ferricyanide reduced·$hr^{-1}$·mg $chlorophyll^{-1}$.

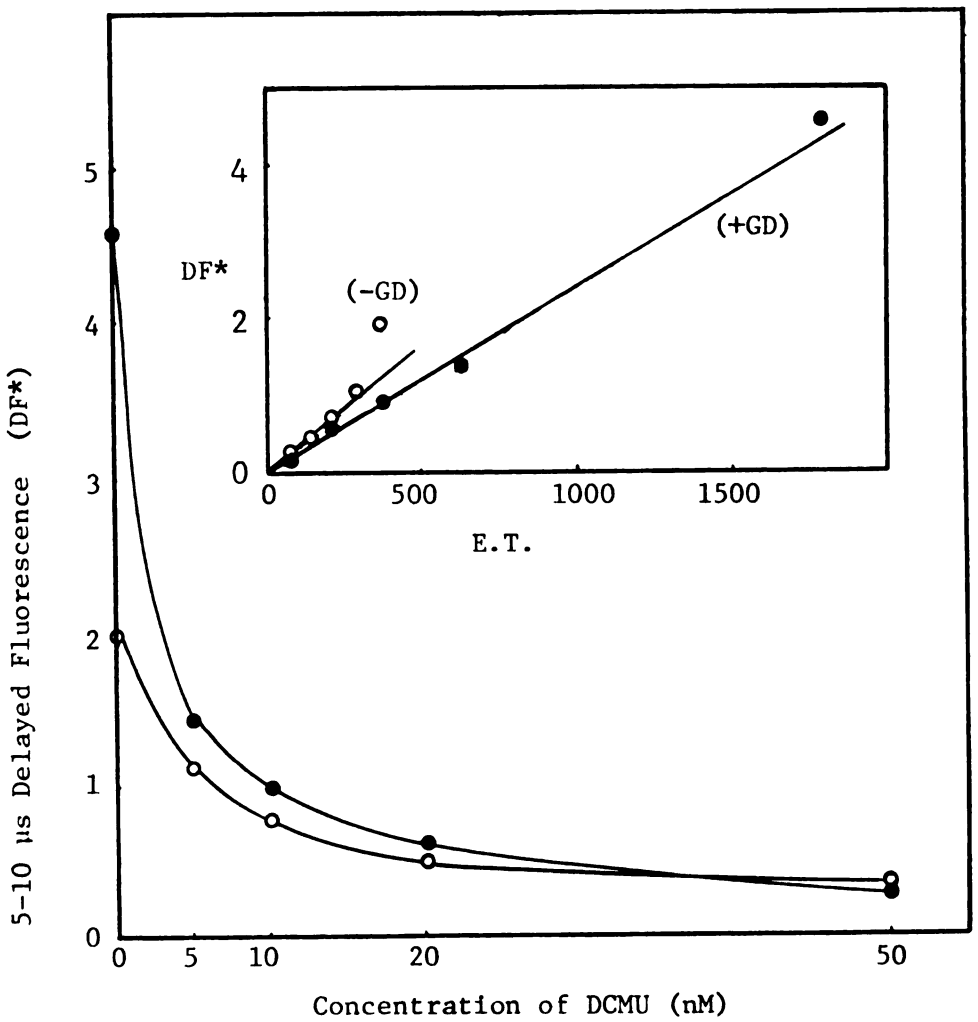
5-10 μs Delayed Fluorescence (DF*)
0
1
2
3
4
5
0
5
10
20
50
Concentration of DCMU (nM)
DF*
0
2
4
0
500
1000
1500
E.T.
(-GD)
(+GD)

Figure 3.

(see Figure 4 of Appendix I). This was true whether or not the chloroplasts had been uncoupled by gramicidin. Dramatic increases in "corrected" delayed fluorescence were observed in hydroxylamine-treated chloroplasts even though the electron transport is inhibited by such treatment. Even without the "correction" which may not be valid, there was little if any inhibition (see Table I). Finally, at pH 6.5, the large increases in the rates of electron transport due to uncouplers were associated with unchanged or decreased intensities of μs delayed fluorescence (see Figure 4).

TABLE I

Effects of an Inhibition of Electron Transport on Delayed Fluorescence when the Inhibition is on the Oxidizing Side of Photosystem II.*

| | | PF ($\times 10^{-3}$) | 5-10μs DF | 5-10μs DF* |
|---|---|---|---|---|
| experiment 1 | $-NH_2OH$ | 163 | 317 | 1.94 |
| | $+NH_2OH$ | 64 | 339 | 5.30 |
| experiment 2 | $-NH_2OH$ | 205 | 436 | 2.13 |
| | $+NH_2OH$ | 86 | 322 | 3.74 |
| experiment 3 | $-NH_2OH$ | 68 | 63 | 0.93 |
| | $+NH_2OH$ | 19 | 64 | 3.37 |

* Chloroplast suspensions with a chlorophyll concentration of 100 μg/ml were treated for twelve minutes in the dark at room temperature in a solution of 0.2 M sucrose, 5 mM HEPES-NaOH (pH 7.5), 2 mM $MgCl_2$, 1.0 mM EDTA, and 3.0 mM $NH_2OH$, an irreversible inhibitor of water oxidation. These chloroplasts were then diluted in the same medium (minus $NH_2OH$), centrifuged, and the pellet washed twice.

Reaction conditions were the same as Figure 1 except that methylviologen was substituted for potassium ferricyanide at a concentration of 200 μM in experiments 1 and 2 and at 50 μM in experiment 3. Absolute values of the directly-measured prompt fluorescence (PF) and delayed fluorescence (DF) are presented along with the computed values DF*. The greater inhibition of prompt fluorescence by the $NH_2OH$ treatment was much greater than the inhibitions of the 5-10 μs delayed fluorescence which suggests that the thermochemical excitation of the reaction center responsible for the delayed fluorescence was actually much more frequent in the presence of the inhibitors.

Figure 4 The rate of electron transport and the 5-10 μs delayed fluorescence intensity at pH's from 6.5 to 9.0 in the presence and absence of uncouplers. Values for the delayed fluorescence intensities (DF) were corrected for changes in the fluorescence efficiency as indicated by changes in the prompt fluorescence (PF) and are plotted in the corrected form, DF*. Reaction conditions as in Figure 1. Buffers were MOPS (pH 6.5, 7.0), Tricine (pH 7.5, 8.0), and TAPS (pH 8.5, 9.0) - all 50 mM. Solid lines indicate intensity of 5-10 μs DF* and dotted lines indicate rates of electron transport. Circles indicate the presence of uncoupler and triangles indicate its absence.

a) Concentrations of $NH_4Cl$ were varied at each pH so that the $NH_3$ concentration was constant at 0.355 mM.

b) Concentration of gramicidin was 5 μg/ml.

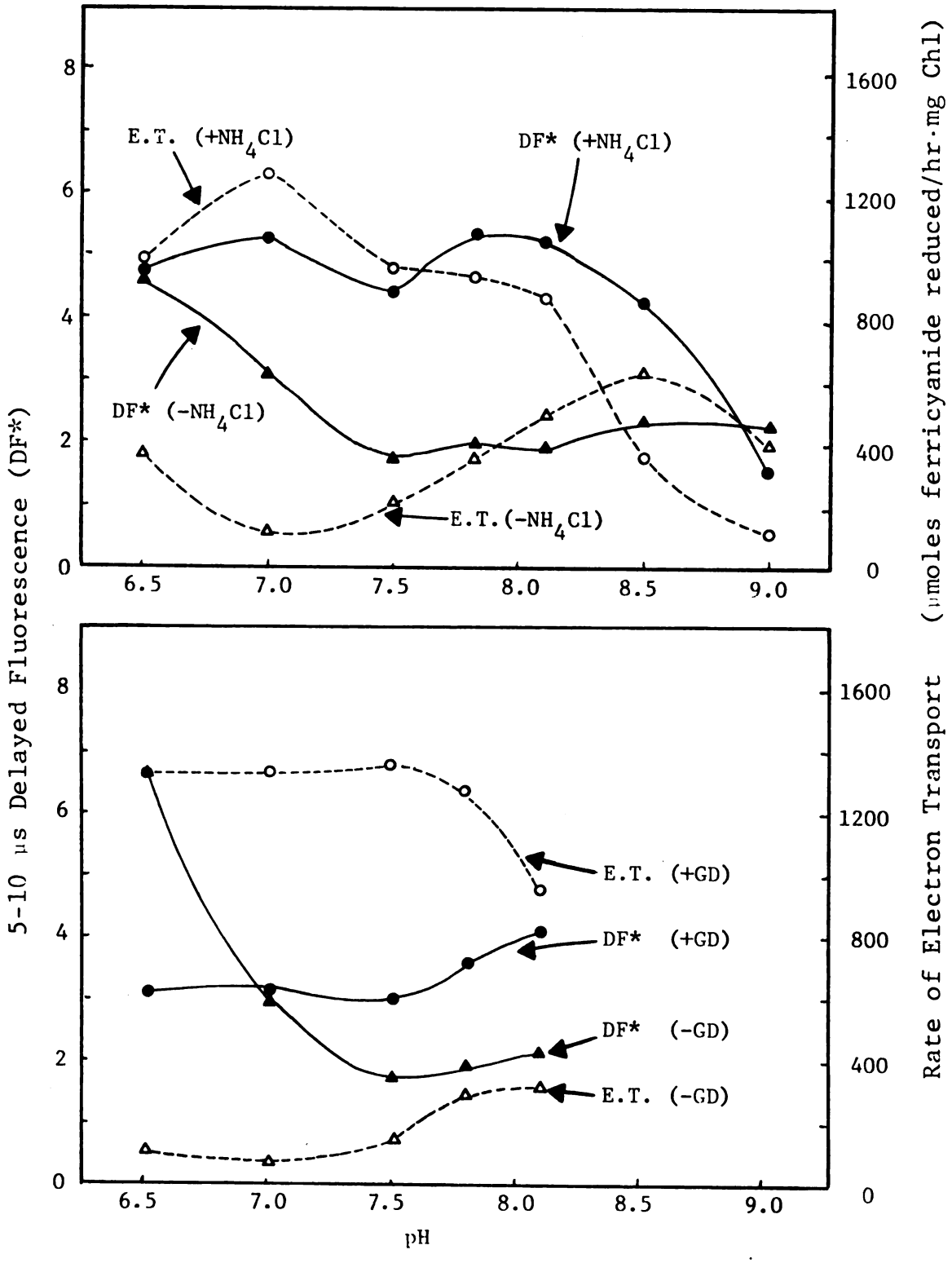
E.T. (+NH4Cl)
DF* (+NH4Cl)
DF* (-NH4Cl)
E.T.(-NH4Cl)
E.T. (+GD)
DF* (+GD)
DF* (-GD)
E.T. (-GD)
5-10 μs Delayed Fluorescence (DF*)
Rate of Electron Transport (μmoles ferricyanide reduced/hr·mg Chl)
pH
0
2
4
6
8
400
800
1200
1600
6.5
7.0
7.5
8.0
8.5
9.0

Figure 4.

# DISCUSSION

## The initial intensity of delayed fluorescence

In the preceding section, it was demonstrated that altering the rate of electron transport can have large effects on the initial intensity of the delayed fluorescence from spinach lamellae. The phosphoroscope technique was employed precisely because it allowed one to measure delayed fluorescence when the energization of the membranes and the redox levels of most of the electron carriers must have been close to their steady-state conditions. The dark period of the phosphoroscope (1 ms) was considerably shorter than the rate-limiting step of photosynthesis (relaxation half-time of 10-20 ms, (Stiehl and Witt, 1968; Emerson and Arnold, 1932). Therefore in the dark, the various pools of electron carriers probably were unable to return to their dark levels. This means that eventually a steady-state level of all of the pools of electron carriers would have been approached. Those other processes which are connected to electron transport (membrane potential, $\Delta$pH etc.) would presumably also reach a steady-state condition. Of course, a steady-state of the intermediates close to the photochemistry can never be reached under these conditions because of the alternating light and dark regime of the phosphoroscope. Redox levels of such intermediates at the end of the dark period may be drastically different from

those at the beginning of the dark period.

One limitation of the phosphoroscope technique that we have employed is that it is unable to measure the prompt fluorescence yield at the end of the dark period. It is thought that since Q is an efficient quencher of fluorescence, the fluorescence yield is a good indication of the levels of $Q^-$ (Duysens and Sweers, 1963). The corrected delayed fluorescence values reported here were on the basis of the fluorescence emitted before the light was turned off and so this correction properly applies only for the delayed fluorescence immediately after light extinction.

Another limitation of this phosphoroscope technique is that for reliable measurements, each point of any decay curve required a separate experiment. This made it impossible to obtain decay curves with enough data points to identify separate exponential phases of the decay (if such separate phases exist). Such distinct phases have been postulated on the basis of experiments with single flashes (see Lavorel, 1975b) and their interpretation will be discussed later.

The recombination hypothesis (see Introduction) suggests that the delayed fluorescence intensity is a function of the abundance of juxtaposed oxidized primary electron donors and reduced primary electron acceptors. The only significant reactions that create these species are light absorption, and the consequent excitation of the reaction center chlorophyll.

Uncouplers are thought to act directly on the various

gradients established across the membrane and indirectly on the rate of electron transport. Figures 1-4 show that uncouplers (and other conditions that stimulate the rate of electron transport) stimulate the initial intensity of the delayed fluorescence. Also several conditions which inhibit electron transport (DCMU, KCN) diminish the 5-10 μs delayed fluorescence to a corresponding extent. To explain these effects it is necessary to consider the points of rate-limitation in electron transport and how alterations in the rates at these points will alter the levels of $Q^-$ and $P680^+$ reached in the light period.

As mentioned above, the rate-limitation of electron transport comes between the two photosystems (at the point of the oxidation of plastohydroquinone) which has a relaxation half-time of around 20 ms (Stiehl and Witt, 1969). The exact nature of this limitation need not concern us here but will be dealt with later. Under the conditions employed here the dark phase of the occulation period (1 ms) was too short to allow much of the light-reduced plastoquinone to be reoxidized and it probably stayed mostly reduced. And since the plastoquinone pool was mostly reduced, it might be expected that those electron carriers which normally donate electrons to oxidized plastoquinone (such as $Q^-$) also stayed mostly reduced. That the levels of $Q^-$ were quite high is born out by measurement of the prompt fluorescence yield at the end of the phosphoroscope light cycle. (Lavorel, 1971).

The oxidized P680 is a different matter however. It must be remembered that electron transport is driven by the light-driven charge separations that occur at each photosystem. However no charge separations take place unless the reaction center is able to accept and use a quantum of energy from the light-harvesting complex. This requires that the reaction center be "open" - a condition in which the primary donor (P680) is reduced and the primary acceptor (Q) is oxidized. As mentioned in the previous paragraph however, under steady-state conditions employed here, the primary acceptor is likely to be mostly reduced and the reaction center associated with it therefore closed. To the extent that electron transport is occurring however, $Q^-$'s are being reoxidized to Q's and, if the corresponding P680's are reduced, a charge separation takes place and new $P680^+$ is formed. The reaction rates of the reduction of $P680^+$ are still uncertain. The most reliable data however suggest that the slowest step in this part of the electron transport chain is the oxygen-liberating step - a step that has a lifetime of approximately 1 ms (Kok et al, 1970). This means that there will certainly be some $P680^+$ at the end of the light period, the amount of $P680^+$ accumulated being dependent on the rate of electron transport. Since the levels of $P680^+$ and $Q^-$ at the end of the light period presumably determine the initial levels of delayed fluorescence, stimulations in electron transport rates might be expected to increase the initial intensity of the delayed fluorescence. Using similar

reasoning, it can be shown that reactions which slow down the electron transport between the two photosystems - such as cyanide treatment and additions of DCMU - might be expected to decrease the initial intensity of the delayed fluorescence. Figure 3 of this thesis and Figure 3 of Appendix I show this to be the case. When electron transport is limited by either of these inhibitors, there is a linear relationship between the initial intensity of delayed fluorescence and the rate of electron transport.

Experiments with $NH_2OH$-treated chloroplasts provide additional support for a model where the accumulation of $P680^+$ plays a major role in determining the intensity of the delayed fluorescence. Hydroxylamine treatment is thought to remove the Mn from the "hole collector" involved in the oxidation of water, thereby eliminating the donation of electrons from water to $P680^+$ (Ort and Izawa, 1973). That is to say, blocking the electron transport on the oxidizing side of photosystem II should give increased levels of $P680^+$ because the $P680^+$ formed in the light cannot be reduced. Since the rate of electron transport between the photosystems is no longer rate-limiting, the levels of $Q^-$ might be expected to be lowered somewhat and the decreased levels of fluorescence tend to support this notion. It is suspected however, that $P680^+$ is itself an effective quencher of prompt fluorescence (Mauzerall, 1972; Butler, 1972) so the decreased levels of prompt fluorescence do not necessarily indicate a significant decrease in the levels of $Q^-$ but may reflect

instead the increased levels of $P680^+$ or may reflect both factors. It should be mentioned that the quenching by $P680^+$ can be reasonably expected to affect the light-induced fluorescence and the chemically induced delayed fluorescence to a similar extent so that the correction for prompt fluorescence which has been used in this thesis is probably still valid and back-reactions responsible for the delayed fluorescence were probably increased by hydroxylamine treatment.

It now seems clear that the initial intensity of delayed fluorescence is a reflection of the rates of electron transfer to and from the reaction center of photosystem II which in turn affect the levels of $Q^-$ and $P680^+$ reached in the light period. It may be however, that these observed relationships apply only under a narrow range of conditions. For instance, lowering the pH from 8.1 to 6.5 in the absence of an uncoupler lowered the rate of electron transport but increased greatly the delayed fluorescence (Figure 4). In addition, the electron transport can be saturated with light but further increases in light intensity still result in increased intensities of delayed fluorescence (Figure 4, Appendix I).

It is possible that the delayed fluorescence at 5-10 μs may not reflect the relative amounts of $P680^+$ and $Q^-$ at the very instant of light extinction. It is still even possible that the effects of uncouplers on the 5-10 μs delayed fluorescence represent inhibitions of some component of a decay which has a time constant much less than 5 μs.

## Decay of Delayed Fluorescence

Most delayed fluorescence experiments heretofore reported can be put into two categories, those using phosphoroscope techniques and those using single flash techniques. Phosphoroscope experiments purport to look at the effect of "steady-state" conditions on delayed fluorescence - usually at a significant length of time after light extinction (milliseconds). Single flash techniques however look at the other side of the coin. Any build up of gradients or filling of electron-carrier pools (such as with closely-spaced flashes) is usually avoided but the time between the measurement and light extinction (microseconds) is several orders of magnitude less than with the phosphoroscope. The flash experiments attempt to look at the fast recovery kinetics of the reaction centers in order to establish the properties of the components associated with photosystem II. These two approaches leave a wide gap between them however, a gap which the experiments detailed here attempt to bridge. The laser phosphoroscope allows the measurement of fast decay kinetics under steady-state conditions. Such determinations are also possible with steady preillumination before a flash or with a rapid series of flashes and such an approach has recently been attempted (Jursinic et al., 1978). The results of these experiments will be discussed further.

In the preceding section we discussed the effect of uncouplers on the initial (5 μs) intensity of delayed

fluorescence and presented a model which attempted to explain these effects on the basis of rates of electron transport determining the levels of $P680^+$ and $Q^-$ formed in the light. In this section the electron transport-centered model is extended to explain the effects of uncouplers on the rate of decay of the intensity of the delayed fluorescence.

The major points to explain are:

a) Uncouplers have no effect on the rate of decay of the delayed fluorescence measured up to 100 μs after light extinction.

b) At times after light extinction greater than 100 μs, the rate of the decay of delayed fluorescence in the presence of uncouplers is greater than the rate of decay in the absence of uncouplers.

Before attempting to explain these observations, it is necessary to discuss briefly the meaning of a decay curve for delayed fluorescence.

As mentioned above, the intensity of delayed fluorescence at any time is thought to be a function of the number of reaction centers at any instant that have both a reduced primary electron acceptor ($Q^-$) and an oxidized reaction center chlorophyll ($P680^+$). Delayed fluorescence thus is considered to be a measure of the back-reaction of $Q^-$ and $P680^+$. If there are no reactions that compete with these back-reactions, the decay of delayed fluorescence would probably follow simple exponential kinetics, and a plot of

the log of the delayed fluorescence intensity versus time after light extinction would give a straight line. One observes however that the decay of delayed fluorescence is not a simple exponential but is more complex. An initially rapid rate of decay gives way to a slower rate of decay. However separate decay phases are not always easy to distinguish (see Figure 1, Appendix I). Deviations from simple exponential may be due to reactions of $Q^-$ and $P680^+$ other then the light-producing back reaction. The reduction of $P680^+$ by electrons from water and the oxidation of $Q^-$ by plastoquinone and ultimately by photosystem I are such reactions.

Detailed analyses of the various phases of the decay of the delayed fluorescence observed after saturating flashes of light have appeared quite often in the literature (Lavorel 1975b; Zankel. 1971) and are perhaps a good beginning point for this section of the discussion. However the conditions most often employed for these studies (single saturating flashes with dark-adapted chloroplasts or algae) are significantly different from those used here (phosphoroscope and steady-state electron transport conditions) so that the observations obtained in the earlier studies may apply in only a general way to the results reported here.

It is well known that the rate of reduction of $P680^+$ in the dark is significantly faster than the oxidation of $Q^-$. The values of these reactions are commonly obtained by such physical mean as rapid light and EPR spectroscopy, prompt

fluorescence, and $O_2$ yield.

$P680^+$ is rapidly reduced by the first secondary donor (Z) and the half-time for this reaction is probably around 30 ns (VanBest and Mathis, 1978). In fact, these two electron carriers may be in direct equilibrium. $Z^+$ is reduced by the "hole collector", with rates of reduction determined by the "S-state" of the hole collector. Half times for this reaction range from 100 μs ($S_o$, $S_1$) to 1 ms ($S_3$) (Babcock et al., 1976). This means that if one was to look at the reduction of $P680^+$, it would have an initial fast phase of reduction which reflects the donation of electrons from those Z's that are reduced. There would also be slower phases that reflect the reduction of $P680^+$ by Z's that were initially oxidized and reduce $P680^+$ after having accepted an electron from the hole collector.

On the Q side: The reoxidation of $Q^-$ is reported to have a half-time of about 0.6 ms (Forbush and Kok, 1968; Stiehl and Witt, 1969) whereas the reoxidation of half of the plastoquinone pool takes a much longer time, about 20 ms (Stiehl and Witt, 1968). The latter reaction is sensitive to uncouplers and Rumberg and Siggel (1969) report that the half-time value decreases to about 6 ms in the presence of 3μM gramicidin at pH 8.0. It should be emphasized that pool sizes are important when considering the oxidation of $Q^-$ by plastoquinone. On the basis of these data (Rumberg and Siggel 1969), it can be calculated that one millisecond after light extinction, approximately 1% of the

plastoquinone pool would have been reoxidized in the absence of gramicidin and over 10% would have been reoxidized in the presence of gramicidin. Estimates vary on the number of active plastoquinone molecules per Q molecule but even with a ratio of 5 to 1 (which means 10 electron equivalents per 1) at the end of one millisecond, the $Q^-$ might be expected to be about 10% reoxidized in the absence of gramicidin and 70% reoxidized in the presence of gramicidin.

To explain the observations of the effects of uncouplers on the rates of decay of delayed fluorescence it is necessary to remember that uncouplers increase the rate of oxidation of plastoquinone. Therefore it seems probable that the intensity of delayed fluorescence is determined by the amount of $P680^+$ during the first 100 μs, and increases in the rate of the oxidation of plastoquinone should have no effect on this initial decay. At longer times however, the amount of $Q^-$ has decayed to a point where it determines the rate of the back-reactions with the rare remaining $P680^+$ centers. Under these conditions, uncouplers which increase the rate of decay of $Q^-$ lower the intensity of the delayed fluorescence.

It seems therefore possible to explain the inhibition of the delayed fluorescence emitted at milliseconds after light off on the basis of their effects on electron transport rather than their effects on ion gradients.

The model presented here implies that membrane potentials have no appreciable effect on the microsecond delayed fluorescence. This corroborates the observation of Jursinic et al.

(1978) that uncouplers had no effect on the microsecond delayed fluorescence observed after a single flash with no preillumination.

In this section of the thesis a model has been presented which attempts to explain the effects of uncouplers on delayed fluorescence on the basis of their effects on the rate of electron transport between the two photosystems. It should be emphasized however that the quantum efficiency of delayed fluorescence is extremely small ($10^{-4}$). It may be that all of delayed fluorescence represents aberrant processes which have little to do with normal photosynthesis.

# SECTION II

# THE EFFECTS OF AMINES AND OTHER UNCOUPLERS ON ELECTRON TRANSPORT, ATP SYNTHESIS, AND $P/E_2$

# INTRODUCTION

This section of the thesis deals with uncoupler effects that are probably more direct than their effects on delayed fluorescence - namely their effects on the rates of electron transport, the rates of phosphorylation and on the magnitude of pH gradients. The literature pertaining to uncouplers is quite extensive and has been periodically reviewed (Good et al., 1966; Gomez-Payou and Gomez-Lujero, 1977; Good, 1977). This section will present the basic phenomena attributed to uncouplers and briefly discuss the accumulating data which suggest that unified theories of amine uncoupling should be reconsidered.

It is impossible to discuss uncouplers in the absence of a discussion of photophosphorylation. The coupling intimated by the term "uncoupler" is between electron transport and the synthesis of ATP. The widely accepted chemiosmotic theory (Mitchell, 1966) states that these two processes are both intimately involved with a pH gradient across the lamellar membrane and, indeed, electron transport generates a pH gradient and ATP synthesis can be induced by a pH gradient. The evidence for these statements will be briefly discussed.

## Electron Transport Generates a pH Gradient

The primary evidence is:

a) If chloroplasts are allowed to perform either noncyclic or cyclic electron transport in a weakly-buffered solution, there is a light-dependent rise of the pH of the external medium (Neumann and Jagendorf, 1964).

b) Monofunctional amines with high pK's are taken up by chloroplasts during electron transport. This has been shown either by monitoring the disappearance of a radioactive amine from the external medium or by measuring the internal concentration of a radioactive amine after rapidly centrifuging the chloroplasts through a layer of silicone (Portis and McCarty, 1976). The uptake of the amines is thought to be a result of the rapid equilibration across the membrane of the lipid-soluble free base and the unequal distributions of the charged and uncharged amines due to the different pH's of the separate sides of the membrane. Assuming ready equilibrium of the uncharged amine, it can easily be shown that $[H^+]_{in}/[H^+]_{out} = [\text{charged amine}]_{in}/[\text{charged amine}]_{out}$.

c) A pH-indicating dye (neutral red) can be allowed to enter the internal space of the chloroplast lamellae and when pH changes in the external medium are prevented by buffering, changes in the dye absorbance are presumed to indicate changes in the pH of the internal space (Auslander and Junge, 1975). This approach is very speculative however because of problems of binding of the dye and the resultant

change of its pK.

The above observations led to the suggestion that both noncyclic and cyclic electron transport involve an electron carrier which accepts a proton when reduced on the outside of the membrane and donates a proton when oxidized on the inside of the membrane. One such electron and proton carrier is presumed to be a plastoquinone because:

a) The number of active plastoquinone molecules is greater than those of any other member of the electron transport chain and this pool may be large enough to span the membrane. It is also possible that the plastoquinone which is lipid-soluble may be able to diffuse across the membrane (Witt, 1979).

b) The kinetics of internal $H^+$ release parallel the kinetics of the oxidation of plastohydroquinone over a range of 20-fold variation (Tiemann et al., 1979).

c) The number of protons taken up from the external phase increases in parallel with the number of electrons taken up by the plastoquinone pool (Tiemann et al., 1979).

## A pH Gradient Generates ATP

Much data has been accumulated that suppports the hypothesis that a pH gradient can drive ATP synthesis (see Jagendorf, 1977). Chloroplasts synthesize ATP from ADP and $P_i$ when a pH gradient is imposed across their membranes. The

gradient can be formed by incubating the chloroplasts at a low pH and then rapidly raising the pH of the external medium (Jagendorf and Uribe, 1966). This acid-base induced ATP synthesis is sensitive to internal buffering, weak amines which store protons giving greater yields of ATP.

Another indication of the connection between the pH and ATP synthesis is the observation that under suitable conditions, ATPase, will hydrolyze ATP with a concomitant "pumping" of protons into the internal aqueous space (Crofts, 1966).

Experiments in several laboratories have shown, however, that whereas a proton gradient of 2 to 3 units is sufficient to drive ATP synthesis, such a large pH gradient is not always necessary and that a membrane potential can contribute much of the energy required for ATP synthesis (Schuldiner et al., 1973; Graan, 1979).

## Uncoupling and the Control of the Rate of Electron Transport

The rate of noncylic electron transport varies with pH and is greatest between pH 8.0 and 8.5 in the absence of uncouplers. This maximum shifts to around pH 6.5 in the presence of uncouplers (Good et al., 1966). It is well known that a main rate-limiting step in electron transport is located between the two photosystems and probably reflects the rate of oxidation of plastohydroquinone. It would not

be surprizing if the step were sensitive to pH because the oxidation of $PQH_2$ releases protons, and a high concentration of $H^+$ (low pH) might exert a back-pressure on this reaction. Some investigators have found that the controlling factor is the internal pH (Rumberg and Siggel, 1969; Siggel, 1974). However their conclusions were based on the assumption that pH gradients are completely abolished by 3 μM gramicidin, an assumption that was not subsequently supported by the pH determinations of Portis and McCarty (1976). Bamberger et al. (1973) have suggested that the controlling value is instead the average of the internal and external pH's. However as was shown by Ort (1976) with dark-adapted chloroplasts, the initial rate of electron transport is rapid but slows to steady-state rates within 50 ms. The addition of a permeant buffer which presumably prevents any rapid decrease in the pH of the internal space, does not significantly delay the time at which this slowdown occurs. Thus the pH of the inner aqueous phase may have nothing to do with the primary control of electron transport.

A general definition of uncoupling is any treatment which inhibits phosphorylation without causing corresponding inhibitions in electron transport, or increases electron transport without causing a corresponding increase in phosphorylation (Good, 1977). However such a definition is not completely satisfactory because the omission of ADP or $P_i$ or the addition of an energy transfer inhibitor, by inhibiting phosphorylation would act as "uncoupling" of

chloroplasts performing phosphorylation at site II only, since the rate of electron transport at this site is usually independent of phosphorylation (Gould and Izawa, 1973). Uncoupling is therefore better defined as the destruction of the energized state of the lamellar membrane (Good, 1977). If, in fact, electrochemical gradients are required for phosphorylation, then uncouplers are agents which make the membrane "leaky" to protons and other ions.

The uncoupling effects of coupling factor removal (which forms nonspecific holes in the membrane- Avron, 1963) and phosphate analogs (which replace $P_i$ to form unstable intermediates with ADP- Avron and Jagendorf, 1959; Avron and Shavit, 1965) are more malfunctions of the coupling factor than of the membrane per se and will not be considered here.

## Uncouplings by Amines

In 1959, Krogmann et al. reported that ammonium salts can act as uncouplers. It is now known that many amines with membrane-permeating unprotonated (hence uncharged) forms can abolish pH gradients across the lamellar membranes of illuminated chloroplasts (Good, 1960; Hind and Whittingham, 1963). The acidification of the inner space which would be anticipated in the absence of an amine is thought to cause a decrease in the internal concentration of the free base of the amine. Since the internal concentration of the free

base has been lowered, a gradient is established and more of the free base diffuses across the lamellar membrane and into the inner space. However, this uptake does not by itself abolish the acidification of the internal space. Since an uptake of the uncharged form of the amine fails to counteract the uptake of positive charges (proton), the internal space becomes positively charged; this charge may be sufficient to drive the charged protonated form of the amine back out of the chloroplast and it is this influx of unprotonated amine and efflux of protonated amine which effectively abolishes the acidification of the interior. In the presence of permeant anions, the uptake of the uncharged amine is accompanied by an uptake of an anion and there may be no increase in the net positive charge of the internal space sufficient to expel the protonated amine. Thus in the presence of permeant anions, amines cause a swelling of the lamellae but often fail to prevent the acidification of the interior, and therefore uncouple less effectively.

## Uncoupling by Weak Acids

Uncoupling can be accomplished by weak acids if their anions are relatively lipid soluble. In this case there is no uptake of the uncoupler but rather the uncoupler shuttles protons across the membrane. Carbonylcyanide phenylhydrazones (e.g. CCCP and FCCP) are perhaps the best examples of these since they uncouple effectively at low concentrations. Indophenols at lower pH's which allow the formation of some

of the red, unprotonated form, also seem to uncouple in this manner but they are not nearly as effective. They are more commonly used as electrons acceptors which only incidentally uncouple and then only partially, depending on pH.

## Uncoupling by ionophores

Ionophores are a group of lipid-soluble molecules with backbones of diverse structures that contain strategically-placed oxygens. The conformation of the molecules are such that the oxygens form a central ring or cavity in which small cations may become sequestered (Pressman, 1976). This sequestering effectively buries the charge of the cation within the ionophore molecule with the result that they can often carry ions while at the same time being lipid soluble. The structure of the ionophore imparts an element of selectivity in the cations that can be transported. Those ionophores of greatest interest to uncoupling can transport protons and other small univalent cations. Nigericin catalyzes an exchange of $H^+$ for $K^+$ across the membrane.

## Uncoupling by Quasiionophores

Though often considered along with ionophores just mentioned, gramicidin is not an ionophore in the stricktest sense. It forms ion-conducting channels across the full thickness of membranes (Pressman, 1976). These pores are

also very specific for inorganic monovalent cations, and, since the action of gramicidin does not depend on its diffusion across the membrane, uncoupling can be very effective.

## Unconventional Uncoupling

Many other substances can uncouple chloroplasts and their mechanisms are often not well understood (for a review, see Good, 1977). The most interesting of these is atebrin, an aliphatic amine uncoupler containing an aromatic ring. It clearly has some mechanism distinct from that of simple amines for several reasons: a) Uncoupling by atebrin is accompanied by a shrinking of the chloroplasts instead of a swelling; and b) Atebrin is reputed to uncouple even while bound to large sepharose beads (Kraayenhof and Slayter, 1975). This second effect has also been found with long alkylamines (n>12) bound to sepharose (Lotina et al., 1979).

There are striking differences among the effects of uncouplers and, although many abolish gradients by increasing the rates of ion diffusion, different types of "leaks" must be involved. Some uncouplers interact in ways which cannot yet be explained.

There seems to be a hierarchy of shrinking and swelling effects: carbonylcyanide phenylhydrazones abolish all volume changes whether or not amines or atebrin are present. Amines cause swelling whether or not atebrin is present. Also the

removal of coupling factor in no way interferes with the shrinking or swelling induced by atebrin or amines although chloroplasts uncoupled by removal of coupling factor alone neither shrink nor swell.

Chloroplasts have different sensitivities to uncouplers under different conditions and changes in conditions may not change these sensitivities in parallel. Under steady-state conditions, low concentrations of CCCP inhibit phosphorylation quite effectively at low light intensities while at higher light intensities the same concentrations have little effect (Saha et al., 1970). Octylamine and FCCP inhibit ATP synthesis with short flashes of light at concentrations that hardly effect steady-state phosphorylation (Ort, 1978) and acid-base phosphorylation driven by small pH changes is much more sensitive to proton-carrying uncouplers than is steady-state phosphorylation (Graan, 1979).

These intriguing differences were a stimulus for the studies reported here on the effects of uncouplers on electron transport and photophosphorylation. Since there is a dearth of reports in the literature on surveys of the effects of uncouplers at different light intensities and pH's etc., such an investigation was though advisable as a starting point.

## METHODS

Isolation of chloroplasts was as described in the Methods section of Section I.

Noncyclic electron transport was measured as the reduction of ferricyanide and was measured as the decrease in absorbance at 420 nm. Illumination was provided by the 500 watt bulb of a slide projector the light of which was filtered by a dilute copper sulfate solution (to screen out infrared radiation) and a red glass filter. Temperature was maintained at 18°C with circulating water from a thermostatted water bath through a solid brass cuvette holder. The red actinic light was kept out of the 420 nm measuring device by the use of a complementary blue filter.

ATP synthesis was measured as the radioactivity remaining after the extraction with organic solvents of the unreacted $^{32}P_i$ as phosphomolybic acid. This was done by removing a 1.0 ml aliquot of the mixture and mixing it with 9.0 ml of cold 10% perchloric acid saturated with hexanol in a 20 mm x 150 mm pyrex test tube. To this was added 1.0 ml of 20% (w/v) solution of ammonium molybdate and the solution was stirred well for 60 s with a glass plunger. After five min, 16 ml of hexanol (saturated with 10% perchloric acid) were added and stirred with a glass plunger. After the two layers had separated the top (organic) layer was removed by suction and the lower layer was subjected

to gravity filtration through pre-wetted filter paper. This filtration step removed particulate matter and any remaining minute drops of the organic (and highly radioactive) layer. To the filtrate was added 0.1 ml more of the ammonium molybdate solution and another 16 ml of the hexanol solution. After 60 s of vigorous mixing, 0.1 ml of 0.1 M $Na_2HPO_4$ was added to the test tube in order to facilitate the removal of the remaining ammonium molybdate. After 5 min, the two layers were again mixed, the phases allowed to separate, and the top layer removed with suction. If the bottom solution showed any traces of a yellow color, it was once more extracted with hexanol and the top layer removed.

Radioactivity in the final aqueous phase was determined by counting the scintillations due to Cerenkov radiation and counts associated with ATP were made by comparisons with accurate dilutions of the $^{32}P_i$ stock.

## RESULTS AND DISCUSSION

As a starting point for these studies of the effects of uncouplers, a series of experiments were conducted to determine the effective concentrations at pH's from 6.5 to 9.0. In these studies, basal (non-phosphorylating) and coupled electron transport, ATP synthesis, and phosphorylation efficiency ($P/e_2$) were determined (Figure 5).

The major observations were :

a) As has long been known, basal and coupled electron transport in the absence of uncouplers have their maximum rates between pH 8.0 and 8.5. $P/e_2$ has a broad maximum between 8.0 and 9.0.

b) The highest rates of electron transport were obtained at pH 6.5 when the concentrations of the uncouplers were high enough to give maximum rates at the pH investigated.

c) All uncouplers gave their largest stimulations of electron transport at the lower pH's. This is, in part, because of basal electron transport is then so slow.

d) At pH's well above their pK's, amines did not uncouple even though they were effective uncouplers at or below their pK's.

e) In the presence of some amines and at some pH's large increases in electron transport were observed with little or no decrease in ATP synthesis.

Figure 5 Effects of uncouplers on electron transport and photophosphorylation at pH's from 6.5 to 9.0. Reaction mixtures contained in 2.0 ml: chloroplasts with 30 µg chlorophyll; sorbitol, 0.4 µmol; $MgCl_2$, 4.0 µmol; potassium chloride, 20 µmol; potassium ferricyanide, 1.0 µmol; ADP, 1.5 µmol; $K_2H^{32}PO_4$, 10 µmol. Buffers used were MOPS-NaOH (pH 6.5, 7.0), Tricine-NaOH (pH 7.5, 8.0), and TAPS (pH 8.5, 9.0) - all 100 µmol.

For determinations of electron transport in the absence of phosphorylation (open circles), the $K_2H^{32}PO_4$ and the ADP were omitted (a,b,c,d) or both the $K_2H^{32}PO_4$ and the ADP were omitted (e,f, g,h,i,j). $P/e_2$ (closed circles) represents the phosphorylating efficiency of the electron transport; the number of molecules of ATP formed for each pair of electrons transferred from water to ferricyanide. The phosphorylation rates (as µmoles ATP formed/hr·mg Chl) are indicated by closed triangles and the rates of electron transport when phosphorylation is occurring (as µmoles ferricyanide reduced/hr·mg Chl) are indicated by open triangles.

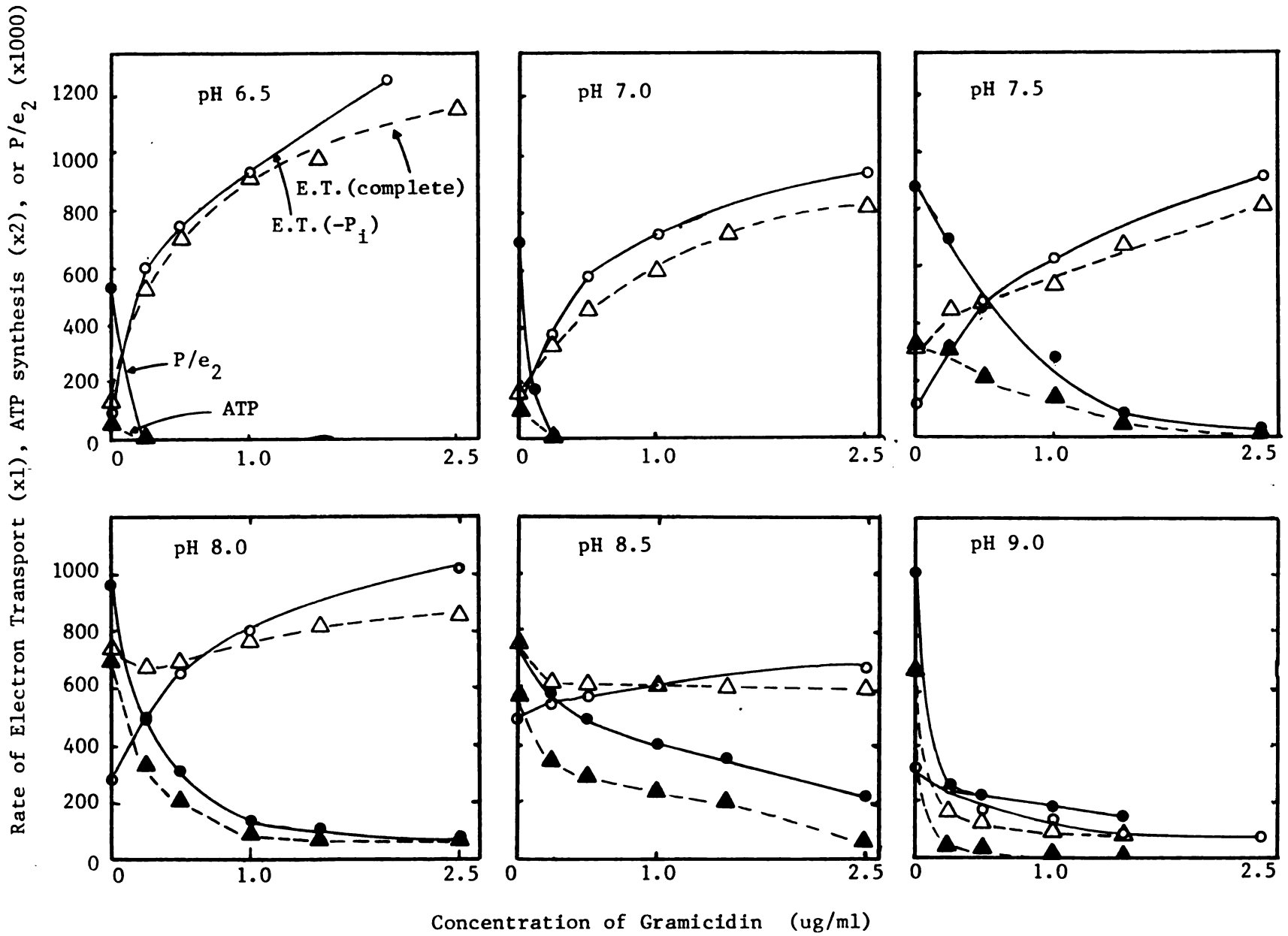
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T.(complete)
E.T.(-P$_i$)
P/e$_2$
ATP
Rate of Electron Transport (x1), ATP synthesis (x2), or P/e$_2$ (x1000)
Concentration of Gramicidin (ug/ml)

Figure 5a.

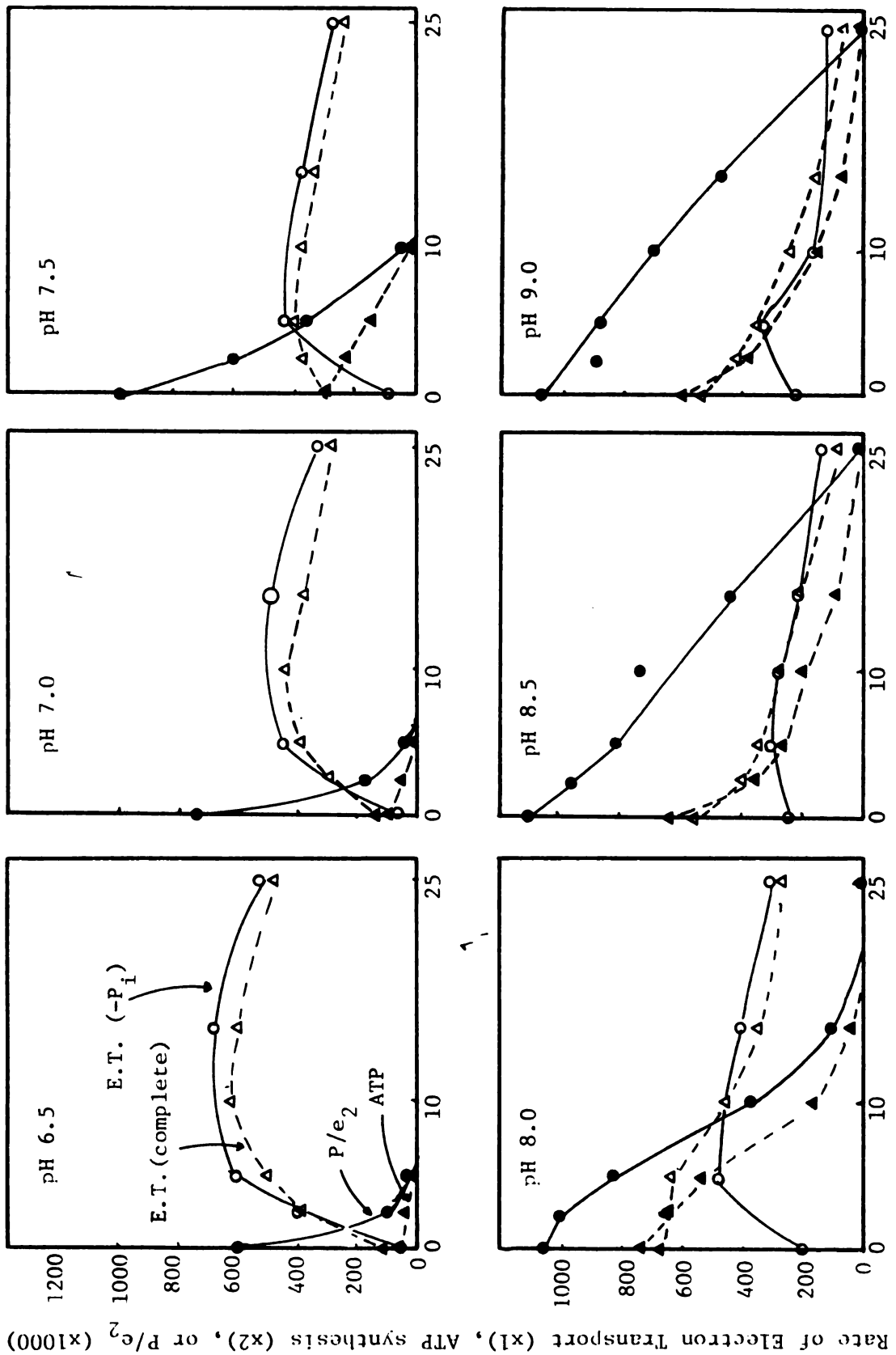
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T. ($-P_i$)
E.T. (complete)
$P/e_2$
ATP
Rate of Electron Transport (x1), ATP synthesis (x2), or $P/e_2$ (x1000)
Concentration of 2,4 lutidine (mM)

**Figure 5b.**

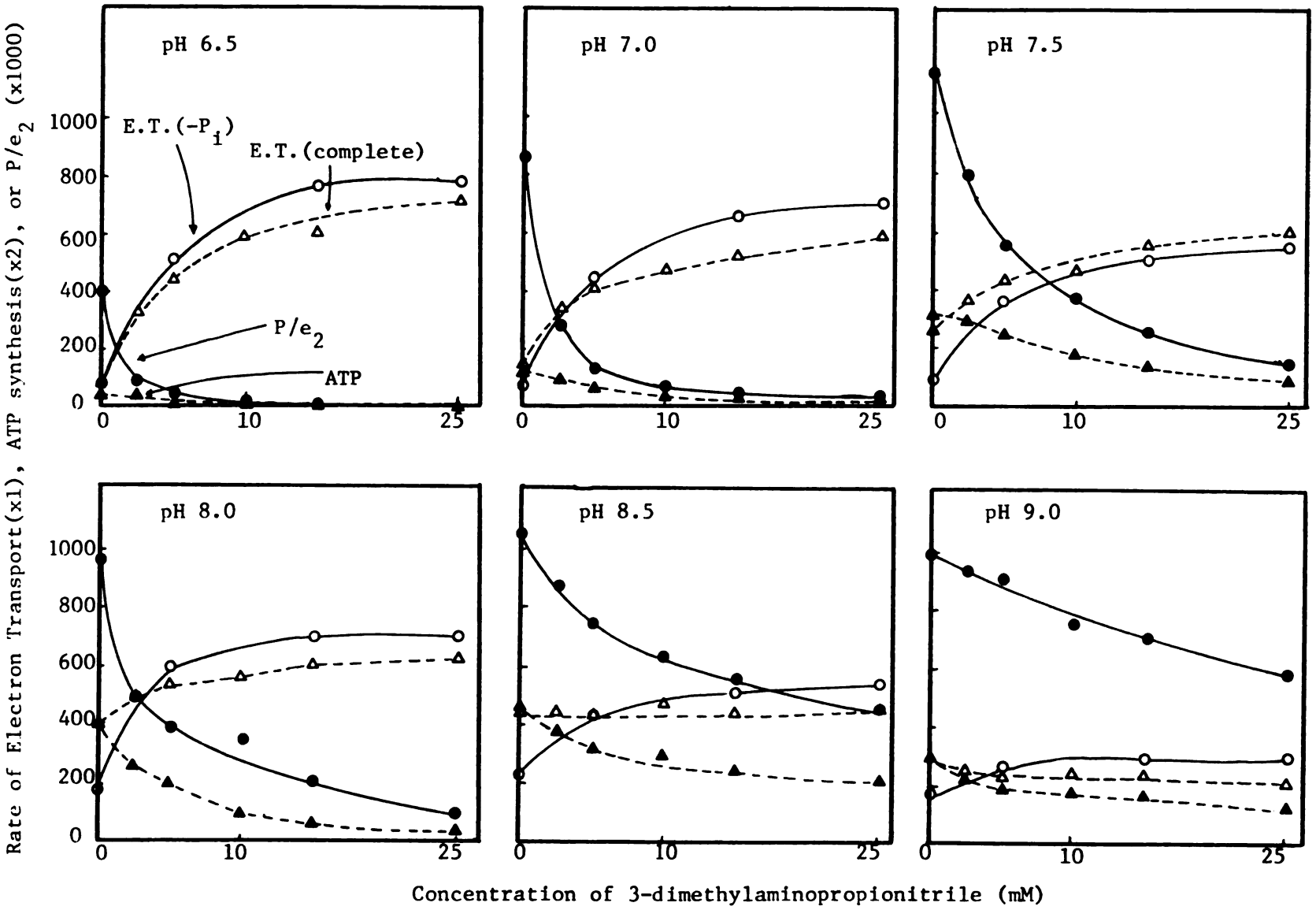
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T.(-P$_i$)
E.T.(complete)
P/e$_2$
ATP
Rate of Electron Transport(x1), ATP synthesis(x2), or P/e$_2$ (x1000)
Concentration of 3-dimethylaminopropionitrile (mM)

Figure 5c.

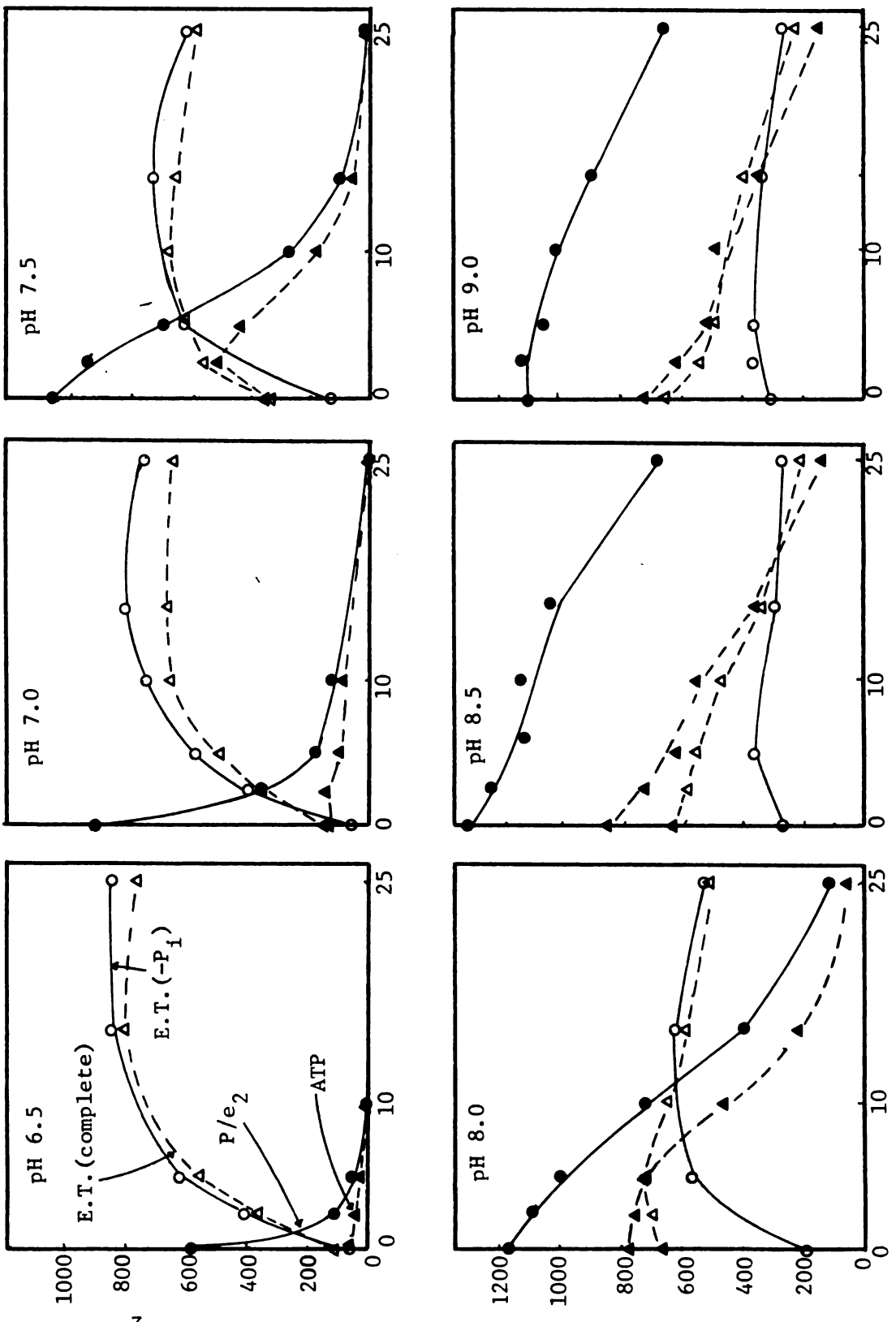
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T. (complete)
E.T. (-$P_i$)
P/$e_2$
ATP
Rate of Electron Transport(x1), ATP synthesis(x2), or P/$e_2$ (x1000)
Concentration of 2,6 lutidine (mM)

Figure 5d.

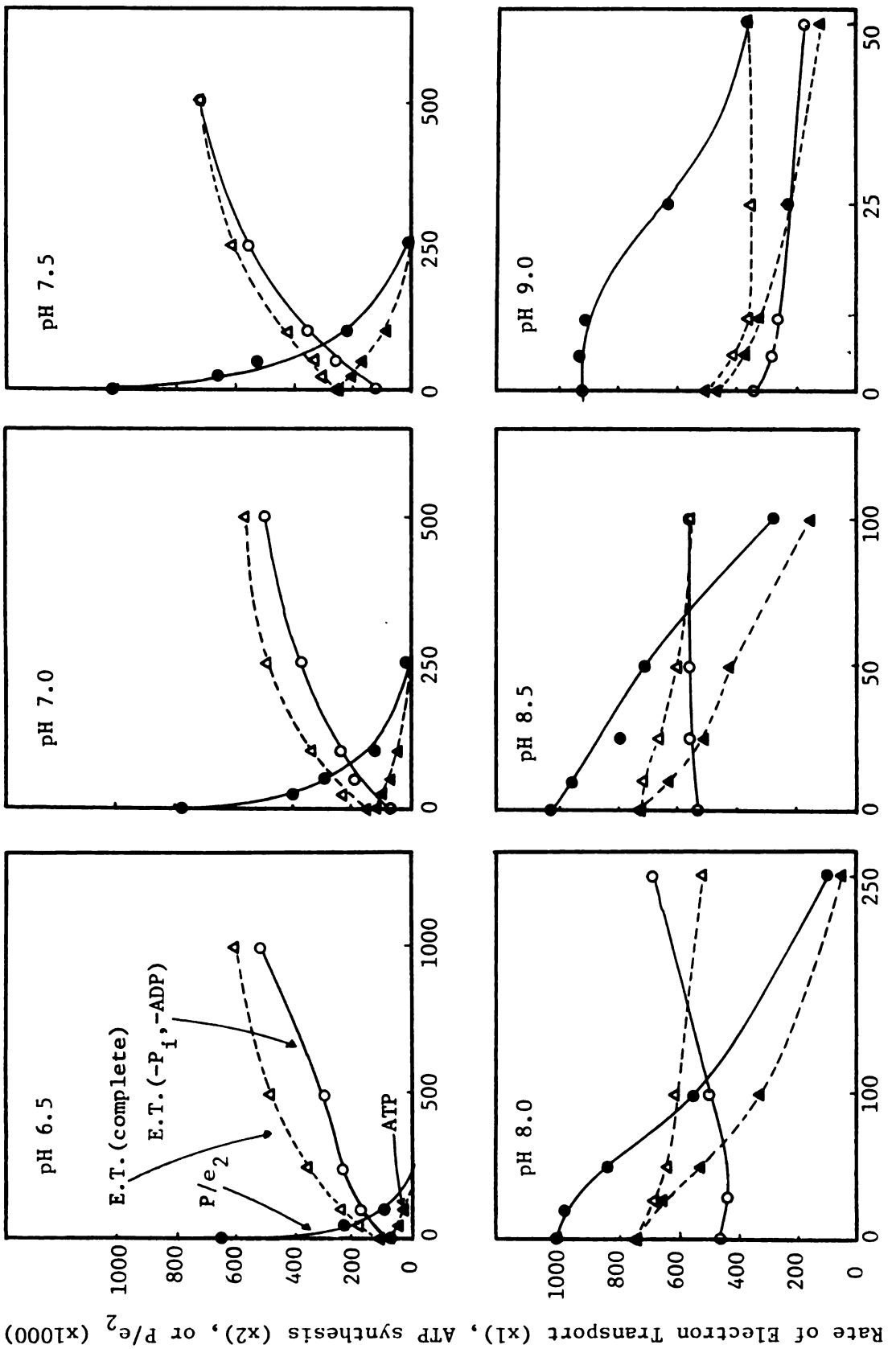
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T.(complete)
E.T.(-$P_i$,-ADP)
P/$e_2$
ATP
Rate of Electron Transport (x1), ATP synthesis (x2), or P/$e_2$ (x1000)
Concentration of octylamine (μM)

Figure 5e.

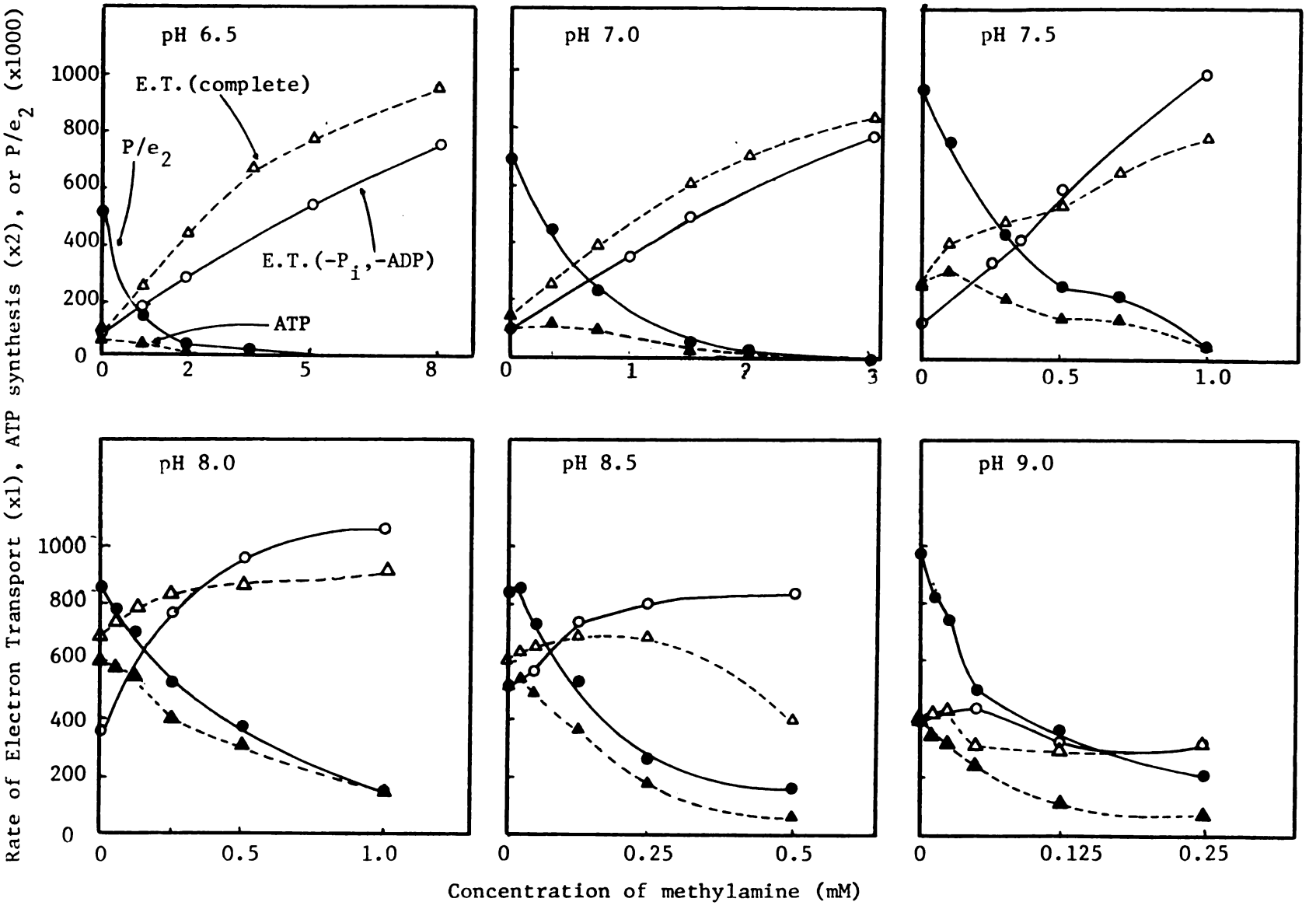
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T.(complete)
P/e2
E.T.(-Pi,-ADP)
ATP
Rate of Electron Transport (x1), ATP synthesis (x2), or P/e2 (x1000)
Concentration of methylamine (mM)

Figure 5f.

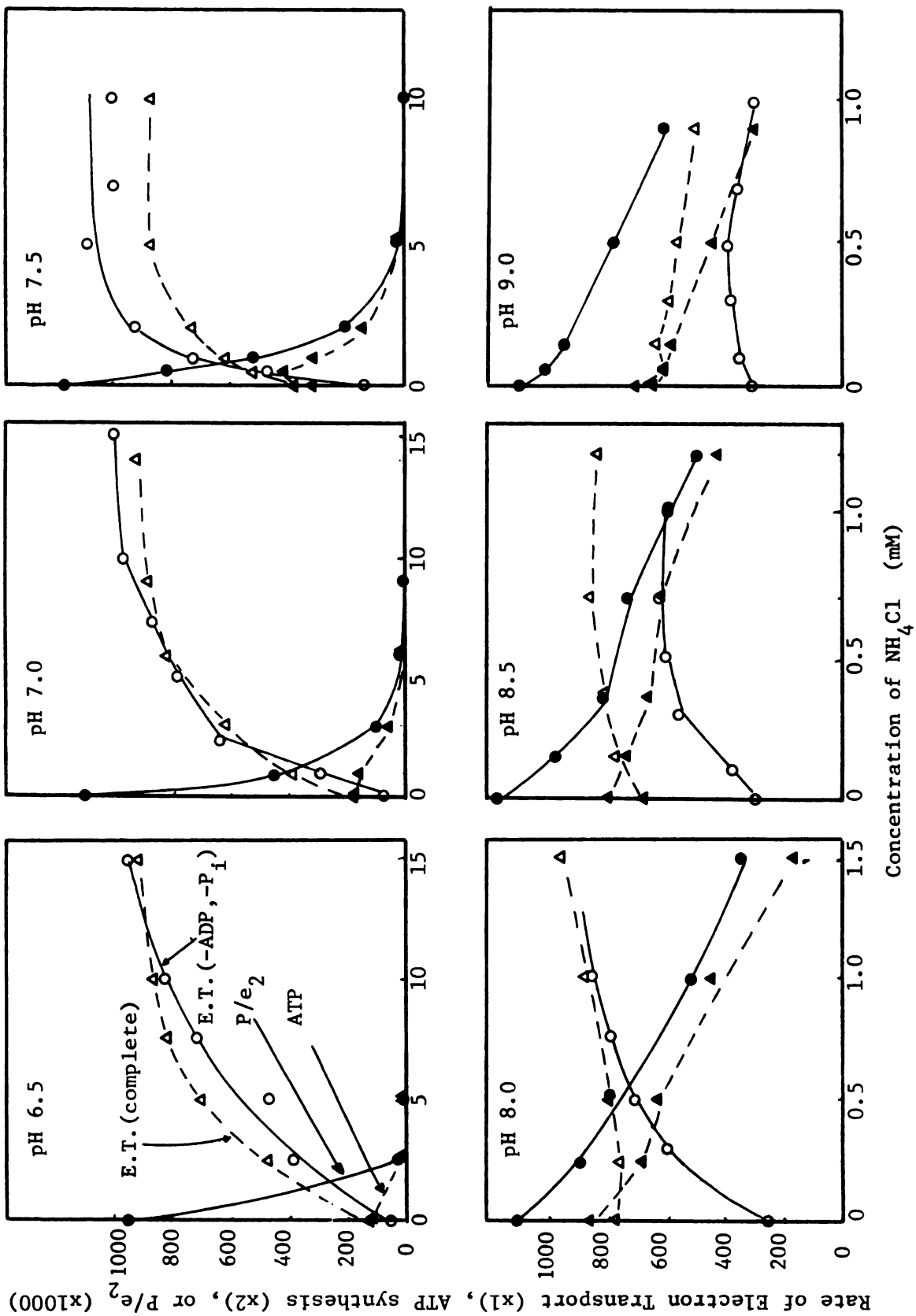
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T. (complete)
E.T. (-ADP, -$P_i$)
P/$e_2$
ATP
Rate of Electron Transport (x1), ATP synthesis (x2), or P/$e_2$ (x1000)
Concentration of $NH_4Cl$ (mM)

Figure 5g.

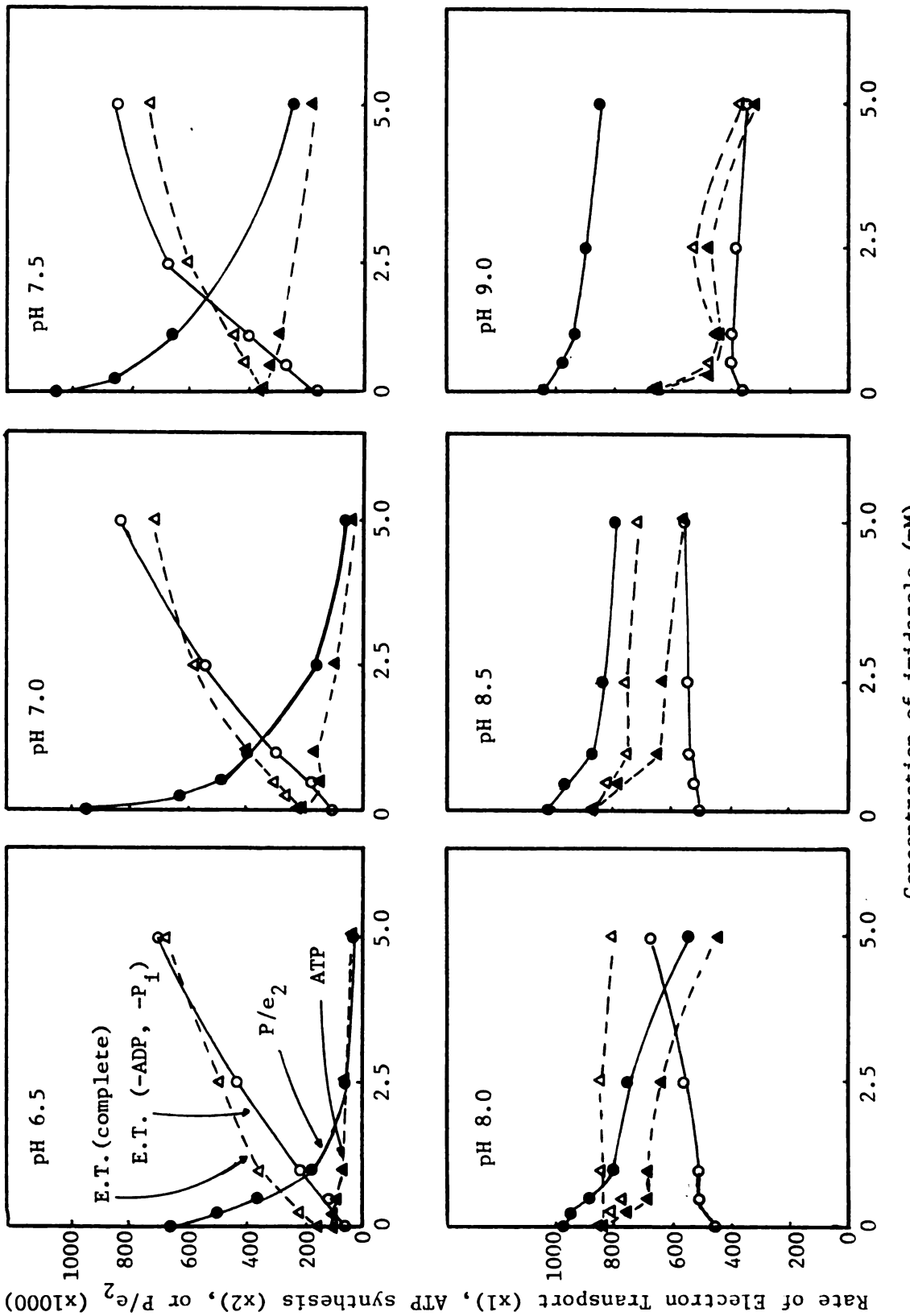
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T.(complete)
E.T. (-ADP, -$P_i$)
$P/e_2$
ATP
0
200
400
600
800
1000
2.5
5.0
Rate of Electron Transport (x1), ATP synthesis (x2), or $P/e_2$ (x1000)
Concentration of imidazole (mM)

Figure 5h.

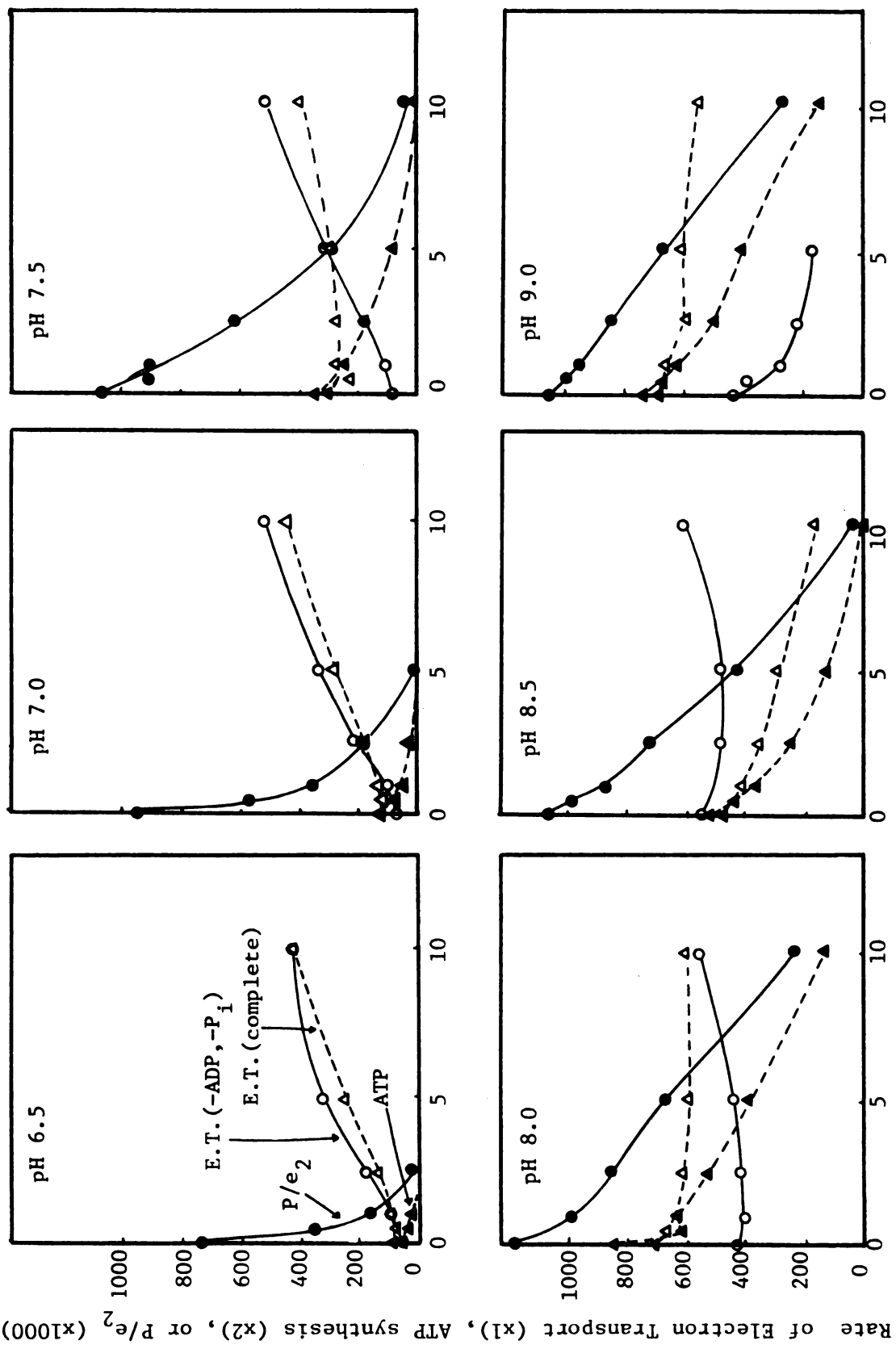
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T.(-ADP,-$P_i$)
E.T.(complete)
$P/e_2$
ATP
0
200
400
600
800
1000
0
5
10
Rate of Electron Transport (x1), ATP synthesis (x2), or $P/e_2$ (x1000)
Concentration of CCCP ($\mu$M)

Figure 5i.

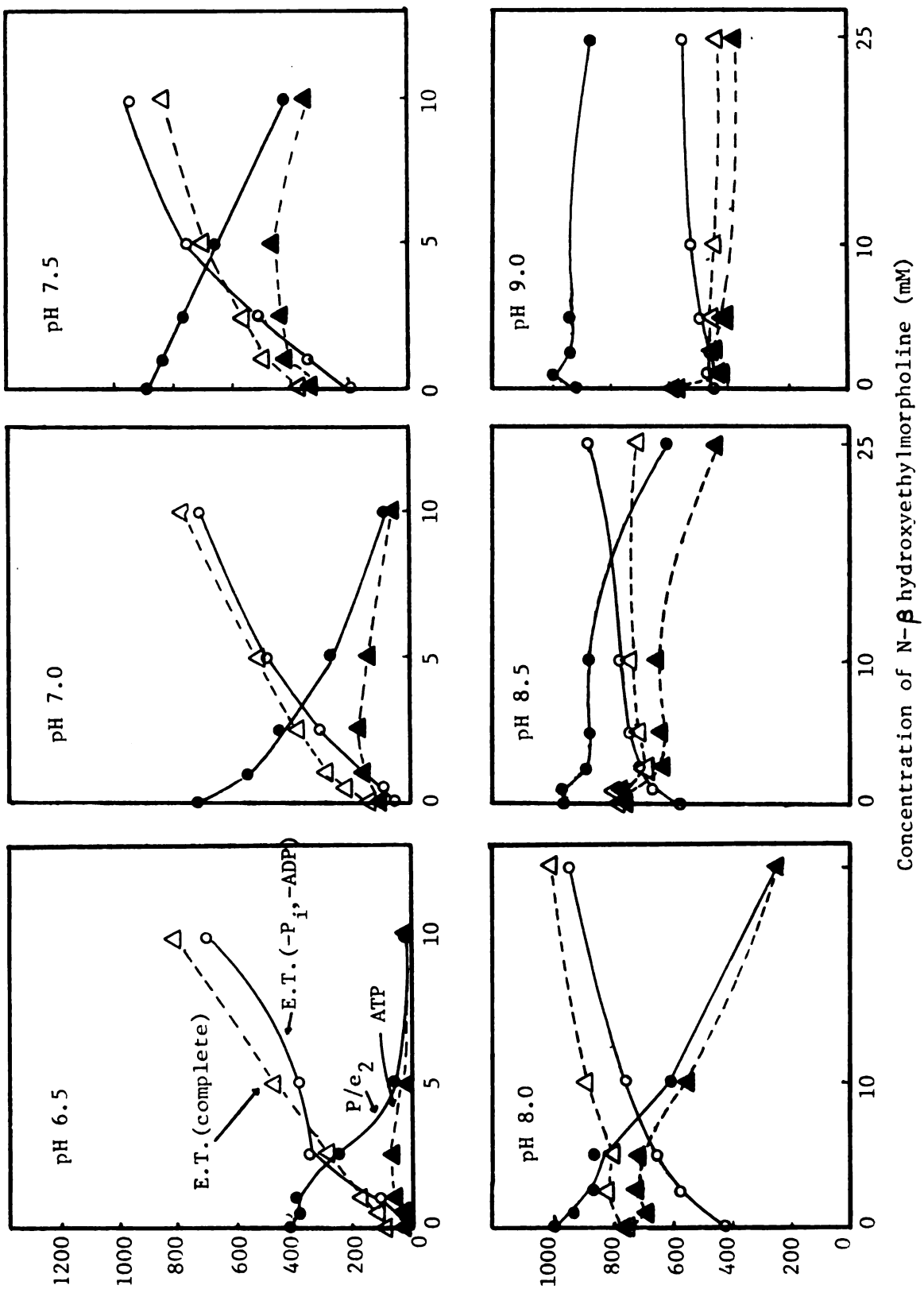
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
E.T. (complete)
E.T. (-$P_i$, -ADP)
$P/e_2$
ATP
Rate of Electron Transport (x1), ATP synthesis (x2), or $P/e_2$ (x1000)
Concentration of N-β hydroxyethylmorpholine (mM)

Figure 5j.

Points a), b) and c) are commonly observed and perhaps need no elaboration beyond the explanation given in the introduction of this section.

Point d) is an indication that amines with low pK's behave differently from those with higher pK's. The few amines mentioned have pK's around 7.0. It has long been thought that weak amines such as those listed are poor uncouplers. If one limits one's observation to high pH's only, this is true. However at lower pH's, large increases in electron transport are possible at concentrations of amine that are not unreasonable. These differences in effectiveness at high and low pH are easily explained however (see Schuldiner et al., 1972). It should be remembered that the uncoupling by amines is due to the different pH's on the two sides of the membrane. This difference in pH normally results in differences in the concentrations of the uncharged form of either side of the membrane and diffusion of this form across the membrane followed by the extrusion of the protonated amine neutralizes the internal pH. At pH's well above the pK of the amine however, the amine is almost entirely in the free base form and drops in the internal pH are not sufficient to lower the internal concentration of the free base sufficiently to cause a massive uptake and release of the amine which seems required for effective uncoupling.

Point e). The fact that conditions which increase the rate of non-phosphorylating electron transport (i.e. uncoupling)

often do not significantly inhibit phosphorylation and can sometimes actually increase the rate of ATP synthesis seems inconsistant with the very concept of coupling. The failure of certain amounts of uncoupled electron transport to decrease phosphorylation can be understood if the actual coupled transport reaction is strongly rate-limiting; that is to say, if all other reactions are in excess. Then the coupled reaction might be in part uncoupled or become non-phosphorylating without diminishing the supply of electrons for the coupled reaction. In this context it should be remembered that the phenomenon we are discussing is most pronounced at low pH's where those electron transport reactions associated with phosphorylation are indeed very slow and probably rate-limiting. However this interpretation does not explain the actual stimulations of phosphorylation observed.

There have been some other reports of small (< 20%) increases in the rate of electron transport with no decrease in phosphorylation with low concentrations of methylamine (see Good et al., 1966) but no reports with the large discrepancies reported here. In fact, at pH 6.5, "uncoupling" by HEM at 2.5 mM gave a 1.85-fold increase in the rate of ATP synthesis with a 3-fold stimulation of electron transport!

Other amines with pK's near 7.0 seem to behave in a similar if not identical manner (see Table II). The weaker amines give much less of a decrease in the rate of ATP synthesis when the electron transport rate is increased 4-fold. The differences between the amines with pK's near 7.0 might

TABLE II. Correlations Between the Stimulation of Electron Transport and the Inhibition of Phosphorylation by Uncouplers at pH 6.5*.

| Uncoupler | pK | Increase in E.T. rate | %Inhibition of Phosphorylation |
|---|---|---|---|
| Octylamine | 10.6 | 4-fold | 100 |
| Gramicidin | - | 4 " | 100 |
| CCCP | - | 4 " | 100 |
| $NH_4Cl$ | 9.25 | 4 " | 82 |
| Methylamine | 10.6 | 4 " | 65 |
| Imidazole | 7.0 | 4 " | 52 |
| 2,4-lutidine | 6.7 | 4 " | 43 |
| 2,6-lutidine | 6.7 | 4 " | 33 |
| DMAP | 7.0 | 4 " | 22 |
| HEM | 7.0 | 4 " | 0 |

* Data were calculated from the results presented in Figure 5

be thought to be due to differences in the lipid-solubility of their uncharged forms. However HEM and imidazole, which gave large differences in the lack of inhibition of ATP synthesis, have the same pK and partition to the same extent between water and petroleum ether (data not shown).

In order to distinguish further the effects of amines with low pK's and high pK's, the effect of light intensity was investigated at pH 7.5 (Figure 6). Surprisingly, light intensity is an important factor in the stimulation of steady-state phosphorylation by amines and not simply through any role such as rate limitation. Even methylamine, which diminished phosphorylation at high light intensities (100% relative units), can stimulate phosphorylation at lower light intensities (16-43% relative units). Ammonia however decreased the rate of ATP synthesis at all light intensities.

The reasons for these stimulations of phosphorylation by amines are obscure and speculations at this point may be premature. It is clear however that uncoupling by amines is not as straightforward as once thought and it is possible to get large differences between uncoupling as determined by increases in electron transport and as determined by an inhibition of ATP synthesis.

Since amines with pK's near 7.0 clearly need more investigation, many of them were checked for their ability to stimulate electron transport at pH 6.5. The results of these experiments are presented in Table III. All of the amines tested could uncouple to some extent. However the

Figure 6 The effects of light intensity on the uncoupling by amines. Reaction conditions in 2.0 ml: chloroplast suspension containing 30 µg chlorophyll; sorbitol, 0.2 M; $MgCl_2$, 2.0 mM; $Na_2H^{32}PO_4$, 10 mM; potassium chloride, 10 mM, potassium ferricyanide, 0.5 mM; ADP, 0.75 mM; Tricine-NaOH (pH 7.5), 50 mM.

Arrows indicate the rates of electron transport and the bars indicate the rates of phosphorylation at the millimolar concentrations shown. Maximum light intensity (100 %) was approximately 400 $Kerg \cdot cm^{-2} \cdot s^{-1}$.

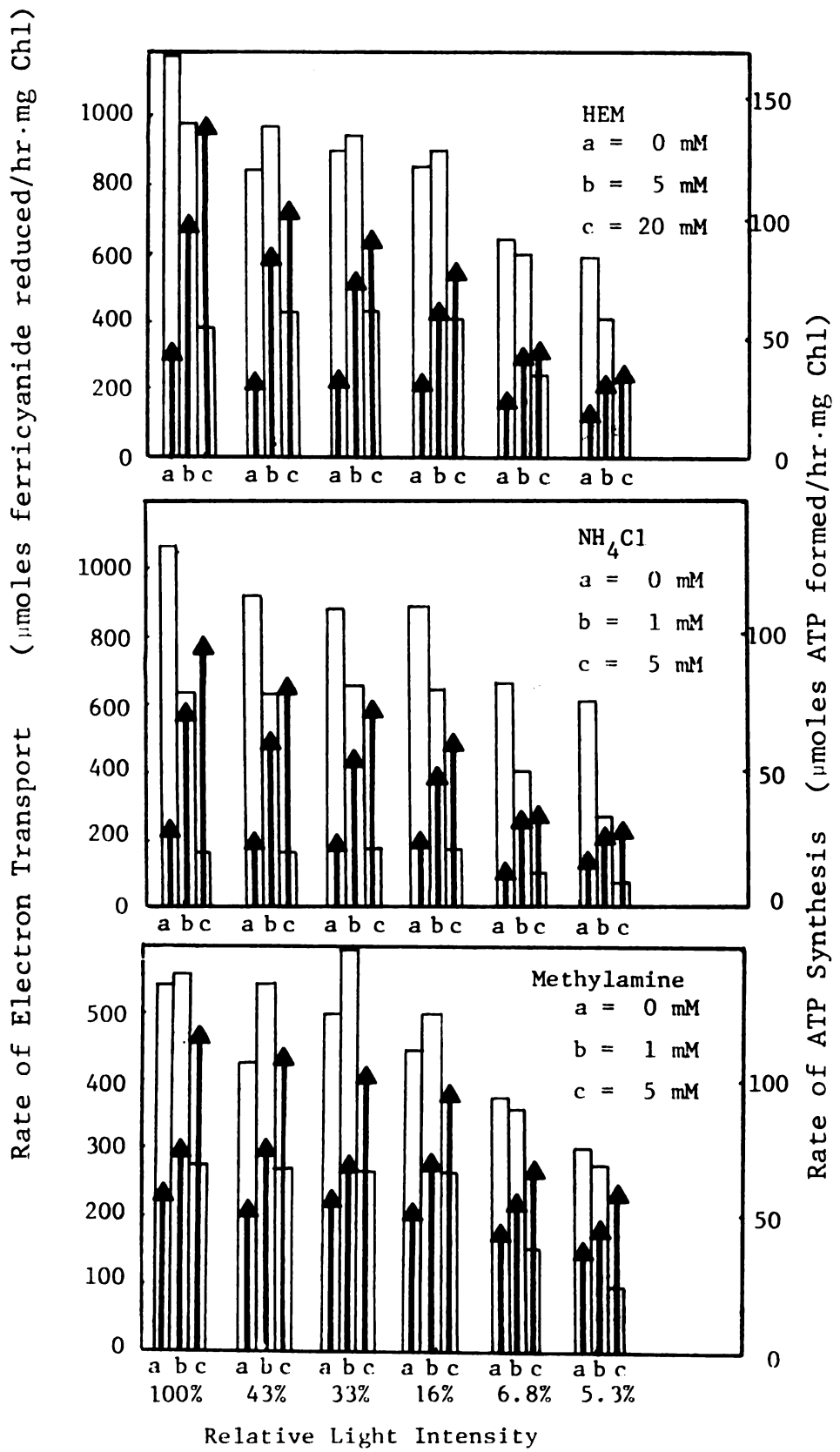
Rate of Electron Transport (μmoles ferricyanide reduced/hr·mg Chl)
Rate of ATP Synthesis (μmoles ATP formed/hr·mg Chl)
HEM
a = 0 mM
b = 5 mM
c = 20 mM
NH4Cl
a = 0 mM
b = 1 mM
c = 5 mM
Methylamine
a = 0 mM
b = 1 mM
c = 5 mM
a b c
100%
43%
33%
16%
6.8%
5.3%
Relative Light Intensity

Figure 6

TABLE III. A Survey of the Effectiveness of Amines with pK's near Neutrality in Stimulating Electron Transport at pH 6.5*.

| AMINE | pK | Electron Transport Stimulation |
|---|---|---|
| 2,4,6-collidine | 7.5 | 11-fold |
| N-methylmorpholine | 7.4 | 26 " |
| 1-ethylimidazole | 7.3 | 18 " |
| N-allylmorpholine | 7.05 | 23 " |
| N-βhydroxyethylmorpholine | 7.0 | 21 " |
| imidazole | 7.0 | 22 " |
| 3-dimethylaminopropionitrile | 7.0 | 15 " |
| bis(2-chloroethyl)amine | 7.0 | 9 " |
| 1-dimethylamino-2-propyne | 7.0 | 22 " |
| 2-aminopyridine | 6.8 | 16 " |
| 2,4-lutidine | 6.7 | 21 " |
| 2,6-lutidine | 6.7 | 12 " |
| 5-ethyl-2-methylpyridine | 6.5 | 3 " |
| 3,4-lutidine | 6.5 | 8 " |
| methylaminoacetonitrile | 5.2 | 5 " |

*Reaction conditions as in Figure 5. Values given for stimulation are a ratio of the maximum uncoupled rate of electron transport to the basal rate of electron transport.

substituted pyridines tended to give progressive inhibitions and should be used with caution.

# LITERATURE CITED

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# APPENDICES

# APPENDIX I

# STIMULATION OF MICROSECOND-DELAYED FLUORESCENCE FROM SPINACH CHLOROPLASTS BY UNCOUPLERS AND BY PHOSPHORYLATION

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# STIMULATION OF MICROSECOND-DELAYED FLUORESCENCE FROM SPINACH CHLOROPLASTS BY UNCOUPLERS AND BY PHOSPHORYLATION

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## Summary

Delayed fluorescence, as measured with a laser phosphoroscope, is stimulated not inhibited by uncouplers during the first 100 $\mu$s after the light is turned off. This is true only when uncouplers cause an increase in the rate of electron transport. When ADP and $P_i$ cause an increase in the electron transport rate, microsecond-delayed fluorescence is also increased. Indeed, there is a complex quantitative relationship between the rate of electron transport and the initial intensity of delayed fluorescence under a wide range of conditions.

Uncouplers or ADP and $P_i$ also increase the rate of decay of delayed fluorescence so that after about 150 $\mu$s they become inhibitory, as already reported by many authors.

Microsecond-delayed fluorescence continues to rise with rising light intensities long after the rate of reduction of exogenous acceptor is light-saturated.

These observations suggest a correlation of the rate of electron transport both with the intensity of the 5—100 $\mu$s-delayed fluorescence and with the rate of decay in the intensity of delayed fluorescence. The data imply that the decrease in intensity of millisecond-delayed fluorescence which has often been noted with uncouplers is probably not due to the elimination of a membrane potential. It seems more likely that the decrease in millisecond-delayed fluorescence is a reflection of the rate of disappearance of some other electron transport-generated condition, a condition which is uncoupler-insensitive. Certainly stimulations of microsecond-delayed fluorescence by electron transport which has been uncoupled by gramicidin suggest that ion gradients are not an essential component of the conditions responsible for delayed fluorescence.

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine. DF*, values of delayed fluorescence corrected for changes in the efficiency of fluorescence of excited chlorophyll and expressed as the ratio of delayed fluorescence counts to prompt fluorescence counts multiplied by 1000.

## Introduction

The delayed fluorescence of green cells and isolated chloroplasts appears to be a reversal of the initial photochemical and thermochemical events associated with Photosystem II [1] (for a review, see ref. 2). This fluorescence persists for milliseconds or longer and is envisioned as arising from a charge recombination within the Photosystem II reaction center involving the reduced primary electron acceptor ($Q^-$) and the oxidized form of some primary electron donor [3].

Uncouplers of photophosphorylation inhibit that part of the fluorescence which is delayed 3 ms or more after the light is extinguished [4]. Since uncouplers also inhibit the development of ion gradients across the lamellar membranes, it was suspected that delayed fluorescence in some way depends on a transmembrane hydrogen ion or salt concentration difference. This suspicion was greatly strengthened by Mayne's 1968 observation [5] that preilluminated chloroplasts emit light when shifted from an acidic to a basic medium and by Miles and Jagendorf's 1969 observation [6] that abrupt increases in salt concentration cause a similar burst of fluorescence (see also ref. 7). Fleischmann [8], Crofts et al. [9] and others suggested that photosynthetic electron transport establishes an electrochemical potential gradient across the membrane and that this membrane potential lowers the activation energy required for the recombination of the electron in $Q^-$ with a hole in an electron donor, $Z^+$. The initial photochemical charge separations of photosynthesis probably occur across the membrane, reducing Q to $Q^-$ toward the outside and oxidizing Z to $Z^+$ toward the inside [10]. If a membrane potential arises (positive to the inside), it would then facilitate the return of an electron from $Q^-$ to $Z^+$. To explain the stimulation of delayed fluorescence by a pH gradient, Crofts et al. [9] suggested that the electron-carrying redox couples $Q/Q^-$ and $Z/Z^+$ are in equilibrium with pools of secondary electron donors and acceptors which are hydrogen-carrying redox couples. These pools are supposed to be themselves in equilibrium with the aqueous phases on the opposite sides of the membrane and their redox potentials dependent on the pH of these phases. Thus, the pH difference across the membrane could increase the availability of either $Q^-$ or $Z^+$ or both.

The first hint that the Fleischmann hypothesis might be wrong came from the observations of Neumann et al. [11] and Felker et al. [12]. They showed that, under certain conditions, uncoupling by ammonium salts enhanced millisecond-delayed fluorescence instead of inhibiting it. However, it was by no means certain that ammonia uncoupling actually abolished the membrane potential. Furthermore, no conditions were found where other uncouplers failed to inhibit millisecond-delayed fluorescence. Even methylamine, which seems to uncouple in the same manner as ammonia, was always inhibitory. Thus the Fleischmann model remained tenable.

We wish now to report an entirely different pattern of uncoupler effects on delayed fluorescence which is observed when the measurements are made a few microseconds after the excitation light is extinguished. The development of a laser phosphoroscope which is capable of making reliable measurements of delayed fluorescence within microseconds [13] allowed us to investigate the

effects of uncouplers in this time range (see Methods).

It should be emphasized that our selection of a phosphoroscope was deliberate and not motivated by the convenience of rapidly summing the signals. We were particularly concerned about the relationship of delayed fluorescence to the cumulative effects of frequently repeated periods of illumination on that energized state of the membrane which is responsible for ATP formation and is abolished by uncouplers. The concern arose from the fact that this state was reputed to be involved in delayed fluorescence [8,9]. Single flash experiments on dark-adapted chloroplasts would have provided us with no information on these matters and, indeed, it is not at all clear why such single flash experiments should be affected by uncouplers at all. It seemed to us that single flash experiments on preilluminated chloroplasts would have suffered from the ambiguities of the phosphoroscope without the advantages. Both the phosphoroscope and the single flash following preillumination have the disadvantage that the delayed fluorescence following the period of illumination may be superimposed on the residual delayed fluorescence associated with the previous period of illumination. On the other hand, the phosphoroscope has the added advantage that we are able to measure electron transport and delayed fluorescence in the same experiment and this is patently impossible with a single flash.

The series of rapidly repeated illuminations with the strong light employed in the laser phosphoroscope provided an average intensity capable of saturating the electron transport system and, indeed, the rate of electron transport with this intermittent light was no less than with continuous light of the same intensity. Therefore, it is reasonable to suppose that the electron carrier pools at the end of each millisecond illumination period were close to the conditions achieved during steady-state electron transport.

We found that, in stimulating electron transport, uncouplers also increase the intensity of the delayed fluorescence measured at any time between 5 and 100 $\mu$s after light extinction. However, the rate of decline in the intensity of the delayed fluorescence with time after light extinction is also increased by uncouplers, so that within milliseconds uncouplers appear to inhibit delayed fluorescence. These observations seem to preclude the possibility that any energized state of the membrane system which is sensitive to uncouplers can be required for the delayed fluorescence reported here, be it a membrane potential, an ion gradient, or a transmembrane pH difference.

## Materials and Methods

Chloroplast lamellae were isolated from commercial spinach by the method of Ort and Izawa [14] and resuspended in a solution containing 0.2 M sucrose, 5 mM HEPES-NaOH (pH 7.4) and 2 mM $MgCl_2$.

Nigericin and gramicidin were added in ethanol. The final ethanol concentration in the reaction mixture was always less than 3%. This concentration of ethanol had no effect on the phenomena being studied.

The laser phosphoroscope employed was a modification of that described by Beall and Haug [13]. Briefly, the light produced by a continuous output argon laser (principle lines at 488 and 515 nm) was focussed by a microscope objective and chopped by a rotating sector at the focal point. This gave a cycle

of 1 ms light and 1 ms dark with a light-dark transition time of less than 250 μs. The light beam was then collimated and directed through the bottom of a 1 × 1 × 4.5 cm quartz cuvette. Prompt and delayed fluorescence were measured at a right angle to the actinic beam by a single photomultiplier tube cooled with solid $CO_2$. A 690 nm interference filter was placed between the cuvette and the photomultiplier to shield it from stray actinic light. The 50 Ω output of the photomultiplier was in discrete pulses 1–3 ns in length, each pulse associated with individual photons striking the cathode surface. To measure the delayed fluorescence, only those pulses generated during a specified period after light extinction were counted. To do this its timing circuit was activated at the instant of light extinction. This timing circuit created a counting "window" by opening a gate from the photon detector to the counter for a prescribed time. Under most circumstances this window began 5 μs after light extinction and ended 10 μs after light extinction. The pulses arriving during 10 000 of these gated counting periods were summed (one counting period every 2 ms for 20 s). The duration and time of commencement of the counting window could be varied independently so that the rate of decay of delayed fluorescence could be determined. When longer delays occurred before the fluorescence was measured, the rate of photon counting was so much slower that the window was extended considerably, to 25 μs when the delay was 500 μs and to 100 μs when the delay was 1 ms. However, in Figs. 1 and 2, the data presented have taken into account this change in counting time. Prompt fluorescence was measured with the same photomultiplier by allowing the gate to open to the counter while the actinic light was still on.

Delayed fluorescence is reported as the ratio of counts during 5 μs divided by the prompt fluorescence in 5 μs for the following reasons; we are interested not so much in the actual delayed fluorescence as in the thermochemical reactions responsible for the excitation of the chlorophyll. Since delayed fluorescence and prompt fluorescence seem to come from the same chlorophyll, it is reasonable to assume that the chemically excited chlorophyll responsible for delayed fluorescence fluoresces with the same efficiency as the light-excited chlorophyll responsible for prompt fluorescence. Thus, dividing by the prompt fluorescence should help to correct for variations in the fluorescence yield of excited chlorophyll although, to be sure, the prompt fluorescence yield was not measured and could not be measured at the same instant as the delayed fluorescence. Nevertheless, these "corrected" values for delayed fluorescence should reflect the recombination reactions we wish to measure more accurately than the delayed fluorescence itself. The "corrected" values have the added advantage of normalizing the results of the various experiments since each data point represents a separate experiment and there are inevitable small variations in the actinic light intensity, chloroplast density and chloroplast condition.

In the same experiments electron transport was measured as the reduction of ferricyanide. This was done by observing the changes in the ferricyanide absorbance at 420 nm. A weak light was passed through a 420 nm interference filter before it passed through the cuvette. This beam was then detected with a photomultiplier which was protected by another 420 nm interference filter to screen out the actinic light. The signal from the photomultiplier was processed by a logarithmic amplifier before it was fed into a strip chart recorder so that

rates of change in the concentration of ferricyanide could be directly determined.

In all cases the values given for delayed fluorescence intensity are the mean of 5 determinations. Prompt fluorescence was measured after 20 s of preillumination and immediately before and after the delayed fluorescence determinations. All experiments were conducted at room temperature, approx. 23°C. The intensity of the light from the laser impinging on the reaction cuvette was approximately 80 mW · $cm^{-2}$. The reaction mixture occupied a cubic volume 1 cm on each side.

The contrast ratio of the measurements of the apparent delayed fluorescence and actual prompt fluorescence observed when there was really no delayed fluorescence at all, was determined by replacing the chloroplast-containing reaction mixture with a solution of chlorophyll in acetone. With this chlorophyll solution, the counts falsely attributed to the 5—10 $\mu$s delayed fluorescence were always less than one per 10 000 counts of prompt fluorescence measured over the same length of time. The intensity of the delayed fluorescence from chloroplasts was routinely ten times or more higher than this noise level.

## Results

Uncouplers of photophosphorylation and phosphorylating conditions (ADP + $P_i$) increase the intensity of delayed fluorescence for the first 100 $\mu$s after the light is turned off (Figs. 1 and 2). Ammonium ions routinely increase the number of photons emitted 5—10 $\mu$s after illumination about 3-fold. Increases due to other uncouplers and phosphorylating conditions are somewhat smaller, viz., 50—100%. These stimulations of delayed fluorescence are real and not artifacts of our method of correcting for the fluorescence efficiency of excited chlorophyll (see Methods), since they are present and of a similar magnitude whether or not such corrections are made. On the other hand, the rate at which delayed fluorescence dies away after the light is off is markedly increased by uncoupling and by ADP and $P_i$. Consequently uncoupler-influenced delayed fluorescence is usually less than in the control after about 150 $\mu$s. As has been noted previously, delayed fluorescence disappears almost entirely in 1 -2 ms with many uncouplers.

It is clear from these observations that the intensity of delayed fluorescence cannot be dependent on any uncoupler-inhibited state of the membrane system. On the contrary, some process which is enhanced by uncouplers or ADP and $P_i$ must be determining delayed fluorescence. Since all uncouplers share with ADP + $P_i$ the ability to increase the electron transport [15] and the ability to increase the intensity of 5-$\mu$s delayed fluorescence, we sought and found a correlation between electron transport rates and the initial intensity of delayed fluorescence.

Table I illustrates the correlation between electron transport and 5-$\mu$s delayed fluorescence. In these experiments, electron transport was varied in three ways: It was greatly diminished by omitting an exogenous electron acceptor or almost abolished with the inhibitor DCMU. In both cases the intensity of $\mu$s-delayed fluorescence was much lowered, in the case of DCMU almost

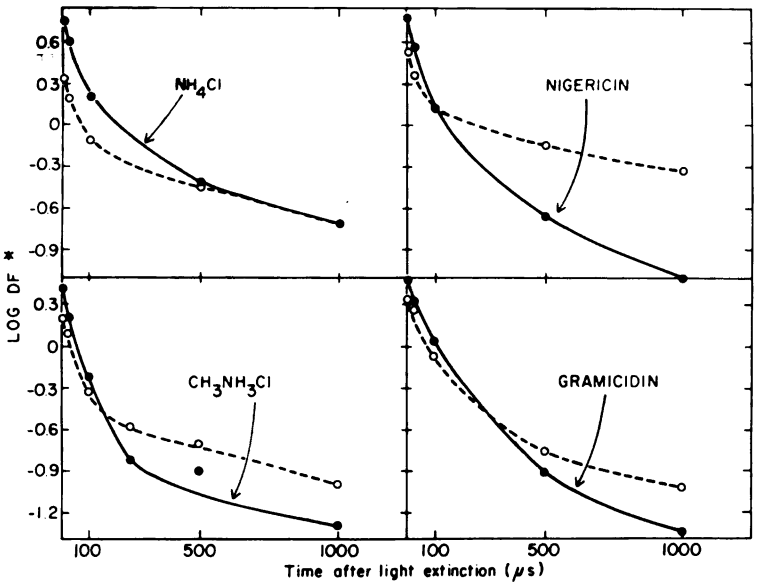


Fig. 1. Delayed fluorescence in spinach chloroplasts and its rate of decay in the presence and absence of uncouplers. Reaction mixtures contained in 1.0 ml: chloroplasts with 5—10 μg chlorophyll; $MgCl_2$, 2 μmol; Tricine-NaOH (pH 7.8—8.1), 50 μmol; potassium ferricyanide, 0.5 μmol; sucrose, 100 μmol. When added: $NH_4Cl$, 5 μmol; methylamine-HCl, 5 μmol; gramicidin, 5 μg; nigericin, 5 μg. Ordinates are log *DF**, where *DF** is 1000 times the ratio of the number of delayed fluorescence photons counted in 5 μs in the dark to the number of prompt fluorescence photons counted in 5 μs in the light. At 500 and 1000 μs after the light was turned off, the periods of counting of delayed fluorescence photons were increased to 25 and 100 μs, respectively, with appropriate corrections. Solid lines represent decays of delayed fluorescence in the presence of the uncoupler and dotted lines represent decays of delayed fluorescence in the absence of the uncoupler. Note that the decay is polyphasic whether or not uncouplers are present. This suggests that different reactions probably limit the back-reaction responsible for the chemical excitation of chlorophyll at different times.

absent. Or electron transport was greatly increased by the use of uncouplers in which case, as already noted, the increase in microsecond-delayed fluorescence was marked. It is particularly important to note that the increase in delayed fluorescence caused by uncouplers, which is noted in the presence of an exo-

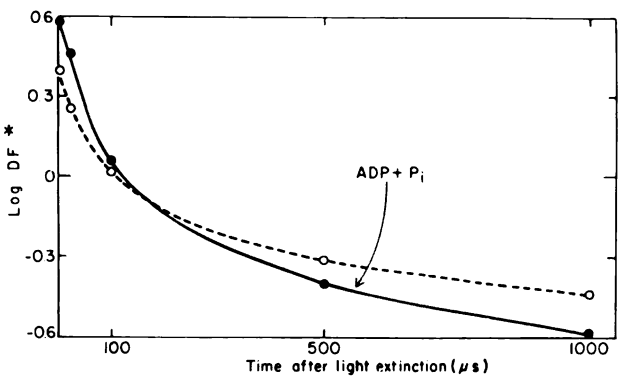


Fig. 2. Delayed fluorescence in spinach chloroplasts and its rate of decay in the presence and absence of ADP and orthophosphate. Reaction conditions and data presentations as in Fig. 1. When added, ADP was 1.5 μmol, and $K_2HPO_4$, 5 μmol.

TABLE I

THE EFFECTS OF FERRICYANIDE, UNCOUPLERS, AND DCMU ON THE RELATIVE INTENSITIES OF 5—10 MICROSECOND-DELAYED FLUORESCENCE AND ELECTRON TRANSPORT

Reaction conditions as in Fig. 1. DCMU when used was 100 nmol. Rates of electron transport are in μmol electrons · $h^{-1}$ · $mg^{-1}$ Chl Rate of electron transport was measured with an oxygen electrode in a parallel experiment as oxygen production in the presence of ferricyanide and as oxygen consumption in its absence.

| Additions | Relative *DF** | Electron transport |
|---|---|---|
| None | 46 | 64 |
| Methlyamine-HCl | 46 | 65 |
| Gramicidin | 51 | 59 |
| Ferricyanide | 100 | 280 |
| Ferricyanide, methylamine-HCl | 187 | 1170 |
| Ferricyanide, gramicidin | 147 | 1240 |
| Ferricyanide, DCMU | 14 | 46 |

genous electron acceptor, does not occur when the electron transport rate is limited by the absence of an exogenous electron acceptor. That is to say, uncouplers do not increase the intensity of the μs-delayed fluorescence when the uncouplers fail to increase the electron transport rate.

Under some conditions, μs-delayed fluorescence is a nearly linear function of the rate of electron transport (Fig. 3). However, this linear relationship may only apply to a rather narrow range of special situations. For instance, the partial inhibition of electron transport by DCMU, which blocks transport before

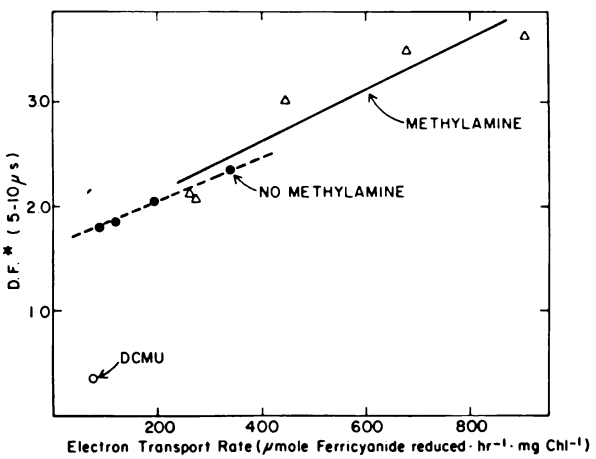


Fig. 3. The relationship of electron transport rate to the intensity of delayed fluorescence. In order to lower the electron transport rate without lowering the light intensity, the chloroplasts were pretreated with KCN, thus inactivating a portion of the plastocyanin [16]. A variety of electron transport rates were achieved by varying the length of the KCN pretreatment (up to 48 min) and by uncoupling with methylamine. △, experiments with methylamine addition. Cyanide pretreatment was in an ice bath in the dark with the following mixture: Tricine-NaOH (pH 8.0), 100 mM; sucrose, 100 mM; $MgCl_2$, 1 mM; KCN, 50 mM; and potassium ferricyanide, 50 μM. Reaction mixture as in Fig. 1. Methylamine-HCl when used, 5 mM. Numbers in parentheses represent minutes of incubation of the chloroplasts in the KCN. The values of the point labelled "DCMU" were calculated from the data of Table I.

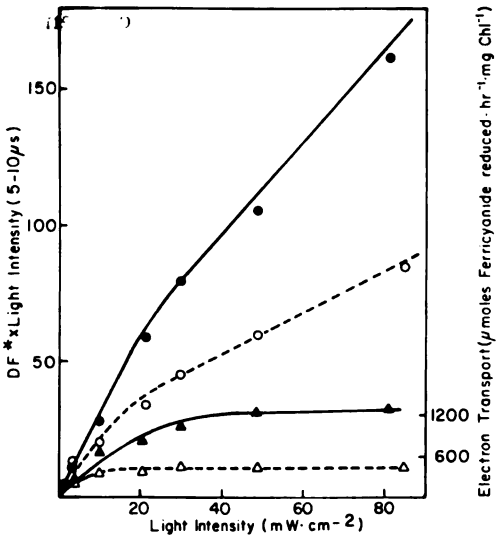


**Fig. 4. Delayed fluorescence and electron transport as functions of the exciting light intensity in the presence and absence of the uncoupler gramicidin. The value for "corrected" delayed fluorescence (*DF**) is obtained by dividing the observed delayed fluorescence by the prompt fluorescence. Since prompt fluorescence under any one set of conditions is usually proportional to the incident light intensity, for the purposes of the comparison made here it was necessary to multiply *DF** by the light intensity. The maximum rate of electron transport in the absence of gramicidin was over 400 μmol ferricyanide reduce · $h^{-1} \cdot mg^{-1}$ Chl, which suggests that these chloroplasts had probably been inadvertantly partially uncoupled.**

plastoquinone, gives much more inhibition of μs-delayed fluorescence than would be predicted from an extrapolation of the straight line in Fig. 3 where the electron transport rate was varied by inhibiting plastocyanin. For that matter, this straight line does not extrapolate through the origin of the graph and, therefore, it would be wrong to conclude that delayed fluorescence even requires the net transfer of electrons to an exogenous acceptor. Furthermore, different uncouplers sometimes give different intensities of delayed fluorescence at the same rate of reduction of the exogenous acceptor (data not shown).

Changing the rate of electron transport by changing the light intensity has very different effects on the delayed fluorescence (Fig. 4). Long after the light intensity has been raised to a level where the electron transport system is saturated, that is to say long after the rate of reduction of exogenous electron acceptor has stopped increasing, the delayed fluorescence continuous to increase. This is true whether or not the chloroplasts have been uncoupled by gramicidin. However, regardless of the light intensity used, the uncoupler does increase the 5—10-μs delayed fluorescence.

## Discussion

The effects of uncouplers on microsecond-delayed fluorescence in chloroplasts have been observed by Jursinic et al. [17] using a single flash with and

without preillumination. These authors observed no inhibition with concentrations of gramicidin sufficient to abolish any membrane potential-induced absorbance change at 518 nm. They also observed that there was no enhancement of 6—100 microsecond-delayed fluorescence in the presence of valinomycin in response to an abrupt increase in KCl concentration. Consequently they concluded that membrane potentials probably did not play any part in providing activation energy for microsecond-delayed fluorescence in their systems.

Our conclusions based on entirely different data are very similar. Uncouplers which should diminish or abolish ion gradients and membrane potentials actually increase microsecond-delayed fluorescence if electron transport is also increased. It should be emphasized that the latter observations are not at variance with the observations of Jurisinic et al. since their gramicidin effects were observed in the absence of an electron acceptor and therefore in the absence of a high rate of electron transport during preillumination. We also found that gramicidin neither increased nor decreased microsecond-delayed fluorescence in the absence of an exogenous electron acceptor (see Table I).

We also report that uncouplers and phosphorylating conditions increase the rate of decay of the delayed fluorescence so that after milliseconds the delayed fluorescence is diminished. Since the decay in the ability of chloroplasts to produce delayed fluorescence is complex, it is difficult to determine the nature of the processes involved in the decay. It seems likely that the delayed fluorescence measured microseconds after light extinction is the result of conditions which are unrelated to the membrane potential but are intimately associated with the rate of electron transport. It seems probable to us that the stimulation of microsecond-delayed fluorescence and the increased rate of decay are both due to some as yet unspecified effects of uncouplers of electron transport, effects that determine the levels of the reactant species (Z, Q, *P*-680) arrived at the light and also determine the rate at which they disappear in the dark by non-radiative mechanisms.

## Acknowledgements

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# APPENDIX II

# ENERGY TRANSFER INHIBITION BY PYROPHOSPHATE

## Appendix II

Pyrophosphate, which has pK's of 5.8 and 8.2 is a commonly-used buffer. In the course of some experiments on ATP synthesis induced by an acid-base shift, one of the students in our laboratory (T.Graan) thought to use it as a buffer. Yields of ATP synthesis were abnormally low under these circumstances, suggesting that the pyrophosphate has some deleterious effects on phosphorylation.

I therefore attempted to establish whether this inhibition occurs with steady-state phosphorylation. The results presented in Figure 1 confirm that pyrophosphate does indeed inhibit steady-state phosphorylation. A concentration of 2.5 mM was sufficient to inhibit phosphorylation 50% and coupled electron transport 38% while having no effect on the basal electron transport or on the electron transport uncoupled by gramicidin (not shown). Such effects are typical of energy transfer inhibitors (such as phlorizin), that is, agents which inhibit the mechanism of ATP synthesis directly without any direct effects on electron transport.

In order to study more fully the nature of this inhibition, competition studies were carried out with $Mg^{2+}$, phosphate, and ADP. In contrast to phlorizin, which competes with phosphate (Winget et al., 1969), pyrophosphate seems to inhibit competitively with ADP (Figure 2).

Figure 1. Effect of pyrophoshate on the rates of phosphorylation and electron transport in spinach lamellae. Reaction mixtures contained in 2.0 ml: chloroplast suspension containing 30 µg chlorophyll, 0.2 M sorbitol, 2.0 mM $MgCl_2$, 10 mM $Na_2H^{32}PO_4$, 10 mM KCl, 0.5 mM $K_3Fe(CN)_6$, 0.75 mM ADP, 50 mM Tricine-NaOH (pH 8.0).

Solid lines indicates rates of electron transport under non-phosphorylating ($-P_i$, filled circles) and under phosphorylating conditions (open circles). Dashed line indicates the effect of pyrophosphate on the phosphorylation rate.

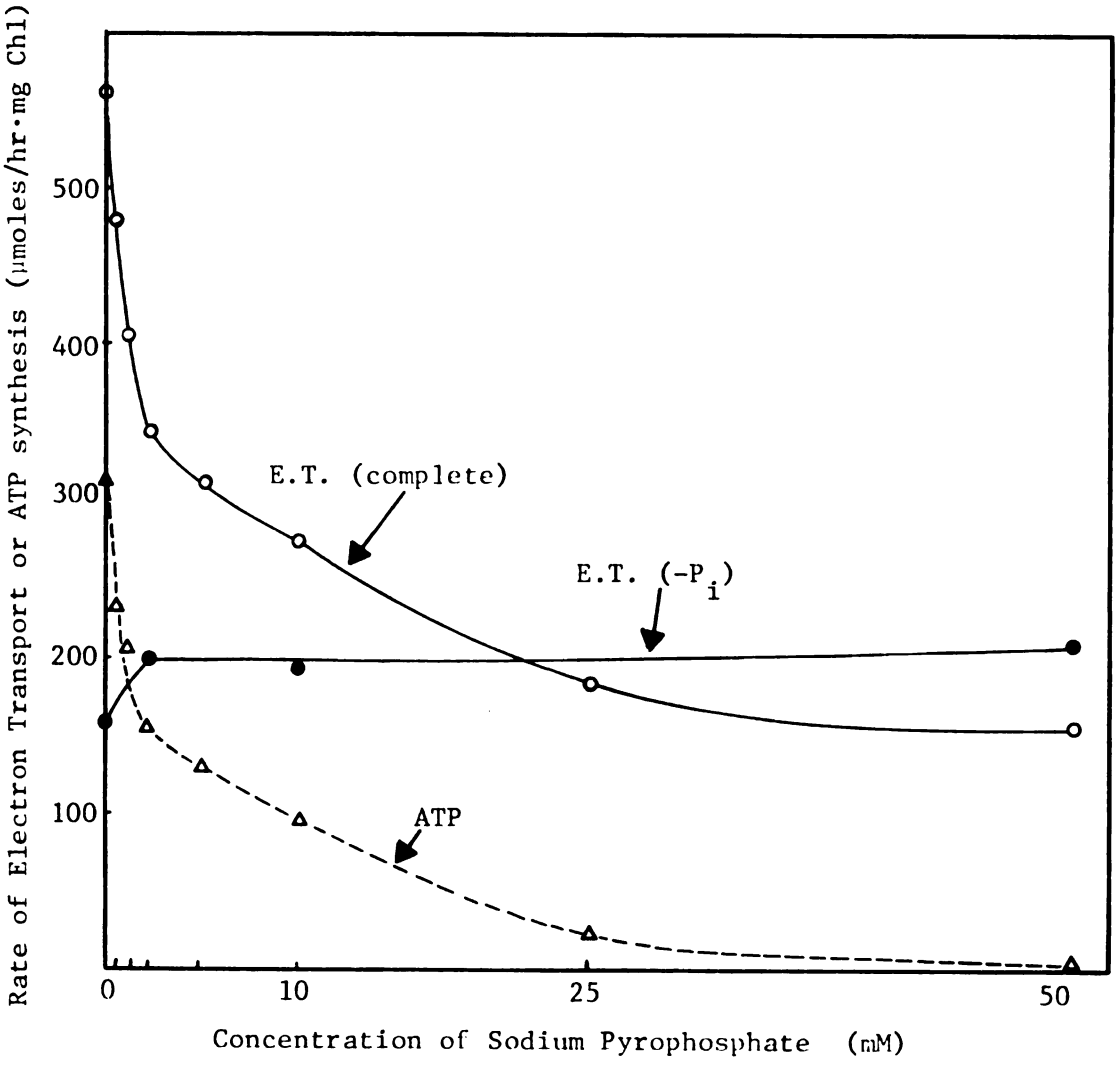
Rate of Electron Transport or ATP synthesis (μmoles/hr·mg Chl)
500
400
300
200
100
E.T. (complete)
E.T. (-P$_i$)
ATP
0
10
25
50
Concentration of Sodium Pyrophosphate (mM)

**Figure 1.**

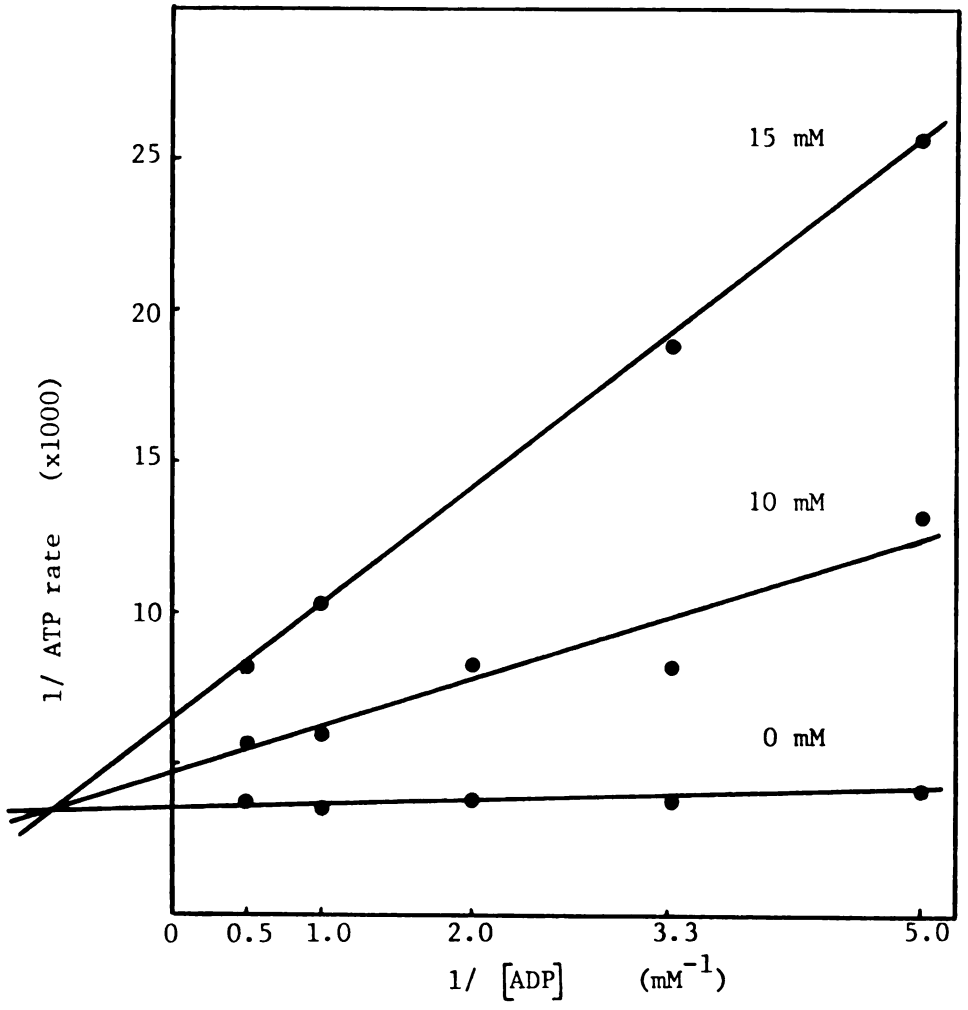


Figure 2. Double reciprocal plot of the inhibition of phosphorylation by different levels of pyrophoshate. Conditions as in Figure 1.

# APPENDIX III

# THE SUITABILITY OF SEVERAL NEW AMINOSULFONIC ACID BUFFERS IN STUDIES OF PHOTOSYNTHESIS

## Appendix III

In 1966, Good et al. introduced a series of new buffers that, because of their high water solubility and low solubility in other solvents, low tendency to bind metals, and pK's in a range important in biological reactions, have become widely used. Several of these buffers were N-substituted aminoethane sulfonic acids and their wide-spread use (especially of HEPES*) suggested the synthesis and use of a series of N-substituted aminopropane sulfonic acids which were less expensive to synthesize (e.g. MOPS, TAPS, HEPPS- Good and Izawa, 1972). However in recent years, it has been determined that one of the precursors used in the synthesis of these latter propane sulfonic buffers (propane sultone) is carcinogenic and hazardous to use, and may soon be commerically unavailable. This led Dr. William Ferguson, a chemist at Research Organics Inc., Cleveland to prepare a new series of buffers based on reactions with the presumably harmless 3-chloro-2-propane-sulfonate. Five new

---

| | |
|---|---|
| *HEPES | N-hydroxyethylpiperazine-N'-ethanesulfonic acid |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| MOPSO | 3-(N-morpholino)-2-hydroxypropanesulfonic acid |
| HEPPS | N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid |
| HEPPSO | N-hydroxyethylpiperazine-N'-2-hydroxypropane-sulfonic acid |
| TAPS | 3-tris(hydroxymethyl)-methyl-3-aminopropanesulfonic acid |
| TAPSO | 3-N-tris(hydroxymethyl)methylamino-2-hydroxy-propanesulfonic acid |
| POPSO | piperazine-N-N'-bis(2-hydroxypropanesulfonic acid) dihydrate |
| DIPSO | 3-N-bis(hydroxyethyl)amino-2-hydroxypropanesulfonic acid |

buffers were synthesized with pK's from 6.9 to 7.9. They were MOPSO (pK 6.95), DIPSO (pK 7.6), TAPSO (pK 7.7) HEPPSO (pK 7.9), and POPSO (pK 7.85).

This appendix reports that these new buffers are satisfactory for phosphorylation and electron transport studies in spinach lamellae. (Table I).

TABLE I. Rates of Electron Transport and ATP Synthesis in Illuminated Spinach Chloroplast Lamellae with Various Buffers*.

| Buffer | Electron Transport | | ATP Synthesis | | $P/e_2$- | |
|---|---|---|---|---|---|---|
| (50 mM) | Initial | After 10 min | Initial | After 10 min | Initial | After 10 min |
| Tricine | 732 | 760 | 395 | 409 | 1.08 | 1.08 |
| HEPES | 728 | 724 | 390 | 385 | 1.07 | 1.06 |
| DIPSO | 820 | 788 | 416 | 433 | 1.01 | 1.10 |
| HEPPSO | 780 | 780 | 421 | 422 | 1.08 | 1.08 |
| MOPSO | 800 | 776 | 418 | 406 | 1.04 | 1.05 |
| POPSO | 692 | 664 | 360 | 338 | 1.04 | 1.02 |
| TAPSO | 832 | 800 | 445 | 403 | 1.07 | 1.01 |
| Tricine | 804 | 760 | 417 | 393 | 1.04 | 1.03 |

* The reaction mixture consisted of chloroplast lamellae containing 20 µg chlorophyll 0.4 mmol sorbitol, 1.0 µmol $K_3Fe(CN)_6$, 4 µmol $MgCl_2$, 1.5 µmol ADP, 20 µmol $Na_2H^{32}PO_4$, 20 µmol KCl, and 100 µmol of the indicated buffer (adjusted to pH 8.0 with NaOH) in 2.0 ml. Electron transport rates are expressed as µmol ferricyanide reduced/hr·mg Chl and ATP synthesis rates are expressed as µmol ATP formed/hr·mg Chl. $P/e_2$ represents the ratio of the molecules of ATP formed to the number of pairs of electrons transported. Temperature 18°C.

# APPENDIX IV

# THE DESIGN OF AN INEXPENSIVE, RELIABLE, AND RAPID-ACTING FRACTION COLLECTOR

## Appendix IV

In the course of some experiments studying amine uptake by the flow dialysis technique, a fast-acting fraction collector was designed. Because of its ease of construction, low cost, and reliability, this fraction collector may be of general interest. The mechanical parts are illustrated in Figure 1.

The samples are collected in test tubes that are held against the crown-shaped collector head by a band of rubber cut from a rubber innertube. The V-shaped notches assure very accurate positioning of the test tubes which may be of any diameter equal to or less than the circumference of the collector assembly divided by the number of notches. The V-shaped notches do not extend the length of the collector head but rather form a collar and a base which are separated by removable spacers of a smaller diameter, thus allowing the assembly to hold short or long test tubes of a wide variety of diameters.

The drive mechanism for the collector is simple and reliable. A 40 cm coiled door spring is attached at one end to a post and at the other end to a cord. This cord is attached to the center shaft of the collector head. Turning the head in a clockwise direction winds the cord on the shaft and extends the spring. The taut spring then provides the driving force turning the collector assembly. Positioning

Figure 1. Diagram of the escapement mechanism which regulates the advancement of the fraction collector.

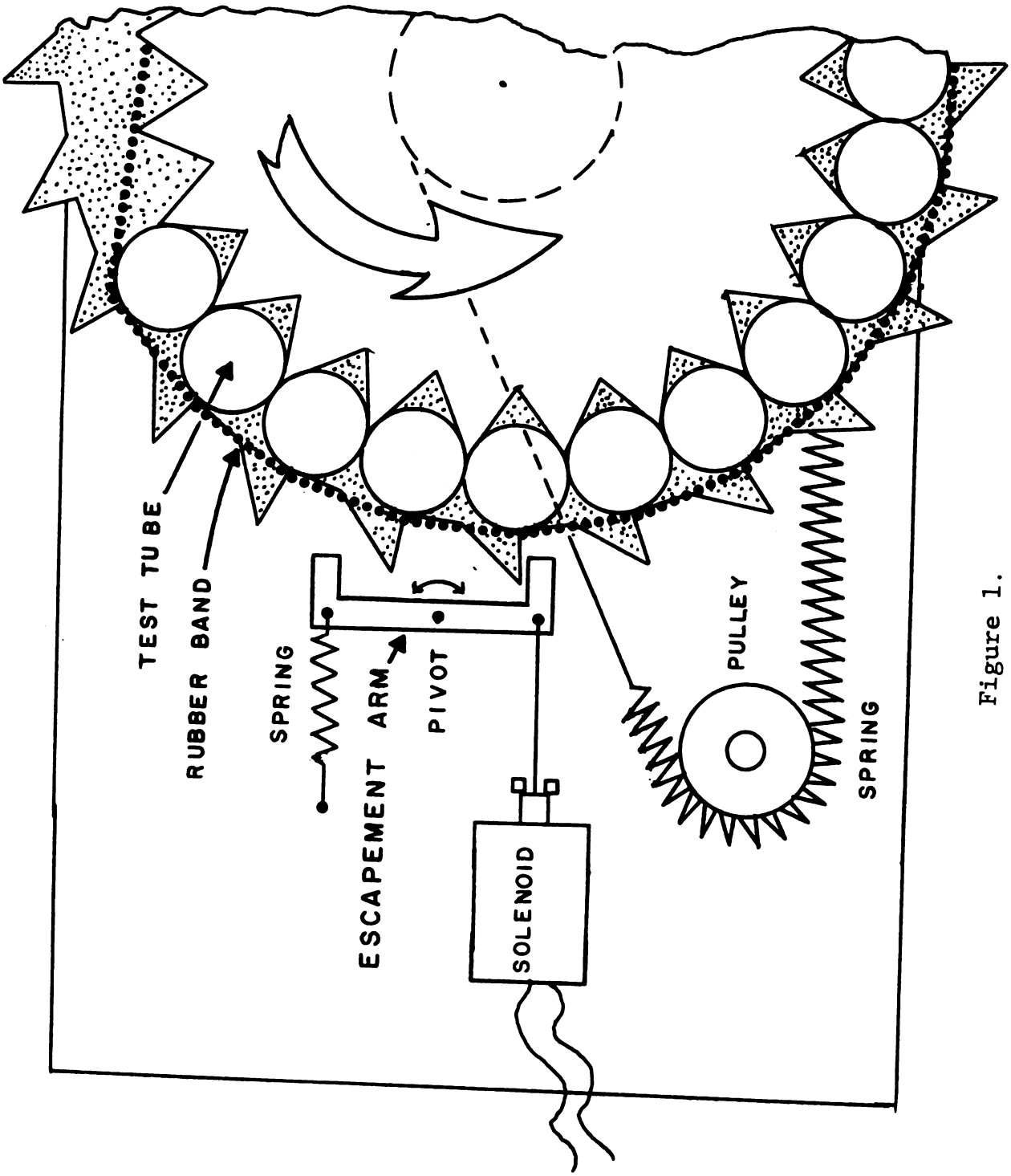
TEST TUBE
RUBBER BAND
SPRING
ESCAPEMENT ARM
PIVOT
SOLENOID
PULLEY
SPRING

Figure 1.

and advancement are performed by a simple escapement mechanism similar to that used in clocks and watches. Under normal circumstances the return spring pulls on the end of the pivoting escapement arm so that the forward end of the arm is caught on a cog of the collector assembly. When it is necessary to advance the fraction collector to the next tube, a solenoid is actuated. This in turn pulls the escapement arm and allows the collector assembly to advance to a position where the motion is stopped by the second arm of the escapement lever. This second arm of the escapement lever prevents the collector assembly from advancing more than one position while the solenoid is actuated. To complete the advancement, the solenoid relaxes and the escapement arm returns to the original position. The duration of a complete advancement cycle takes only a few tenths of a second at most.

The design of the circuit that actuates the solenoid is shown in Figure 2. Fractions can be collected for a preset length of time or for a present number of drops. The drop detector consists of a Light Emitting Diode (LED) and a phototransitor with a gap between them through which a drop can pass. The action of the descending drop disperses the light beam and momentarily lowers the voltage at the output of the phototransistor. This signal is then amplified by a transistor ($Q_2$) and is fed into a comparator (one quarter of an integrated circuit, 324). The integrated circuit compares this voltage to a reference voltage. When the voltage from $Q_2$ is greater than the reference voltage the output of the

Figure 2. Schematic diagram of the fraction collector circuit. All of the components were housed within an aluminum box except the drop detector assembly and the solenoid. For a discussion of the circuit, see text.

| | |
|---|---|
| $Q_1$ | -NPN phototransistor (Radio Shack 276-130) |
| $Q_2$, $Q_4$, $Q_5$ | -NPN transistor (MPS 2222A or equivalent) |
| $Q_3$ | -NPN power transistor (TIP 33) |
| Solenoid | -type B22 (12 volt, 2.3 A) |
| LED Display | -Seven-segment, common anode (Radio Shack 276-053) |

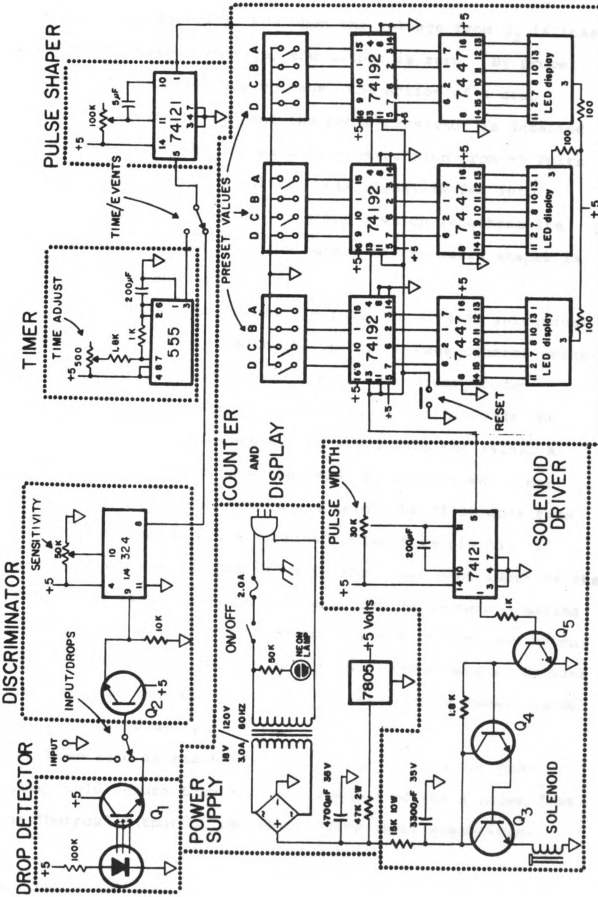
DROP DETECTOR
INPUT
INPUT/DROPS
DISCRIMINATOR
SENSITIVITY
POWER SUPPLY
18V /120V 60HZ 3.0A
+5 Volts
7805
ON/OFF
2.0A
COUNTER AND DISPLAY
PULSE WIDTH
SOLENOID DRIVER
SOLENOID
TIMER
TIME ADJUST
555
PULSE SHAPER
74121
TIME/EVENTS
PRESET VALUES
74192
7447
LED display
RESET

Figure 2.

comparator is +5 volts, but when the voltage from $Q_2$ is less than the reference voltage, the output is zero. By proper setting of the reference voltage, the action of a drop passing between the LED and the phototransistor is detected at the output of the 325 as a brief excursion from +5 Volts to zero and back to +5 volts. This signal is fed into a one-shot multivibrator (pulse shaper) which lengthens its duration and inverts it. The output of the pulse shaper is fed into the counting circuit.

The heart of the counting circuit is a 74192 integrated circuit. This is a presettable Up/Down decade counter which can be set at any number from 0 to 9 as a binary-coded decimal (BCD). A momentary grounding of pin 11 loads the number that is in switches A,B,C, and D into the 74192. A pulse at pin 4 then causes the 74192 to count down to one unit. If the previous number was zero, the 74192 goes from zero to 9 and sends out a "borrow" pulse from pin 13. If this borrow pulse is then fed into the count down input of the next 74192, it is possible to count from 99 to zero. Adding a third 74192 allows one to count from 999 to zero and so on. The 7447 integrated circuit is a binary-coded decimal decoder which decodes the BCD output of the 74192 for a common anode 7-segment LED display.

When zero is reached at all of the 74192's the next pulse would return them all to 9's and send out a pulse from the "borrow" output of the third 74192 (most significant

digit). However if this pulse is fed into the "load" inputs of all of the 74192 integrated circuits, the counters recycle to the present values rather than 999. This borrow pulse also activates the solenoid-driving circuit by triggering the one-shot multivibrator, a second 74121. The setting of the pulse-width potentiometer determines the duration of the solenoid actuation. The pulse from the one-shot multivibrator makes transistors $Q_3$, $Q_4$, and $Q_5$ conducting and discharges the 3300 μF capacitor through the solenoid.

The power supply section of the circuit provides for a +5 volt output (regulated by a heat-sinked integrated circuit - 7805) and charges the 3300 μF capacitor to about 22 volts.

For time-based fraction collection, a one Hz pulse is generated by a 555 integrated circuit. This pulse is fed into the same pulse shaper as the drop-detecting circuit and the counting proceeds as described above.