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ESTIMATION OF POOL SIZES AND KINETIC CONSTANTS

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INTRODUCTION

In chloroplasts electrons are driven from water to NADPH or other electron acceptors through a potential difference of > 1.2 V by means of two series connected photoacts. The photoproducts are processed in dark reactions which operate at both ends of the two photosystems and in the connecting chain. This essentially linear electron transport chain is linked to an ATP generating process which consumes part of the chemical potential generated by light. The electron transport chain contains a dozen or more components, each in very small concentration compared to the total chlorophyll (Chl). Observation of these components and that of the end products is done by a variety of direct or indirect methods, discussed in other parts of this volume. We will be concerned here with the nature and number of the electron transport components; particularly the interpretation of kinetic data concerning these intermediates. Any models presented are for illustrative purposes and are not an attempt to present a true and complete story.

Because of the ease with which chlorophyll can be determined, concentrations of the electron transport components are usually expressed in terms of total chlorophyll. One should realize that chlorophyll content varies with plant material and its pretreatment.

In strong, rate saturating light, green plants, algae and well prepared spinach chloroplasts evolve up to 300-400 moles of O_2 per mole chlorophyll per hour.[1,2] This corresponds to .1 O_2/Chl·sec or approximately .5 electron equivalent/Chl·sec. Roughly then, assuming that 2 excitations

are needed to move an electron through the chain ($8\ h\nu/O_2$) we compute that at optimal rate each chlorophyll undergoes one useful excitation per second; i.e., at optimal rate the turnover time of the chlorophyll is 1 sec.

Instead of giving continuous strong light, Emerson and Arnold[3,4] illuminated algae with repetitive short bright flashes. They found that the individual flashes lost effectiveness in removing electrons from water if they were spaced closer than ~ 10 msec and that flashes, bright enough to saturate the photochemical events, could not process more than 1 electron per ~ 500 Chl. Optimal results in similar experiments[5] range to values of ≃ 5 msec and 1 eq/400 Chl, which is in rough agreement with the turnover time/Chl given in the preceding paragraph. The interpretation is that there is one electron transport system for each 400 Chl in the chloroplast. This is substantiated by the observation that certain important constituents such as cytochrome f[6] and ferredoxin[7] occur in an abundance of about 1/400 Chl. If we now assume that the total chlorophyll is equally divided between the two photosystems, the picture emerges (Fig. 1) of separate electron transport chains which each comprise 2 photosystems (I and II). Each photosystem contains some 200 light harvesting pigment molecules from which excitations flow to a trapping center where the photoconversion occurs. Arrival of a quantum in each reaction center results in a charge separation. In system I an electron moves from the primary photooxidant P700 (a special chlorophyll complex of medium potential) to the primary photoreductant "X" which has a very low midpoint potential but is not further identified. In system II the electron is transferred from an unidentified high potential oxidant "Z" to a medium potential reductant "Q", possibly a quinone. In

subsequent dark steps Z^+ is reduced, ultimately by water, while O_2 is set free and X^- is reoxidized - ultimately in whole cells by CO_2. $P700^+$ is reduced by Q^- via the interconnecting reaction chain - probably including the site which generates ATP. The ATP is utilized in the fixation of CO_2. In isolated chloroplasts one can arrange conditions so that only part of the chain is operative and suitable artificial electron donors or acceptors can replace the natural ones.

Of the 4 primary photoproducts only P700 can be monitored directly. This can be done by spectroscopically viewing the loss or gain of absorption at 700 nm upon oxidation or reduction. The redox state of Q can presumably be viewed by the fluorescence yield of system II, an indirect but technically rather simple method. No direct observations have been made of Z, X or most of the other components of the chain except for cytochromes and quinones which undergo adequate absorption changes upon oxidation or reduction. Thus, much of the kinetic information is obtained by indirect approaches in which one monitors products such as O_2, reduced acceptors, ATP, or pH.

We will discuss methods for determining the nature and number of components in the electron transport chain and the rates at which the equivalents are transferred from one component to another. First we will discuss the primary photochemical donors and acceptors, then the pools of components in the chain and finally some aspects of kinetics applied to photosynthetic systems.

Observation of P700 and Its Immediate Electron Donors.

Light absorbed by system I causes the photooxidation of P700 which is characterized by a loss of optical density[8] and the formation of an EPR signal.[9] The photoconversion can take place in a single very brief flash and at temperatures as low as that of liquid helium.[10] This indicates a primary event; i.e., not a transfer of electrons in a thermal dark reaction. Under appropriate conditions one can slowly reduce P700 in dark and subsequently view the time course of its photooxidation in weak light. The kinetics of this conversion also indicate that the oxidation of P700 is a primary event: If the acceptor X is maintained in the active oxidized state, the rate of P700 photooxidation at any moment is proportional to the amount of reactive P700 (P700 still reduced) resulting in a first order time course of this photooxidation. The rate of this oxidation is proportional to the number of incident photons per unit time so that the half-time of the photooxidation is inversely proportional to intensity. The observations are consistent with:

$$\frac{dP}{dt} = - \alpha_1 I \phi_1 P/Ptot,$$

where ϕ_1 is the quantum yield when all P is reduced, α_1 the fraction of photons absorbed by system I, I the number of incident photons, P the amount of reduced P700 and P_{tot} is the total amount of P700. Assuming that the extinction coefficient of P700 is the same as that of the light harvesting chlorophyll, one computes from the maximum change in optical density upon photooxidation an abundance of the order of one P700/400 Chl.

In green cells, cytochrome f and plastocyanin act as electron donors to $P700^+$ and occur in a similar concentration, probably in a 1:1 ratio.[6,11]

It is somewhat difficult to isolate the photochemical conversion of P700 since these donors react rapidly with P700$^+$, even at low temperatures.[12] For instance, at -196°C (in liquid N_2) light photooxidizes both P700 and cytochrome f (denoted "C") and even here the transfer can occur in a few milliseconds.[13] The ability of cytochrome to react rapidly with P700 at low temperature indicates that collision chemistry is not involved in the transfer and that the two components are arranged as pairs in close proximity to each other.

The Photoreductant of System I.

The chemical nature of the primary electron acceptor in system I is unknown ("X"). One *a priori* expects that its abundance is equal to that of P700 and that the two form a complex. X^- is a strong reductant which can reduce many electron acceptors including low potential dyes like methylviologen. The semi-quinone of viologen has a strong absorption band so that one can spectroscopically view its formation after a short light flash - and its subsequent disappearance due to reoxidation by O_2. As expected, the reaction $X^- + V + H^+ \rightarrow VH + X$ proceeds faster the more viologen (denoted V) is present. At a concentration of 10^{-3} M the reaction is half completed in less than a millisecond. Indophenol dye reacts with even greater rapidity.[14] The rate of the natural reoxidation of X via ferredoxin and NADP reductase to NADP is not known; however, measurements with chloroplast fragments and exogenous donor indicate it is greater than 100/sec.[15] For chloroplasts in the absence of an exogenous electron acceptor there remains a rather variable residual rate of electron transport in air (Mehler reaction),[16] indicating that X^- reacts with O_2 in about one sec.

The Photoreductant of System II.

The chemical nature of the primary electron acceptor "Q" in system II is also not known. It is speculated that it may be a plastoquinone. Therefore indirect means must also be used to determine the redox state of this component. These methods are based on the assumption that photochemistry can only proceed when the acceptor is in the oxidized, active state.

Fluorescence, being relatively easy to measure, is often used as a tool for monitoring the state of Q. The reasoning used is as follows: A quantum absorbed by one of the chlorophylls in a unit wanders through the pigment array until it encounters an active reaction center where it carries out a photoconversion. These events must take place in less than $\sim 10^{-8}$ sec or else the excitation is degraded into heat or reemitted as ($\sim$ 700 nm) fluorescence. Blocking of the photochemistry is therefore accompanied by an increase in fluorescence; and fluorescence changes occur in a manner complimentary to the photochemical rates.

Photochemistry in the two systems is blocked if any of the two donors or two acceptors are in the inactive state. However, no significant fluorescence changes have been observed which are conclusively attributed to the state of the system I traps, a fact for which we have no ready explanation. Furthermore, fluorescence changes are usually attributed exclusively to the state of the system II acceptor Q, since it is assumed that under most conditions the high potential photooxidant Z remains in its reduced, photoactive state.

The sum of the quantum yields for fluorescence f, degradation into

heat H, and photochemistry r must be equal to unity $(f + H + r = 1)$. The fluorescence yield then increases as the system II traps close; i.e., $f \to 1 - H$ when $r \to 0$. Most fluorescence analyses assume that f is proportional to H. If this postulate is made, $r = 1 - F$; where $F = f/f_{max}$ and f_{max} (see Fig. 2) is the fluorescence for all traps closed, $r = 0$. With this postulate, changes in photochemical yields are equal to normalized negative changes in fluorescence yields. We can compare with the photochemical rate rather than the yield, since the quantum yield for photochemistry is the rate/absorbed intensity; the latter expressed in quanta/unit time. For a given intensity the rate R is proportional to the quantum yield; i.e., R = constant x r. In addition to the variable fluorescence f described above, there is a constant fluorescence yield f_o, which is unaffected by the photochemistry. A problem encountered is the question whether f_o is the fluorescence due to some small amount of disconnected pigment (dead fluorescence) or is due to fluorescence in competition with photochemistry. For either case $R/R_{max} = 1 - F$, where R_{max} is the maximum rate and f and f_{max} are measured from f_o. For the latter case (no dead fluorescence) f_o must be included in f for computation of the quantum yield r.

The variations in fluorescence do tend to behave anti-parallel to the rate of electron transport of system II; i.e., variations of the rate of photochemistry as measured by the rate of oxygen evolution follow the properly normalized variable fluorescence yield.[17] With an artificial electron donor such as hydroxylamine replacing water, the fluorescence transient is not changed,[18] which supports the notion that one exclusively views the phenomena on the reducing side of system II.

It is important to note that although the assumption that H is proportional to f does appear to be valid in many cases, and is used extensively in interpreting fluorescence data, the literature abounds with evidence that the situation is not so simple. One exception is seen in whole cells when considering long term effects (~ 1 min or longer).[19-21] Another exception to the simple behavior is observed at the onset of illumination after a dark period where F shows a peculiar rapid rise.[22] Still another problem encountered is the inability to induce the full fluorescence rise in less than about 50 msecs, no matter how intense the light.[23] It should be also mentioned that we have only considered non-cyclic flow through system II. The O_2 evolution system is rather fragile and Z^+ is a labile photoproduct which can oxidize substances other than water, probably including Q^- so that a cyclic flow in system II may occur. In spite of these reservations fluorescence has been a very useful tool for the study of Q. Our application in the following will be for situations in which the deviations probably play little or no role.

Assuming that Z is always reduced, the rate of system II photochemistry is proportional to the amount of Q in the active, oxidized state. This gives $Q/Q_{max} = R/R_{max} = 1 - F$. Actually the relation between Q and R (and therefore F) is not quite linear.[24] This non-linearity is ascribed to a certain degree of cooperation between system II pigment units - an absorbed quantum which finds the nearest center closed having a chance to travel to a neighboring unit. The reader is referred to the literature for this complicating phenomenon.[24,25] For simplicity we will adhere to the linear relation between Q, F and R.

The poison DCMU presumably prevents the normal rapid reoxidation of Q^- by its reaction partner A, discussed below. A slow dark oxidation remains however, so that in darkness Q^- is returned to Q in about a second. If one now admits a light beam, the incoming quanta progressively convert Q to Q^- and the fluorescence yield rises (Fig. 2). Since in the presence of DCMU Q^- is reoxidized slowly, reoxidation during this conversion can be neglected in all but the weakest intensities. Assuming that Z remains reduced, we expect that the photochemical rate is proportional to oxidized Q and the conversion should, as in the case for P, proceed in a first order manner:

$$- \frac{dQ}{dt} = \alpha_2 I \phi_2 Q = Q_{tot} \frac{dF}{dt} .$$

Due to the excitation energy transfer between units mentioned above, this expectation proved only approximately fulfilled.[24]

In the presence of DCMU the conversion $Q \rightarrow Q^-$ as defined by fluorescence behaves as a purely photochemical act: a) the rate is inversely proportional to light intensity, b) most of the conversion can be carried out by a single 10 μsec light flash, provided it is bright enough to deliver sufficient quanta, c) the phenomenon still occurs if the sample is dark restored at room temperature and then prior to illumination is cooled with dry ice.[26] Since the DCMU rise curve reflects the rate of photoconversion of the system II traps in a sample, its half-time can be used as a rough measure of the effective system II intensity of a light beam.

Pools: General Considerations.

In speaking of pools we are concerned with the number of electrons that can be stored in either part of or the whole of the chain between Q and P. Since we have not been able to measure all of these storage components directly, indirect methods have been used.

In order to discuss these methods, we will first consider the problems involved in measuring the capacity of ordinary swimming pools. Suppose that we have, as in Fig. 3, two pools, A and B. We can measure their total capacity by emptying the pools and then measuring the total amount of water needed to fill the pools; alternatively we can fill the pools and measure the amount of water we can pump from them. It may be easier to measure the rate of water flowing through our pumps, in which case we can integrate this rate over the time necessary to fill or empty the pools: Pool size = $\int$ Rate dt. Since our pools are leaky we must pump fast enough so that we can neglect the water that leaks out. We could check this by doubling our pumping speed and seeing if we get the same result.

If we wanted to measure the capacity of A alone, we must try to fill fast enough so that flow from A to B is negligible, and similarly to get the size of B we must empty B fast enough so that little water flows from A. Again we can check by varying the pumping speed.

We can now discuss photosynthetic systems by replacing our pools with electron transport components, the water by electron equivalents, the pumps by the photoacts and the connection between the pools by some (at times) rate limiting step. We can measure the number of equivalents put into an

emptied pool by the amount of either added or natural system II donor oxidized during the filling. We can measure the number of equivalents removed from the pool from the behavior of system I acceptors. We can also make pool size determinations from the rate of flow at either end.

Different pools can be studied by altering the light intensity (rate of pumping) and the rate limiting steps. If we find that over a wide range of light intensities the pool size determinations are independent of light intensity, then we feel we are viewing a true pool of intermediates.

We can empty the pool with far-red light which is almost exclusively effective for system I. (Care must be used to make this light not too strong, particularly without a system I acceptor, or the small system II component can become appreciable.) No light is exclusively effective for system II and we have to resort to rate limitations for filling.

Pool Size Determinations - Rate Limitations.

Since rate limitations are used to separate pools we will discuss these limitations before going on to specific examples of pool size determinations. The rate limitations depend very much on the system being studied. It is not always clear where the limitation occurs and indeed this is one of the problems with pool size determinations; i.e., to know what pools are being measured.

The rate limitation in isolated chloroplasts without acceptor is in the transfer of electrons from $X \rightarrow O_2$ (Mehler reaction); i.e., at 3

in Fig. 1. This rate is on the order of 1 electron/sec. Therefore, bright light can be used to fill (reduce) the entire chain in well washed chloroplasts.

This rate limitation is removed by the addition of an electron acceptor such as methyl viologen. Now the rate limitation depends on the state of the ATP generating system. If the ATP apparatus is functional but "constipated" (a state which can be obtained by not supplying phosphorylation substrates such as ADP, P_i, etc.) then there is a limitation at the ATP site (2 in Fig. 1) of about 10/sec.[1,27] Therefore, for well prepared chloroplasts with an acceptor but no ADP, etc., bright light probably fills all pools up to an ATP site and empties all pools on the other side.

The limitation at the ATP site is relieved if the ATP process is short circuited by an uncoupler. As will be discussed later, A possibly does not get fully reduced in uncoupled chloroplasts (with acceptor); i.e., A becomes reduced until the transfer time of $Q^- \rightarrow A^-$ equals the transfer time of $A^- \rightarrow B^-$. (The $Q^- \rightarrow A^-$ rate is proportional to A oxidized while the $A^- \rightarrow B^-$ rate is proportional to A reduced.) The rate limitation is therefore possibly at A (1 in Fig. 1). The steady-state rate for such uncoupled chloroplasts is about 100 equiv./sec.[1] This is in agreement with the time for reduction of P700 after the cessation of bright light of $\sim$10 msec and with the turnover time for a component of A, plastiquinone, which will be discussed later.

The situation in whole cells is not entirely clear. The rate in whole cells is comparable to that in uncoupled chloroplasts (with acceptor),[1] indicating that the cells behave almost like such chloroplasts. This is to

be expected since both electrons from system I and the ATP are being efficiently utilized. (Efficient utilization of ATP should give the same result as short circuiting the ATP system.) However, limitations might arise at the ATP site, in a way that balances the ATP production and CO_2 reducing processes.

Pools as Viewed from the System I Side.

As was indicated above, with strong white light we can fill the pools from Q to the ATP site in isolated chloroplasts in the presence of a system I acceptor (such as methyl viologen). We can then use weak to moderate far-red light (effective mainly for system I) to empty it, while measuring the rate of acceptor reduction. (The modulated polarograph[28] arranged to observe methyl viologen reduction rate is best used for this.) The integral $\int Rdt$ is then a measure of the A pool. In the presence of an acceptor, the rate R_1 of photooxidation on system I should be proportional to the amount of P700 in the reduced state. Figures 4 and 5 show that the rate of methyl viologen reduction does indeed parallel the behavior of reduced P700.[27] Initially P700 is fully reduced and the (modulated) system I rate is maximal. At the end of the transient P700 becomes largely oxidized and the rate very low because the reduced pool is depleted.

Since X is kept oxidized by an acceptor, $R_1 = \alpha_1 I\phi_1 P/P_{tot}$ and $\int PIdt$ is proportional to the total number of equivalents. This integral is I times the area under the curve of P vs. t given in Fig. 5. For a fixed far-red measuring beam the area is therefore proportional to the number of equivalents. If all the electrons are measured one should get the

same area for different light intensities if one plots P against I x t (i.e., photons) instead of t. As indicated previously, such a check of using different light intensities is necessary to insure that all of the electrons are counted and that the measurements are not limited by some dark reaction or leaking. The pools measured as in Figs 4 and 5 are independent of light intensity over a large range of intensities. If the measurements are made in an intensity range such that dark reactions have no influence (are fast compared to the quantum flux), the kinetics of the P700 changes (the shape of the transient) should also be independent of intensity when plotted against I x t. It is conceivable that dark reaction times alter the kinetics but not the area or total equivalents counted. No such kinetic changes have been reported, perhaps due to the fact that emptying times have been rather slow (seconds).

The areas shown above give a relative measure of pool size. It is sometimes difficult to determine absolute magnitudes of the pool. To be meaningful these magnitudes must be compared to some standard such as total chlorophyll or number of a particular molecule such as P700. The areas above are referred to the number of P700 molecules in the following manner: Measurements[29] (which will not be discussed here) indicate that after about 20 min. in the dark only P700 and another partner molecule are reduced. The area obtained when P700 is oxidized by far-red light is then two equivalents. All other areas can then be related to this "standard". Such measurements indicate a pool of $\sim$ 10 equivalents/P700.

Care must be exercised with the use of artificial electron acceptors since these may entertain a cyclic electron flow. For example, a reduced

system I acceptor may donate its electron directly or indirectly back to $P700^{+}$ in competition with the flow from the pools. Viologens ($\rightarrow O_2$), ferricyanide and ferredoxin ($\rightarrow$ NADP) are commonly used acceptors which probably do not cycle.

Pools as Viewed from the System II Side.

Pools can also be measured from the system II side of the chain by giving light strong enough to fill (reduce) the chain up to some rate limiting step and counting the number of equivalents put into the system to reach the steady-state. The rate R of oxygen evolution can be measured while filling. The number of equivalents put into the system is then $4 \int_0^\infty R dt$. (The factor 4 comes from 4 equivalents per O_2 molecule.) The rate of filling must be much faster than the rate limiting step or subsequent flow negates the results. For example, the Mehler reaction may add oxidizing equivalents into the system if the measurement is too slow. The influence of this and of other dark reactions can be checked as for P700 by doing the experiments at different intensities and plotting the results in terms of I x t.

Alternatively, since the rate of system II is related to fluorescence yield, the integral $\int_0^\infty (1 - F) dt$ is also proportional to the number of equivalents put into the chain. This is the area above the rise curve for fluorescence (see Fig. 6). The area so obtained in the absence of an acceptor is about 20 times larger than that obtained in the presence of the poison DCMU.[30] Assuming that only Q is reduced in the presence of DCMU, while the whole chain is reduced in its absence, there are 20 equivalents in the chain

for each Q. Again the I x t law is fulfilled - be it now only over a restricted range of intensities where the dark reactions can keep pace with the quantum flux. Therefore it appears that a large pool of intermediates, made up largely of secondary reductants is viewed. The complex time course of F and changes of kinetics with light intensity show that this pool is heterogenous.

Figure 6 illustrates how the equivalents reduced during the rise were quantitated relative to the total chlorophyll.[31] If, after F_{max} was attained, a small, measured amount of acceptor (ferricyanide) was added, the fluorescence showed a temporary dip. The area bounded by this dip now reflected the number of added FeCn equivalents all of which were reduced by the light. Therefore this added area could be used as a standard. The ratio of the area over the rise curve and the calibrated (standard) area yielded the size of the total internal reductant pool. With measurements of absorbed intensity the quantum yield of the conversions could be estimated. It was found to be high ($\sim$ 1 eq/2 $h\nu$). Estimates of this type for the pool of internal reductants yielded values $\sim$ 1/35 Chl, an order of magnitude greater than that of the trapping centers. This is in agreement with the pool size found (see above) using DCMU as a reference. The pool size of plastoquinone determined by absorption changes at 254 nm appears to be about 14 equivalents (7 molecules) per chain.[32]

Kinetics: General Principles.

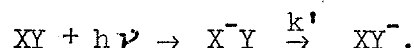
The behavior (redox state) of a component or pool X is governed by the flow of equivalents into it (f_{in}) and the flow out (f_{out}). The rate of

change of X^- is the sum of these*. In kinetic analyses of X one determines f_{in} and f_{out} and their functional dependences, so as to understand the mechanisms of the underlying processes. The flow rates depend on the redox state of X itself, of its electron donor (D) and of its electron acceptor (A). For example, the flow to a completely reduced component is zero. If the component is a primary photochemical acceptor or donor, the rate depends on light intensity (I). In addition the flow depends on other environmental factors, such as temperature, state of the membrane, pH, etc. We have then

$$\frac{dX^-}{dt} = f_{in}(X^-, I, A, D) - f_{out}(X^-, I, A, D).$$

Techniques used in determining these functions involve a change of the system and a study of the subsequent time course of X and (when possible) other parameters. Isolation of each of the two functions (f_{in} and f_{out}) can often be achieved by blocking one of the transfers. For example, the flow from Q can be blocked chemically with DCMU, or flow from a component might be blocked by reducing its acceptor (A). Particular transfers may be lacking in mutants. If one of the rates (for example f_{in}) is light driven, the dark reaction (f_{out}) can be followed after removing the light.

To illustrate we will take a simple example in which light (I) reduces X and the electron is then transferred in a dark reaction to some component Y:



$$XY + h\nu \rightarrow X^-Y \xrightarrow{k'} XY^-.$$

Let us assume that f_{in} is proportional to X and I and that f_{out} is proportional to the number of donors X^- and acceptors Y. We further assume that Y is held

*The capital letters will be used to denote concentrations of components and the superscript minus to designate the reduced state.

constant (for example by an excess of external oxidant). Then f_{out} is proportional to X^- alone and

$$\frac{dX^-}{dt} = \alpha \phi I (X_T - X^-) - kX^-,$$

where (since Y is constant) $k = k'Y$ and the subscript T denotes total concentration of the component. For $X^- = X_o^-$ at $t = 0$, the solution to this equation yields:

$$X^- = X_F^- - (X_F^- - X_o^-)e^{-Kt},$$

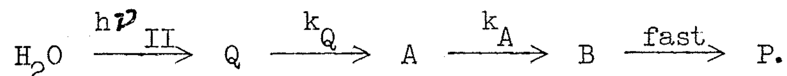
where X_F^-, the steady-state level ($t \rightarrow \infty$) of X^-, is given by

$$X_F^- = \frac{\alpha \phi I}{k + \alpha \phi I} X_T$$

and $K = \alpha \phi I + k$. Fig. 7 illustrates the behavior of X^- at various light intensities for $X_o^- = 0$. The decay in the dark is given by $X^- = X_o^- e^{-kt}$. The dashed lines of Figure 7 indicate the behavior when the light is extinguished after various levels of X^- are reached. There are several points to note. The steady-state level X_F^- asymptotically reaches X_T with increasing light intensity ($\alpha \phi I >> k$). Since initially $X_o^- = 0$, the dark decay f_{out} is initially zero. The initial rate of f_{in} therefore is proportional to light intensity. The rise and decay are first order. One can demonstrate this for the rise by plotting $\log(X_F^- - X^-)$ vs. time. This gives a straight line with slope $-K$. Similarly the decay gives a straight line with slope $-k$ when the $\log X^-$ is plotted as a function of time. Also, since the dark decay does not depend on how X^- is reached, the decays represented by the two dashed curves in Fig. 7 should be superimposable if one of the curves is shifted in time.

Let us now turn the problem around, and assume that we are able to follow the rise and decay of X^- and want to determine f_{in} and f_{out}. To check whether these functions satisfy the first order behaviour described above, we can look at log plots and we can check whether we find the appropriate intensity dependence of the steady-state level and the initial rate.

As an example of the above considerations we will discuss some data on the redox state of plastoquinone in the presence of a system I acceptor and an uncoupler. Changes in optical density at about 254 nm have been attributed to changes in the redox state of plastoquinone.[33] These changes[34,35] appear rapidly ($\leq$ 50 μsec) and have kinetics which suggest that pool A is plastoquinone. Although the data and interpretation may be obsolete in light of newer information,[32] they are presented because of pedagogical merits. The model assumes that at saturating light intensities all Q is reduced and that the time for the initial reduction of Q can be neglected. The rate between Q and A then is proportional to A. A, in turn, transfers its electrons to B which, because of rapid oxidation by the rest of the chain in the presence of acceptor and uncoupler, is always in the oxidized state. We then have



$$H_2O \xrightarrow{h\nu\ II} Q \xrightarrow{k_Q} A \xrightarrow{k_A} B \xrightarrow{fast} P.$$

The rate of reduction of A is given by:

$$\frac{dA^-}{dt} = k_Q\,(A_T - A^-) - k_A A^-,$$

where $k_A = k_A' \cdot B_T$, and A_T and B_T are the total amounts of A and B in

a chain. With the appropriate substitution of symbols, this is the first order equation given previously in the section. The solution to this equation with $A_o^- = 0$ at $t = 0$ is

$$A^- = A_F^- (1 - e^{-(k_Q + k_A)t}),$$

where $A_F^- = \left[k_Q/(k_Q + k_A)\right] A_T$ is the steady-state level of A^-. If the light is extinguished after A^- has reached a certain level, A^- should go oxidized with a rate $dA^-/dt = - k_A A^-$. The plastoquinone changes reported for these two situations follow this first order behaviour, with $(k_Q + k_A) = 46/sec$ in the "light on" experiment; and $k_A = 11/sec$ in the "light off" experiment. Therefore $k_Q = 35$ sec. The maximum amount of reduced A^- in saturating light is then $A_F^- = (35/46)A_T$ or about 80%. A comparison of absorption changes of plastoquinone and P700 (making assumptions concerning extinction coefficients) sets the number of A molecules (A_T) at 13 equivalents per P700 molecule or chain. Therefore about 10 A equivalents per chain are reduced in the steady-state.

The model suggests that at the onset of illumination all Q's are reduced very rapidly (≤ 50 μsecs) and further reduction of the system cannot take place until electrons go to A. The time for transfer from Q to A would be proportional to the amount of oxidized A, (A^+). In the steady-state the rate is $k_Q A^+ = (35/sec)(3) \simeq 100/sec$. Therefore the time is about 10 msec, again reminiscent of the time in the Emerson-Arnold experiment and consistent with the time for reduction of P when light is extinguished. Since in the steady-state all rates must be the same, in particular the rate of transfer

from A ($k_A A^-$) must be $\simeq$ 100/sec. For A completely oxidized, the transfer rate $Q \rightarrow A$ is $(35/\text{sec})(13) \simeq 500/\text{sec}$ corresponding to a time of $\sim$2 msec.

Since $e^{-.69} = 0.5$, the half time for the total plastoquinone change is given by $t_{1/2} = .69/(k_A + k_Q)$ _ 15 msec. That is the time for 5 of the A equivalents to be reduced.

So far we have limited our discussion to first order reactions. Other types of reactions such as second order undoubtedly occur. We refer the reader to textbooks on kinetics for this. However, such discussions are of limited use for the understanding of more complex, interrelating processes. It should be emphasized that precise measurements over a large range are required to decide between a sequence of first order reactions and a second order or more complicated process.

For the case where the decay is not solely a function of X^-, other parameters must be varied in order to obtain the functional dependences of f_{in} and f_{out}. No longer can decays be superimposed. f_{in} can be determined by adding f_{out} to $\frac{dX^-}{dt}$ at each point; i.e., f_{in} at some point is the slope of the rise curve at that point minus the magnitude of the initial slope of the decay curve when the light is extinguished at that point.

It is not always necessary to directly measure a component in order to determine its kinetic behavior. The next section gives an example of how the kinetic decay of an unidentified component which limits the formation of oxygen precursor is determined indirectly from oxygen evolution.

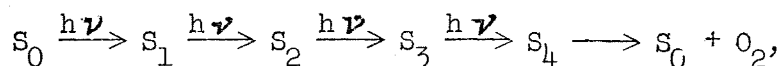
We have tacitly ignored differences between separate and interacting chains in the above kinetic discussions. This was possible because the

behavior for a simple, first order reaction is the same for both. This is not true in general. Some examples of separate chain behavior will be given in the last section.

O_2 Evolution.

Intermediates of the oxygen evolution process - precursors of O_2 - are unstable so that initially after a dark period precursors rather than oxygen are made.[36] Accordingly, if after a dark period a continuous light is given, the O_2 rate is initially zero and then rises to the steady-state value. Such rise curves of the rate follow the I x t relation; i.e., the oxygen deficit at the onset of illumination reflects a finite pool - a pool of O_2 precursors which are present during the steady-state but absent after a dark period. Most of this deficit is removed by a single flash. In other words, the deficit pool is of the order of one equivalent per trapping center.

The simplest kinetic interpretation[37] of the O_2 evolution process is a linear accumulation of four + charges by a catalyst S, connected to each individual trapping center,



$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_4 \longrightarrow S_0 + O_2,$$

where the subscript of S designates the amount of accumulated charge. The S_3 and S_2 states are unstable, being deactivated (reduced) to S_1, a stable state: $S_3 \longrightarrow S_2 \longrightarrow S_1$. Therefore after a long dark period the traps are either in the S_0 or S_1 state. Each of a series of brief flashes adds one oxidizing equivalent to each trapping center and brings S in the next higher oxidation state. After deactivation the first two flashes yield no oxygen

but bring the S_0 or S_1 states into S_2 or S_3 states. The third and fourth flashes now produce oxygen.

Photoact II cannot be repeated unless Q and Z are regenerated. If we assume a one to one correspondence between S, Q and Z, the time between flash pairs is a measure of the slowest regeneration time in the sequence $S \longrightarrow A$. For example (Fig. 8), flash #2 lost half its effectiveness if spaced closer than 200 μsecs to the 1st flash. The regeneration occurred in a second order manner. The regeneration time necessary for flash #3 to be effective after flash #2 is somewhat longer. Thus with the assumption of a one to one correspondence, the recovery of both donor and acceptor for system II, (under certain conditions) takes place in $\leq$ 200 μsecs. Under other conditions this recovery should be slower, reflecting the dependence of the transfer time from Q to A upon the redox state of A.

We could expect that optical density changes associated with either a primary donor or acceptor to system II would occur rapidly upon illumination. From the paragraph directly above, we would also expect a rapid decay of these changes. Fast changes in optical density associated with system II which may be related to the primary acceptor or donor have been observed at 682 nm[38,39] and 325 nm.[33] These changes were observed with the use of repetitive flashes of about 10-20 μsecs duration in the presence of acceptor (benzyl viologen) and uncoupler (NH_4Cl). The negative absorption change at 682 appears to be that of a chlorophyll. Because of its rapid return ($\sim$ 200 μsec) it can be separated kinetically from changes due to P700, which restore much slower. We have observed (unpublished) a similar rapid return of the fluorescence yield, which was raised by a brief flash.

Separate Chains and Equilibria.

As we mentioned before, the small temperature effect upon the $C \rightarrow P^{+}$ transfer shows that we are not dealing with conventional reactions in solution where all reactants can meet each other, but that the components are built together in a rigid matrix - so that each P700 communicates with only one cytochrome donor molecule. It is believed that the other components in the chain behave at least partly in this manner; i.e., to a large degree each chain operates independently of the others. The behavior of such systems is different enough from that of molecules in solution that a few comments are in order.

One feature of independent chains is that the various reactions will show first order kinetics rather than second or higher order behavior; i.e., in the reaction between P700 and cytochrome the rate will not be proportional to the product of the two concentrations $(P^{+} \cdot C)$. For each chain in the $P^{+}C$ state the electron transfer from $C \rightarrow P^{+}$ has a characteristic probability of occurrence $k(\sec^{-1})$, and viewing the overall system the rate will be the product of k and the number of $P^{+}C$ states in the sample.

Another feature of independent chains is that normal equilibrium considerations may not be applicable. An example of this is obtained from the comparison of the steady-state reduction levels of P700 and cytochrome f in weak light of various wavelengths. In weak light, quanta arrive in the traps so infrequently that the dark reactions have ample time to proceed and equilibrium conditions are expected:

$$C + P^{+} \underset{k'}{\overset{k}{\rightleftarrows}} C^{+} + P \qquad \frac{C^{+}}{C} \cdot \frac{P}{P^{+}} = k/k' = K$$

One now observes for example[40], that at a wavelength where cytochrome *f* is ~ 50% reduced P700 is ~ 80% reduced and computes a value

$$K = \frac{0.5}{0.5} \cdot \frac{0.8}{0.2} = 4$$

In darkness, however, with the same chloroplasts, one meets the situation that virtually all P700 is reduced and all cytochrome *f* is oxidized, which suggests a very much higher equilibrium constant.

There are three possible explanations for this apparent change of K. The first is that the same entities are not measured in the two experiments. Spectral measurements indicate that they are. The second is that the equilibrium constant changes, perhaps due to some change in membrane structure. Attempts to see changes caused by the additions of uncouplers have failed, indicating that the high energy intermediate associated with ATP formation and associated phenomena are probably not involved. The third is that in at least one of the experiments equilibrium is not obtained. The rapidity of the reaction between C and P is such that equilibrium should have been obtained. In addition the results were independent of light intensity over a wide range.

The concept of "independent chains" not in equilibrium with one another can account for such an apparent change of K: At any moment the individual chains in the sample can have four possible states P^+C^+, P^+C, PC^+, PC. If we assume a high inherent K value, state P^+C with a life time of 1 msec does not accumulate, so that we have a distribution between only three states with either 0, 1 or 2 electrons in the complex (P^+C^+, PC^+, PC). While the high inherent K, observed in darkness, does not allow the occurrence of P^+C chains, the lack of electron transfer between chains allows a

simultaneous accumulation of states P^+C^+ and PC. Since the entire system and not individual chains are viewed, the presence of both P^+ and C at the same time gives the appearance of a low K. Malkin,[41] who treated this matter in detail, computes that for a 2 component system, like the one discussed, an irreversible step ($K = \infty$) would appear to have a varying K which, in the range of easy measurement, would appear to be as low as 4. He further calculated that this distortion rapidly decreases with an increase of the number of steps and intermediates in the chain.

A similar low apparent equilibrium constant in the light has been observed between Q and P. In the steady state, the rate of input to system II must equal the output of system I and $R_2 = R_1 = \alpha_2\phi_2 IQ = \alpha_1\phi_1 IP$ in which R_1 and R_2 are the rates of the two systems. Thus, considering the overall system (the total of all reaction chains) we will find a wavelength dependent ratio of open traps in the two systems; $Q/P = \alpha_1\phi_1/\alpha_2\phi_2$. If, in addition, we assume equilibrium, the added constant fixes the levels of P and Q; i.e., both P and Q are independent of light intensity. These relationships are observed experimentally for light intensities weak enough so that neither the rate of transfer between Q and P nor the rate of transfer at the ends of the chain is limiting. Therefore the redox state of the two traps in continuous light of various wavelengths should reveal the value of K_{QP}. One can use one of the several procedures discussed above to determine the states P and Q. The results of such measurements[42] suggested an unexpectedly low value for K_{QP} (≤ 10). These low computed K values are clearly in conflict with estimates of K_{QP} in darkness and the respective midpoint potentials (the difference in midpoint potentials ΔE_m at room temperature is given by

$$\Delta E_m = RT \log K_{QP} = 60 \log K_{QP}$$

where the potential is expressed in millivolts. In this instance, due to the large number of components between Q and P, the discrepancy does not appear due to non-equilibrium between chains.

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Fig. 1 - Schematic for the non-cyclic electron flow from water to CO_2. Dashed lines indicate components omitted from the scheme.

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Fig. 4 - Rate of viologen reduction as a function of time for three different intensities of far-red light for emptying the pool.

Fig. 5 - Time course for absorption of P700 in the presence of far-red light after the pool was filled with a long flash (top) or partly filled by a brief flash (bottom).

Fig. 6 - Fluorescence yield (arbitrary units), for isolated chloroplasts as a function of time. The shaded area A is obtained in the absence of an acceptor. An additional area B was obtained when the acceptor ferricyanide was added at time designated by arrow.

Fig. 7 - X^- as a function of t for simple first order behavior. Solid lines, light of given intensities turned on at $t = 0$ with $X_o^- = 0$. Dashed lines, light turned off at arrow.

Fig. 8 - Oxygen yield from the third of a sequence of three flashes as a function of time between the 1st two flashes.

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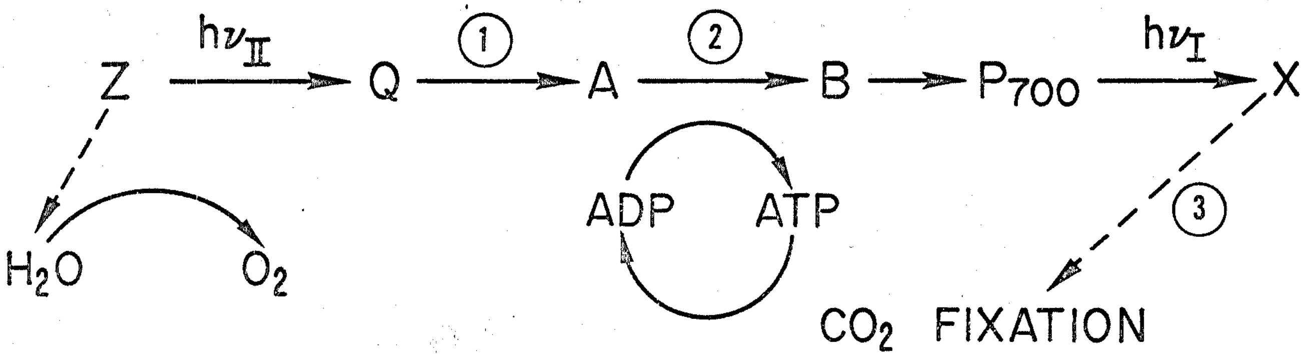

Fig 1

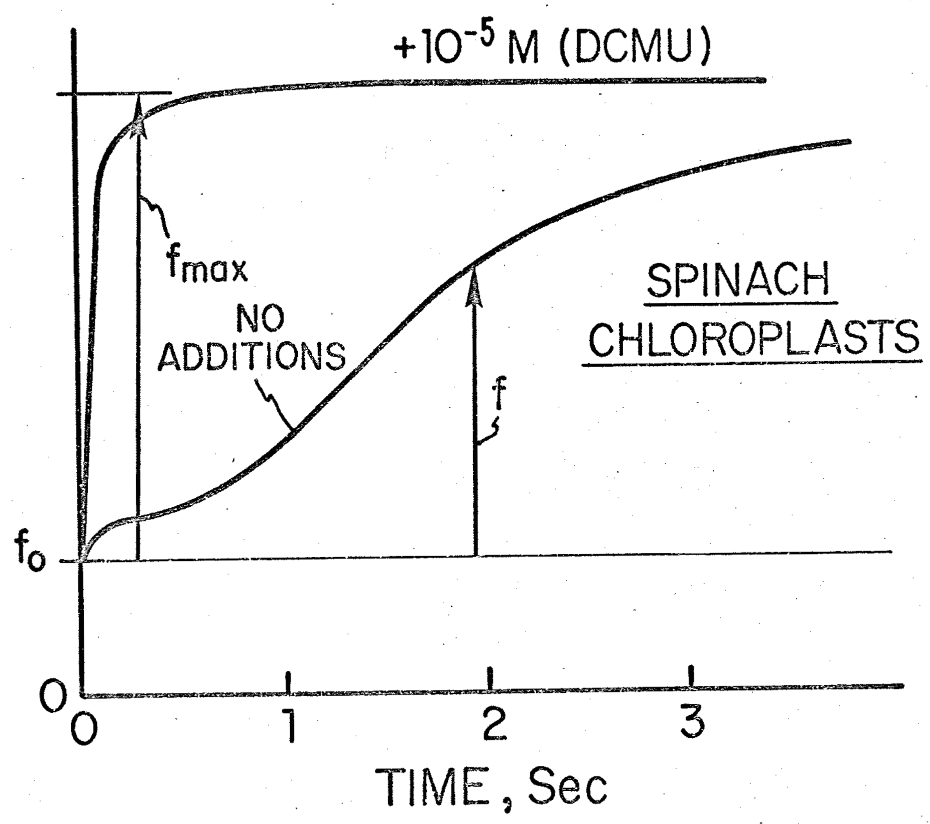

FIG 2

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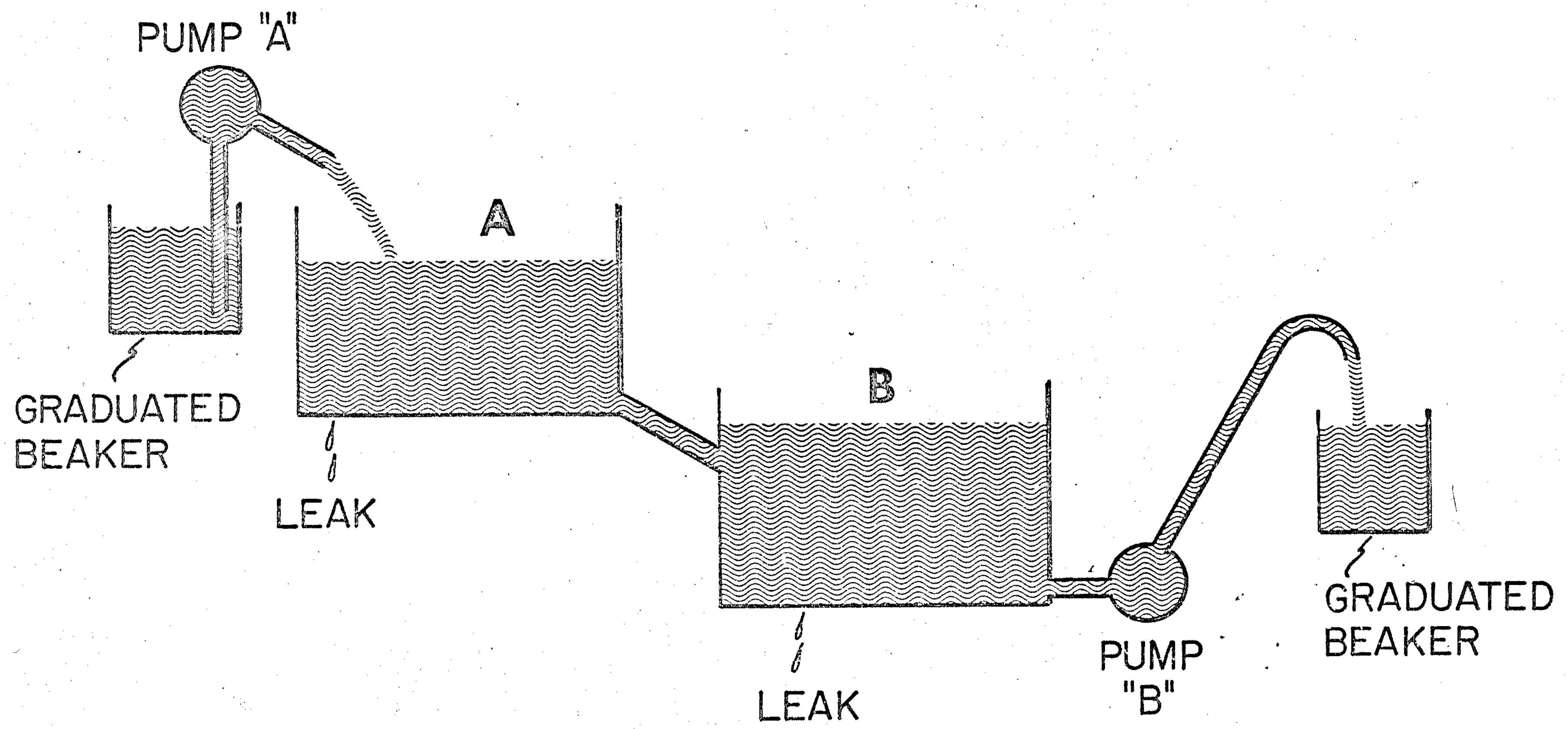
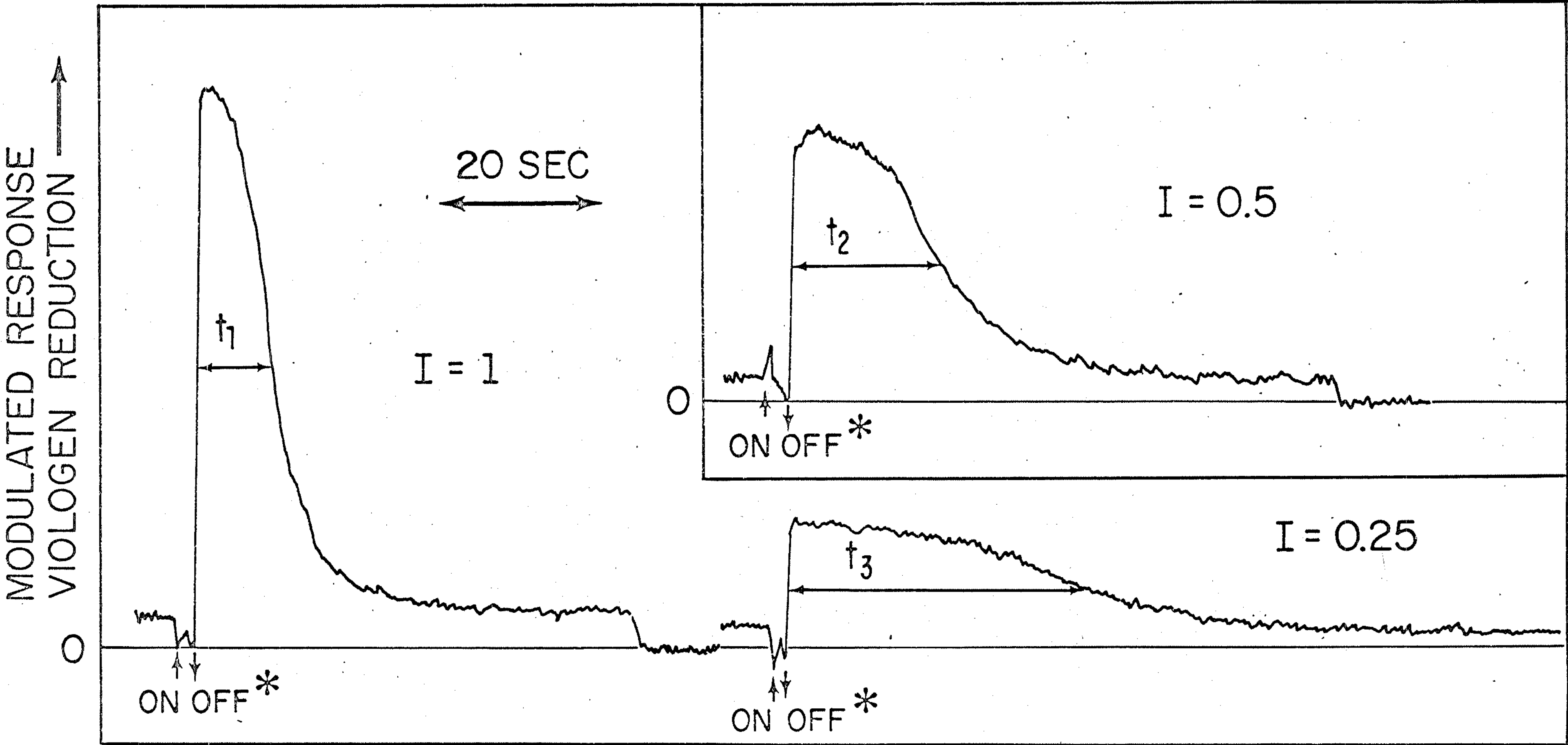


FIG 3



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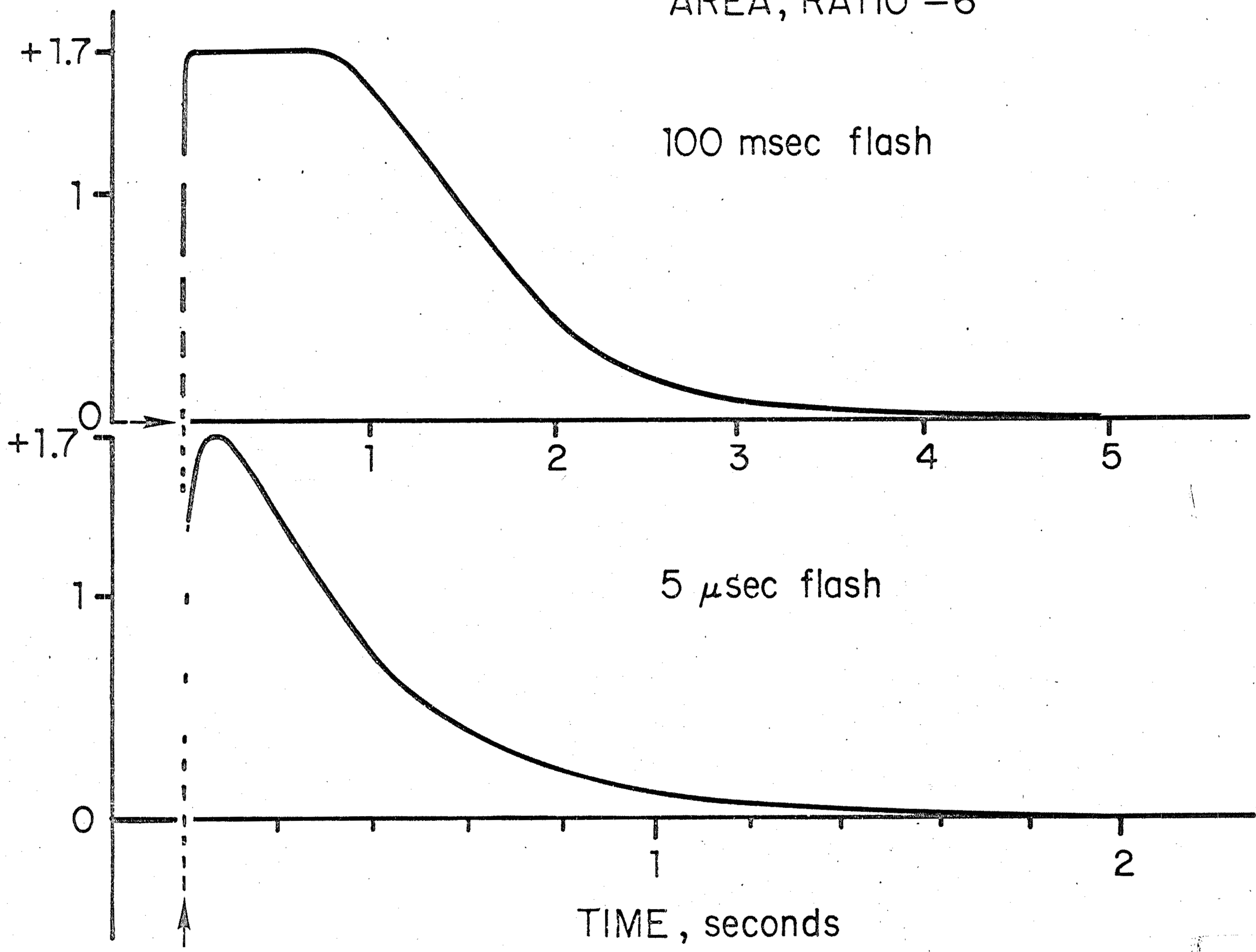
ΔT/T × 10³
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AREA, RATIO ≃6
+1.7
1
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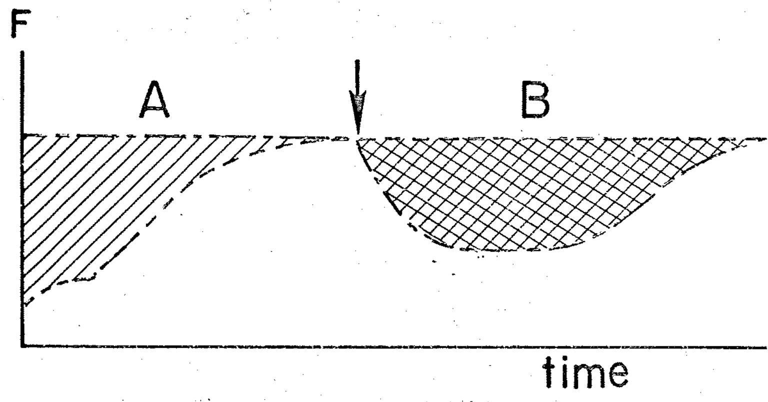

FIG 6

Fig 6

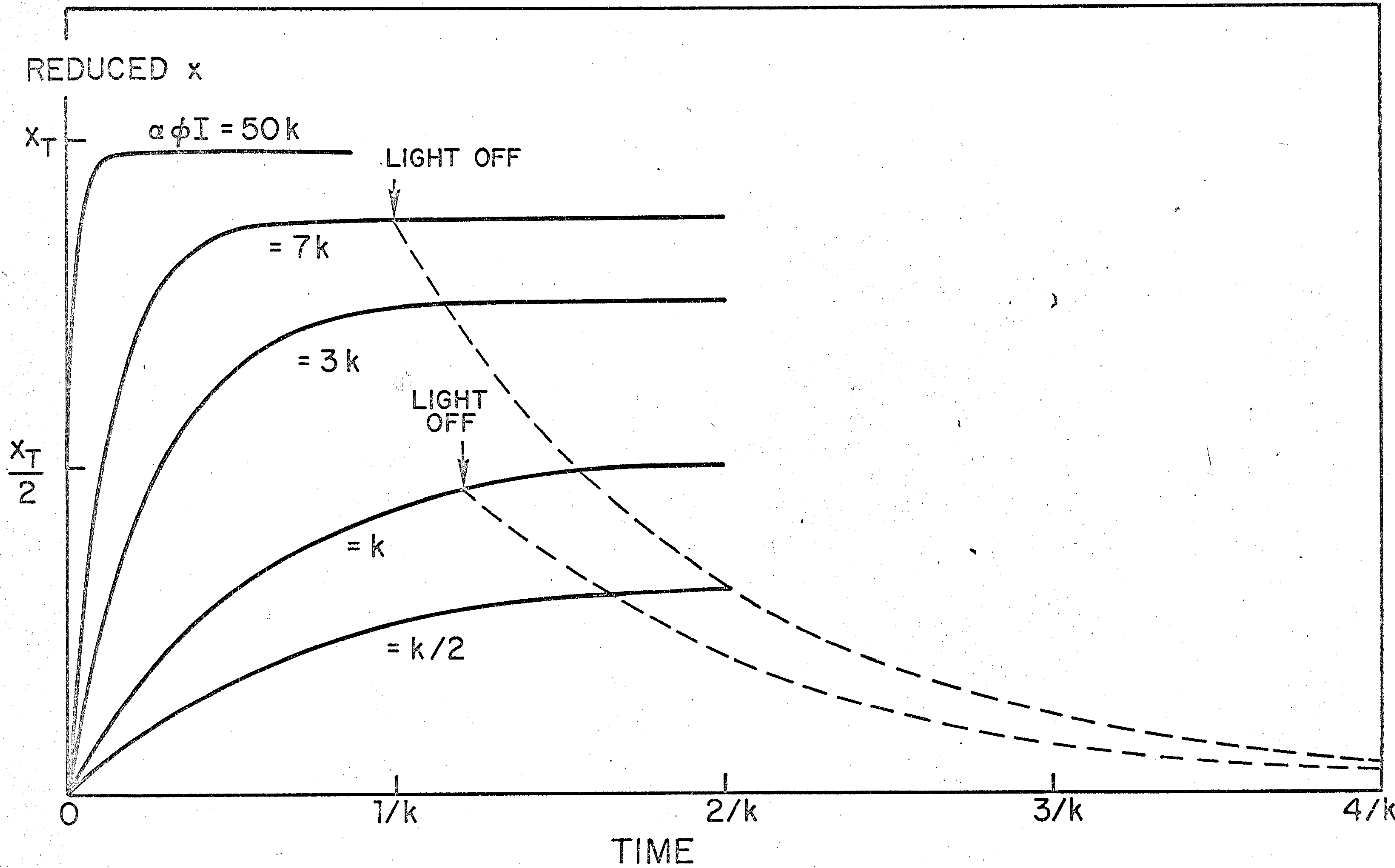

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FIG 7

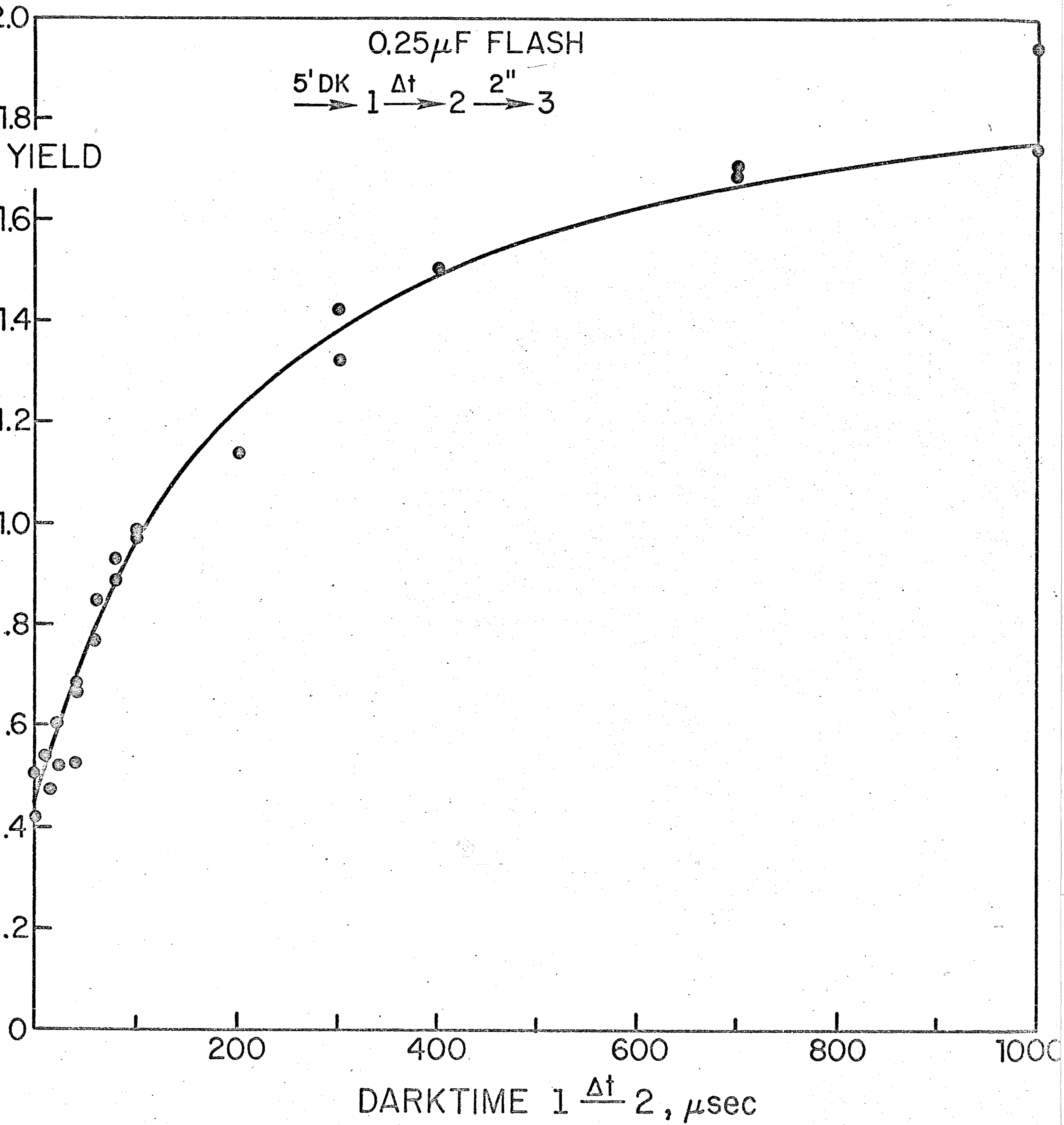

Fig 8