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Kenneth Sauer and Roderic B. Park

May 1965

THE HILL REACTION OF CHLOROPLASTS--ACTION SPECTRA AND QUANTUM REQUIREMENTS[*]

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UCRL 16104

Chloroplasts isolated from spinach carry out the photosynthetic Hill reaction yielding molecular oxygen with quantum requirements as low as 2 quanta per electron transferred to 2,6-dichlorophenolindophenol or 3 quanta per electron transferred to ferricyanide. Factors which favor an efficient reaction with low quantum requirements are (1) low incident light intensity; (2) the presence of adenosine diphosphate, magnesium ion and phosphate; (3) addition of a phosphorylation uncoupler, such as methylamine; (4) optimal initial ferricyanide concentration ($\sim 3 \times 10^{-4}$ moles/liter); (5) excitation wavelengths between 640 and 660 mμ; and (6) the use of young (4-6 week old) spinach leaves grown under winter day length and temperature conditions.

The quantum requirement as a function of wavelength for both Hill oxidants is reasonably independent of wavelength from 635 to 660 mμ, but shows a marked increase (10-fold) starting at wavelengths greater than 690 mμ. The action spectrum of the Hill reaction is characteristic of pigment system II, including chlorophyll <u>b</u> and at least part of chlorophyll <u>a</u>. No significant enhancement was observed for DCPIP[1] photoreduction when 650 mμ light was combined with 688 mμ light.

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[1] Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

The results are shown to be in accord with a two-pigment system mechanism of photosynthesis in which there is no transfer of electronic excitation energy from one pigment system to the other. Evidence is presented that chemical intermediates on the pathway to oxygen evolution may have lifetimes of many seconds.

Chloroplasts isolated from higher plants are capable of carrying out the photochemical evolution of oxygen from water using a variety of oxidizing agents such as ferricyanide, quinones, dyes such as 2,6-dichlorophenolindophenol and, under appropriate circumstances, nicotinamide adenine dinucleotide phosphate (Hill, 1937; 1939; Holt and French, 1948; Arnon et al., 1957). This reaction, known as the Hill reaction, is of interest because it produces a substantial conversion of light energy into chemical potential and because its mechanism is believed to be related to at least part of the energy conversion pathway of photosynthesis.

The Hill reaction has been studied under a variety of experimental conditions. It can be coupled to the photophosphorylation of adenosine diphosphate to adenosine triphosphate (Arnon et al., 1958; Avron and Jagendorf, 1959), and the rate of the ferricyanide Hill reaction leading to oxygen evolution is known to be enhanced in the presence of the phosphorylation cofactors and/or substances which are potent uncouplers of phosphorylation (Arnon et al., 1958; Krogmann et al., 1959; Good, 1960). The wavelength dependence of the Hill reaction corresponds very closely to the absorption spectrum of photosynthesis (Chen, 1952), except at wavelengths longer than 680 mμ where the quantum requirement shows a pronounced increase (Biggins and Sauer, 1964). The quantum requirement for the ferricyanide Hill reaction at 675 mμ decreases sharply with decreasing light intensity and extrapolates at zero light intensity to a value of 2 quanta per electron transferred to the oxidant (Lumry et al., 1957).

In this study the absolute quantum requirement for the Hill reaction has been studied as a function of several interacting parameters: light intensity, wavelength of excitation, the presence of phosphorylation cofactors and of a phosphorylation uncoupler, and the growth conditions of the plants from which chloroplasts are obtained. The intensity dependence of the quantum requirement of the Hill reaction is found to be virtually removed by the addition of either ADP, Mg^{++} and phosphate or the phosphorylation uncoupler methyl amine. Quantum requirements extrapolated to zero light intensities, however, are independent of whether the phosphorylation cofactors or methyl amine are present, regardless of the wavelength of the exciting light. These zero-intensity quantum requirements are very close to 2.0 quanta per electron transferred to DCPIP (3.0 quanta per electron for ferricyanide) from 635 to 660 mμ. The quantum requirements increase at wavelengths longer than 690 mμ to greater than 20 quanta per electron at 730 mμ. The action spectrum for the Hill reaction is characteristic of a pigment system containing chlorophyll b and part of the chlorophyll a. The results are consistent with a two-pigment system mechanism of photosynthesis in which there is no interaction between the pigment systems at the level of electronic excitation. Evidence is presented that intermediates on the pathway to oxygen evolution may have lifetimes of many seconds.

EXPERIMENTAL PROCEDURES

Preparation of Chloroplasts.— Chloroplasts prepared from spinach obtained from several sources were compared. For most of the work reported here, leaves of Spinacia oleracea, var. Bloomsdale, were obtained from plants grown from seed in a growth chamber. Plants were grown in soil under a 10 hour photo-period with 80°F. day temperature and 55°F. night temperature. Plants were harvested between 4 and 8 weeks following germination. Typically, 10 gm of leaves were homogenized for 20 seconds in 50 ml of 0.5 M sucrose, 0.1 $\underline{M}(K)PO_4$, pH 7.4 buffer in a micro

Waring blendor attachment. The resultant homogenate was strained through 8 layers of cheesecloth, and centrifuged according to Park and Pon (1961) to isolate once-washed chloroplasts. These chloroplasts were suspended in 3 to 5 ml of 0.5 M sucrose, 0.05M(K)PO_4, pH 7.4 and stored at 0°C. until used. In some cases commercial spinach, fully grown, was used for the sample material. These instances are noted in the text.

Reagents.— Stock solutions of ferricyanide, ferrocyanide and DCPIP (K&K Laboratories, Jamaica, New York) were always prepared freshly on the day of the experiment. The stock DCPIP solution was prepared in 0.05 M(K)PO_4 buffer at pH 7.45, and the concentration was confirmed by absorbance measurements after the undissolved dye had been removed by centrifugation. The extinction coefficients of Armstrong (1964) were used. ADP was obtained from the Pabst Brewing Co., Milwaukee, Wisconsin, and DCMU from duPont de Nemours & Co., Wilmington, Delaware. Methyl amine hydrochloride was dissolved in distilled water and titrated to pH 7.4 with dilute NaOH.

Reaction Mixtures.— For the ferricyanide Hill reaction, the standard reaction mixture contained the following in μmoles/ml: potassium phosphate, pH 7.45, 35; sucrose, 350; potassium ferricyanide, 0.26; potassium ferrocyanide, 0.26; methyl amine hydrochloride or sodium chloride, 10.0. For the indophenol Hill reaction, the standard reaction mixture was the same, except that 0.03 μmoles/ml of DCPIP was used in place of the ferricyanide and ferrocyanide. A sufficient amount of the chloroplast preparation was added in the dark at the start of each measurement to give an absorbance of chlorophyll at 678 mμ of 0.3 to 0.7 (1 cm path). Each experiment was performed using 2 ml of reaction mixture which was divided between the sample cuvette and the reference cuvette in the spectrophotometer. Variations of these conditions are noted in the text.

Apparatus.— Experiments were carried out using a Cary model 14 Spectrophotometer modified so that the absorbance of the Hill oxidant could be monitored continuously while the sample was being irradiated from the side with longer wavelength light. The basic techniques have been described elsewhere (Sauer and Biggins, 1965). Ferricyanide concentration was monitored at 420 mμ (ε_{420} = 1000 l-mole^{-1}-cm^{-1}) using a broad-band interference filter in front of the photomultiplier, and DCPIP concentration was monitored at 580 mμ (ε_{580} = 19,800 l-mole^{-1}-cm^{-1} at pH 7.4) using a Corning 4-96 filter.

As in the previous study, the cuvettes used had four clear sides and a rectangular internal cross-section 3 mm x 10 mm. The measuring beams passed through the 10 mm path length and the actinic light incident at right angles on the sample cuvette traversed a 3 mm light path in the reaction mixture. Light intensity measurements were made using a calibrated silicon solar cell (Hoffman, Type 120 CG) placed at the site of the cuvette, and corrections for reflection losses were applied. The inner filter effect was taken into account in calculating quantum requirements for the DCPIP Hill reaction (Biggins and Sauer, 1964).

RESULTS

Light Intensity, Methyl Amine and Phosphorylation Cofactors.— The ferricyanide Hill reaction shows a linear increase in quantum requirement with increasing incident light intensity, as was first shown by Lumry, Wayrynen and Spikes (1957). The dependence is very similar to that observed for NADP reduction, reported previously (Sauer and Biggins, 1965). The presence of methyl amine, a phosphorylation uncoupler, at concentrations of 10^{-3} moles/l or of ADP, Mg^{++} and P_i or both together serves largely to remove the intensity dependence. As seen in Fig. 1, the presence of methyl amine (or of phosphorylation cofactors, not shown) has no effect on the quantum requirement extrapolated to zero light intensity. In other words, light saturation in the Hill reaction occurs at much higher intensities when phosphorylation either occurs rapidly or is uncoupled.

Fig. 2 shows that a similar effect of methyl amine occurs for the indophenol Hill reaction. In the absence of the uncoupler, the intensity dependence of the quantum requirement is less pronounced than is the case for the ferricyanide Hill reaction. This may be a reflection of the fact that oxidized indophenol itself is a strong phosphorylation uncoupler (Gromet-Elhanan and Avron, 1963; 1964; Shen et al., 1963). The addition of DCMU, a substance known to inhibit electron transport leading to oxygen evolution, was found to increase the quantum requirement for ferricyanide reduction over 100-fold at 678 mμ at a DCMU concentration of 1.9×10^{-5} moles-liter^{-1}, both in the presence and absence of methyl amine.

Comparison of measurements made using a number of chloroplast samples prepared by identical procedures either from growth chamber spinach or from mature spinach obtained commercially showed the latter to be much less reproducible than the former. Preparations from commercial spinach frequently exhibited quantum requirements three or four times as great as the low values observed uniformly for growth chamber spinach. There appeared to be no consistent correlations with season, condition of the leaves, etc. The effects of light intensity and phosphorylation uncouplers were always observed with the commercial spinach as well as with the growth chamber spinach, however.

Initial Concentrations of Ferricyanide and Ferrocyanide.— In view of the observation by Lumry and Spikes (1957) that the rate of the ferricyanide Hill reaction is dependent on the initial concentration of ferricyanide, we examined this reaction parameter in the presence of the phosphorylation uncoupler, methyl amine. Our observations were, in brief, that an optimum ferricyanide concentration for this system occurs around 2.6×10^{-4} moles-liter of ferricyanide. At half this initial concentration rates were about 15% less, and at 2-1/2 or 5 times greater concentration they were about 30% less.

Brewer and Jagendorf (1965) have observed a preservative effect resulting from the use of equimolar ferrocyanide/ferricyanide in the initial reaction mixture. We did not observe any noticeable improvement in quantum requirement resulting from the addition of ferrocyanide initially; however, there did seem to be much less scatter in the results when ferrocyanide was present. For this reason we included it in the reaction mixture for all the ferricyanide Hill reaction studies.

The rates of the ferricyanide Hill reaction were generally measured during the first 60% of conversion of the available oxidant. In a test experiment at a single light intensity, the rate of the reaction was found to be nearly constant to over 90% conversion. For both the Hill oxidants studied, a slow back reaction was observed in the dark following illumination. This back reaction was generally greater after a large fractional conversion of the available oxidant. Experiments were designed so that the back reaction was always significantly less in magnitude than the photoreduction, and appropriate corrections to the forward rate were made. The extent of the back reaction varied in an incoherent fashion from one chloroplast preparation to another, and often for successive experiments with a single preparation. Frequently no back reaction at all was observed.

Action Spectra.— As in the previous study (Sauer and Biggins, 1965), quantum requirements measured over a 10-fold range of incident monochromatic light intensity (the overall range of intensities used was 0.04 to 3.5 nanoeinsteins-cm^{-2}-sec^{-1}) were extrapolated to zero light intensity. These zero-intensity values are plotted against wavelength of the exciting light in Fig. 3 for ferricyanide and for indophenol as the Hill oxidant, using the same chloroplast preparations in each case. Results from three different chloroplast preparations are shown; in each case methyl amine was present in the reaction mixture. With DCPIP as the Hill oxidant, a quantum requirement of 2.0 quanta per electron transferred to DCPIP was observed repeatedly in the wavelength region 640 to 660 mμ. This requirement increased to 3.0 near 680 mμ and then increased sharply at wavelengths longer than 690 mμ. The same general behavior was observed for the quantum requirement of ferricyanide reduction by spinach chloroplasts; however, the quantum requirements were uniformly 20 to 40% greater than for DCPIP reduction. Monochromator wavelength bandwidths of 7 mμ were used for most of the results reported in Fig. 3.

In one experiment, the effect of bandwidth was studied at 690 mμ. This is at the steepest point of the absorption spectrum, where the consequences of a broad bandwidth would be expected to be most pronounced. The measured quantum requirements differed by only 10% when a 10 mμ bandwidth was compared with one of 17.5 mμ.

Two-Wavelength Studies.— Experiments were carried out to determine whether the DCPIP Hill reaction of chloroplasts exhibits two-wavelength enhancement effects of the type described by Emerson et al. (1957). Preliminary studies were carried out using 650 mμ and 688 mμ wavelengths as λ_2 and λ_1, respectively. The two beams were combined using a half-silvered mirror and were incident on the reaction mixture from the same direction. The experiment was conducted by presenting first one wavelength to the sample, then adding the second and then removing the first. In other experiments the order was reversed. If the three rates measured are designated R_1, R_{12} and R_2, where the subscripts refer to the wavelengths as indicated above, then a sensitive measure of enhancement is given by the ratio $(R_{12}-R_2)/R_1$. Our observations averaged to 1.03 ± 0.08 for this ratio for a series of nine experiments. There was no significant effect resulting from a three-fold variation of the intensity of either wavelength of light. The intensities chosen were such that the ratio R_2/R_1 varied from 0.05 to 1.1. Our preliminary conclusion is that, within the experimental uncertainties, there is no Emerson enhancement for the reduction of DCPIP by spinach chloroplasts.

Very Low Light Intensities.— In several experiments the quantum requirement for the indophenol Hill reaction remained constant even when the incident light intensity was reduced to less than 0.1 nanoeinstein-cm^{-2}-sec^{-1}. Results of one such experiment at 650 mμ are shown in Fig. 4. At the lowest intensity studied, 0.040 nanoeinstein-cm^{-2}-sec^{-1}, a quantum requirement of 1.94 quanta per electron was obtained. The reaction rate was compared with dark rates immediately before and after the measurement of the rate in the light. The conditions of the experiment were such that 31.6% of the incident light was absorbed in the 3 mm illumination pathway: 8.0% by the chloroplasts and 23.6% by the indophenol. This corresponds to an absorption of 0.0106 nanoeinstein/sec by the chloroplasts per ml of the suspension. The absorbance of the chloroplast suspension alone was 0.361 at 678 mμ for a 1 cm pathlength. We have previously observed that when chloroplasts are extracted with 80% acetone-20% water the absorbance of the extract at 662 mμ is identical (± 1%) to that of the chloroplasts at 678 mμ. Using the extinction coefficients of McKinney (1941) and assuming a chlorophyll a/chlorophyll b ratio of 160/70 (Park and Biggins, 1964), we calculate a chlorophyll a concentration of 4.7×10^{-9} moles/ml and a chlorophyll b concentration of 2.05×10^{-9} moles/ml in the chloroplast suspension described above. Therefore, at the lowest incident intensity studied, each chlorophyll (a or b) molecule absorbed a light quantum on the average of once every 630 sec. If one assumes that the chlorophyll molecules in a photosynthetic unit operate in concert and further, that there are 230 chlorophyll (a and b) molecules in such a unit (Park and Biggins, 1964), then each photosynthetic unit absorbed a quantum on the average every 2.7 sec. Since a minimum of four electrons are required from water for each oxygen molecule produced, and since the measured quantum requirement per electron transferred was about 2, indicating that only half the absorbed quanta lead to photochemical events, this means that about 22 sec would be required to produce an oxygen molecule at each photosynthetic unit without measurable loss in efficiency. The presence of very long-lived intermediates is suggested by these calculations, unless the "photosynthetic units" in turn act cooperatively, or the unit is much larger than that above.

DISCUSSION

The occurrence of a linear dependence of the quantum requirement for a photochemical reaction on incident light intensity is indicative of a rate-limiting dark reaction (Lumry and Rieske, 1959; Sauer and Biggins, 1965). At high light intensities an increasing fraction of absorbed quanta are quenched and do not lead to the normal photochemical products. The present study suggests that this rate-limiting step is closely associated with the site at which phosphorylation is coupled to the electron transport chain. A simplified scheme illustrating these interactions is given in Fig. 5. Chlorophyll molecules excited by light will normally reduce substance X to X^- by some unspecified mechanism. A rate limiting reaction between X^- and Y is postulated to be coupled to the phosphorylation step, regenerating oxidized X in a form such that it can be reactivated by the next light act. The reduced product Y^- is ultimately oxidized by the terminal electron acceptor, e.g. ferricyanide. If the total pool of substance X is of limited size and the reoxidation of X^- is sufficiently slow, then at high light intensities most of it will occur in the reduced form X^-. There is little X present to react with the repeatedly excited pigment molecules. These will be quenched by some dissipative process and produce an increase in the observed quantum requirement for the Hill reaction. The rate-limiting step becomes more rapid when the phosphorylation cofactors ADP, Mg^{++} and phosphate are present, or when the phosphorylation uncoupler methyl amine is added. This latter substance presumably produces a short-circuit for electrons at the site of coupling to phosphorylation. Thus the rate-limiting step, which otherwise leads to the strong dependence of quantum requirement on incident intensity, is bypassed. The observation that the intensity dependence is substantially less pronounced when DCPIP rather than ferricyanide is used as the chloroplast Hill oxidant is consistent with the fact that the oxidized dye is known to be an effective uncoupler of photophosphorylation.

Since the phosphorylation of ADP is an endothermic process under physiological conditions ($\Delta G' \cong 9$ kcal/mole), it is necessary to drive it with a step in the electron transport chain which is sufficiently exothermic to provide the necessary free energy. Such a step must be thermodynamically irreversible, in the sense that the oxidized and reduced cofactors in the electron transport chain (X and Y in Fig. 5) are not directly coupled to one another. If they were reversibly coupled to one another then a short-circuit would exist and it would be impossible for them to transfer energy to the phosphorylation cofactors. It is realistic to expect that such an energy-transferring step in the electron transport sequence will be slow (rate-limiting) in the absence of the phosphorylation cofactors. The function of methyl amine may then be in coupling the oxidized and reduced intermediates at the site of phosphorylation in a reversible, or nearly reversible, fashion. Under these circumstances the energy is dissipated in the short circuit caused by the methyl amine, and the endothermic phosphorylation reaction cannot be coupled in. This removes the rate-limiting step, and causes the virtual disappearance of the intensity dependence of the quantum requirement.

The quantum requirement for the Hill reaction extrapolated to zero light intensity exhibits a wavelength dependence characteristic of pigment system II (Duysens et al., 1961; Duysens and Amesz, 1962). (A similar wavelength dependence has been reported previously [Biggins and Sauer, 1964] but the magnitudes of the quantum requirements observed were much larger. This resulted from the fact that those measurements were made at a single, high light intensity at each wavelength. The results showed that the addition of small amounts of DCPIP to the ferricyanide Hill reaction resulted in an increase in quantum yield, which is in agreement with the present study). An optimal efficiency (minimal quantum requirement) is observed in the wavelength region from 635 to 660 mμ. This efficiency decreases gradually at longer wavelengths and beyond 690 mμ it decreases sharply, falling off 10-fold at 720 mμ. The absorption spectrum of the pigment system responsible for the Hill reaction can be obtained by multiplying the quantum yield at each wavelength by the total pigment absorption. Some justification of this approach is presented, in view of a recent publication of action spectra of the ferricyanide Hill reaction by Horio and San Pietro (1964). We believe that the approach of those authors, in comparing percentage activity spectra with absorption spectra in order to determine the relative photochemical effectiveness of various absorption ing unity for either the Hill reaction or the ascorbate/DCPIPH$_2$ reduction of NADP at wavelengths below 680 mμ where both pigment systems absorb appreciably. The kind of evidence available is such that it can always be argued that the photoreactions are occurring at less than half their optimal efficiency owing to losses in activity of the isolated chloroplasts. Nevertheless, until it can be demonstrated that the sum of the quantum yields at any wavelength for a system I sensitized reaction and a system II sensitized reaction is significantly greater than unity, there is no indication from this source that electronic energy transfer can take place in either direction between the two pigment systems. The authors are not aware of any such evidence existing at the present time.

It is an explicit assumption of much of the published work on the Hill reaction in photosynthesis that light absorbed by pigment system II alone is responsible for the photo-reaction when ferricaynide or DCPIP are the oxidants. Unfortunately, the measurement of action spectra alone will not decide the point in the case of higher plants, contrary to the conclusion of Horio and San Pietro (1964). If both pigment systems must be activated, then relatively high quantum requirements would be expected at long wavelengths where system II absorbs weakly. There is no corresponding spectral region in higher plants where system I absorbs relatively weakly, hence we have no sensitive test of whether it is involved in a given process. This is exemplified by the observation that the action spectrum for NADP reduction using water as reductant, where almost certainly both pigment systems are involved, exhibits a pronounced increase in quantum requirement at long wavelengths (Sauer and Biggins, 1965), very similarly to the action spectra for the Hill reaction of the present study and quite unlike the system I action spectrum for NADP reduction using ascorbate/indophenol as reductant in the presence of DCMU (Sauer and Biggins, 1965). One consequence of the participation of both pigment systems in a given reaction is the presence of enhancement effects, such as have been shown for overall photosynthesis (Emerson <u>et al.</u>, 1957). In the case of the Hill reaction there is a decided conflict in the literature. The Emerson enhancement has been reported by two groups: in one case for the Hill reaction using benzoquinone as oxidant with intact Chlorella (Govindjee et al., 1960; Govindjee and Rabinowitch, 1961) and in the second for the ferricyanide Hill reaction with isolated chloroplasts (Bishop and Whittingham, 1963). On the other hand, two other groups find enhancement to be absent for the Hill reaction, under conditions where enhancement could be observed for overall photosynthetic oxygen evolution, for either Chlorella or plant chloroplasts using ferricyanide or benzoquinone as oxidants (Mayne and Brown, 1963; Gibbs et al., 1963). Furthermore, Fork (1963) has shown that far-red enhancement observed for transient endogenous oxygen evolution in the absence of ferricyanide can no longer be observed when substrate levels of ferricyanide are added to the same photosynthetic system. Our results must also be counted as negative evidence on the occurrence of enhancement in the reduction of DCPIP by chloroplasts. The results are, however, preliminary and need to be extended to a wider variety of conditions and to be compared with situations where enhancement can be clearly demonstrated.

Long-lived intermediates have been sought in photosynthesis on numerous occasions. There is much evidence from studies of absorption changes, fluorescence transients, and alternating wavelength or intermittent illumination experiments to support the occurence of transient species with lifetimes of many seconds. It is much more difficult to demonstrate that these phenomena reflect substances which participate directly in electron transport related to photosynthesis.

In our studies the high sensitivity of our method of following the chloroplast-sensitized photoreduction of DCPIP permits measurements to be carried out at light intensities considerably below those normally used. The observation that the quantum requirement of DCPIP reduction is constant down to these very low intensities has a direct bearing on this problem. The production of one molecule of oxygen from water requires the transfer of four electrons from water to the acceptor DCPIP, and each molecule of DCPIP must receive two electrons to become reduced to the stable product $DCPIPH_2$. If, as is generally assumed, the reaction requires at least 4 quanta to transfer the four electrons, then at very low light intensities some of the intermediates produced may be required to have lifetimes of many seconds before the next quantum is absorbed by the pigment system. The reaction

$$2DCPIP + 2H_2O \dashrightarrow 2DCPIPH_2 + O_2$$

is endothermic by about 4 x 0.60 volt-equivalents or 55 kcal/mole. Each einstein of red light contains only 40 kcal of energy; hence, it is impossible energetically for the entire reaction to be carried out as a consequence of the absorption of a single quantum. A mechanism involving two quanta per O_2 produced is energetically feasible. It would require the formation of at least one metastable intermediate in the process. The calculations given above show that from the results of our experiment this postulated intermediate would have a lifetime of at least 11 sec at the lowest incident intensity studied if the approximately 230 chlorophyll molecules contained within a single quantasome act as a discrete unit. If a minimum of four quanta are absorbed per O_2 literated, then this lower limit is increased to 22 sec and at least three distinguishable intermediate states are involved.

Intermediates of such long lifetimes ought to be detectable directly using appropriate modern techniques, even though their steady-state concentrations would be quite small. If there is a set of such potential intermediates associated with each quantasome or "photosynthetic unit", then under steady illumination an average of 1/2 to 3/4 of the total should be in one or another of the activated forms at any one time. Indeed, the measurements of long-lived absorption changes, fluorescence transients, etc. may very well be reflecting the presence of some of these intermediates.

Two alternative explanations of these low intensity experiments need to be considered. In the first place, if there is efficient electronic energy transfer from the pigment molecules of one photosynthetic unit to those of neighboring units, then of course the number of absorbing molecules feeding energy into a single photochemically active site will be greatly increased and the necessary minimum lifetime of the intermediates will be correspondingly decreased. There is as yet no direct evidence to support such long range electronic energy transfer. Just the converse is indicated by the experiments of Emerson and Arnold (1931; 1932), Kok (1956), and others, which suggest that the photosynthetic units do act discretely and without cooperation. Such long-range energy transfer would be made highly unlikely if it can be conclusively shown that there is no electronic energy transfer between the two pigment systems within a single photosynthetic unit, as we have suggested above. The second case that requires consideration is the possibility that the photosynthetic units can cooperate at the chemical level. This would occur if the intermediates produced are sufficiently mobile to migrate rapidly from one unit to another, or if coupling factors are available which can transport the chemical potential from one site to another.

It is important at this point to distinguish between two different types of long-lived intermediates which have been invoked. The first gives rise to the intensity saturation for short light flashes followed by long dark intervals, observed by Emerson and Arnold and by Kok. This intermediate, characterized as X in Fig. 5, becomes deactivated in about 30 msec at room temperatures. It has the proper characteristics to account for the intensity dependence of the quantum requirement of NADP reduction using water as reductant (Sauer and Biggins, 1965) and for the similar effect for the ferricyanide Hill reaction as observed in this study. The second type of intermediate may have a much longer lifetime (>1 sec) and is responsible for the observation that the quantum requirement for DCPIP reduction remains constant to very low light intensities. This second class of intermediates is further distinguished from the first in that their decay is basically a photochemical process rather than a dark reaction. As successive quanta are absorbed by the photochemical apparatus the first intermediate is converted to a second, etc. and eventually to molecular oxygen. The following hypothetical scheme illustrates this point

$$2H_2O \xrightarrow{h\nu} A_{(HO\cdot OH_2)} \xrightarrow{h\nu} B_{(O\cdot OH_2)} \xrightarrow{h\nu} C_{(O\cdot OH)} \xrightarrow{h\nu} O_2$$

where A, B, C are partially oxidized, stable intermediates on the way from water to molecular oxygen. The actual intermediates present in the photosynthetic process need not involve oxygen atoms except at the final step, of course. Such intermediates will not limit the photochemical rates, in general, since successive light absorption acts will always find them ready to react further. Thus, flashing light experiments will not detect their presence, and consequently the demonstration of non-interacting photosynthetic units by flashing light experiments does not contradict the possibility that these photoreactive intermediates may migrate or cooperate over relatively great distances in the chloroplast. Therefore, the evidence at hand will not permit us to rule on the possibility that long-lived intermediates produced at one photosynthetic unit may migrate to another unit where they become further activated by light on the way to forming molecular oxygen or fully reduced DCPIP. Migration of this type would serve to reduce appreciably the calculated minimum possible lifetime of the proposed intermediates. One test of whether such migration occurs can be made by examining the dependence of the quantum requirement at low light intensities for suspensions of small lamellar fragments, where the average path between photosynthetic units is drastically increased. Comparison of the calculated minimum lifetimes from such experiments with diffusion times in solution should produce an answer to the question of whether [illegible] migration of active intermediates from one photosynthetic unit to another does occur.

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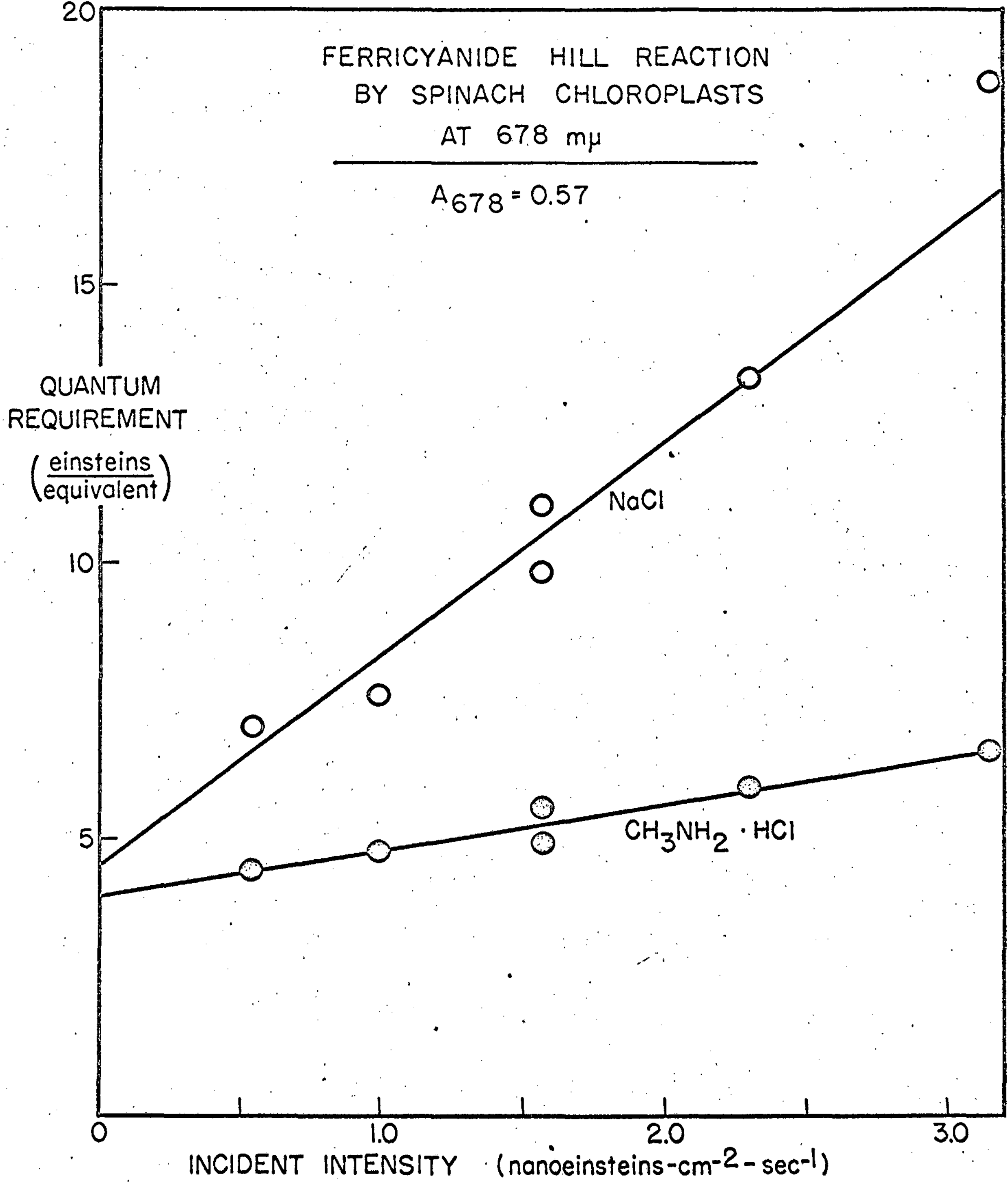
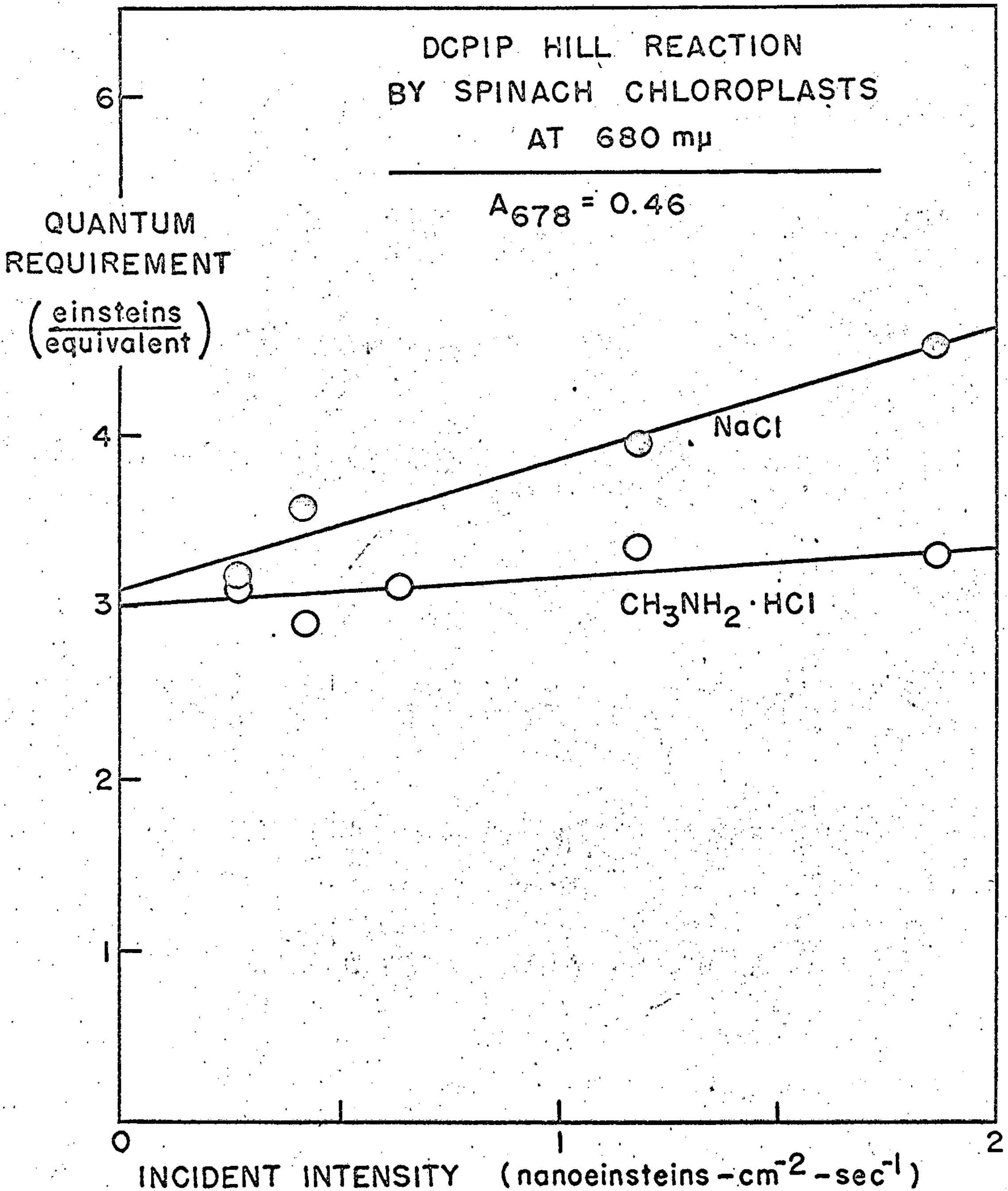


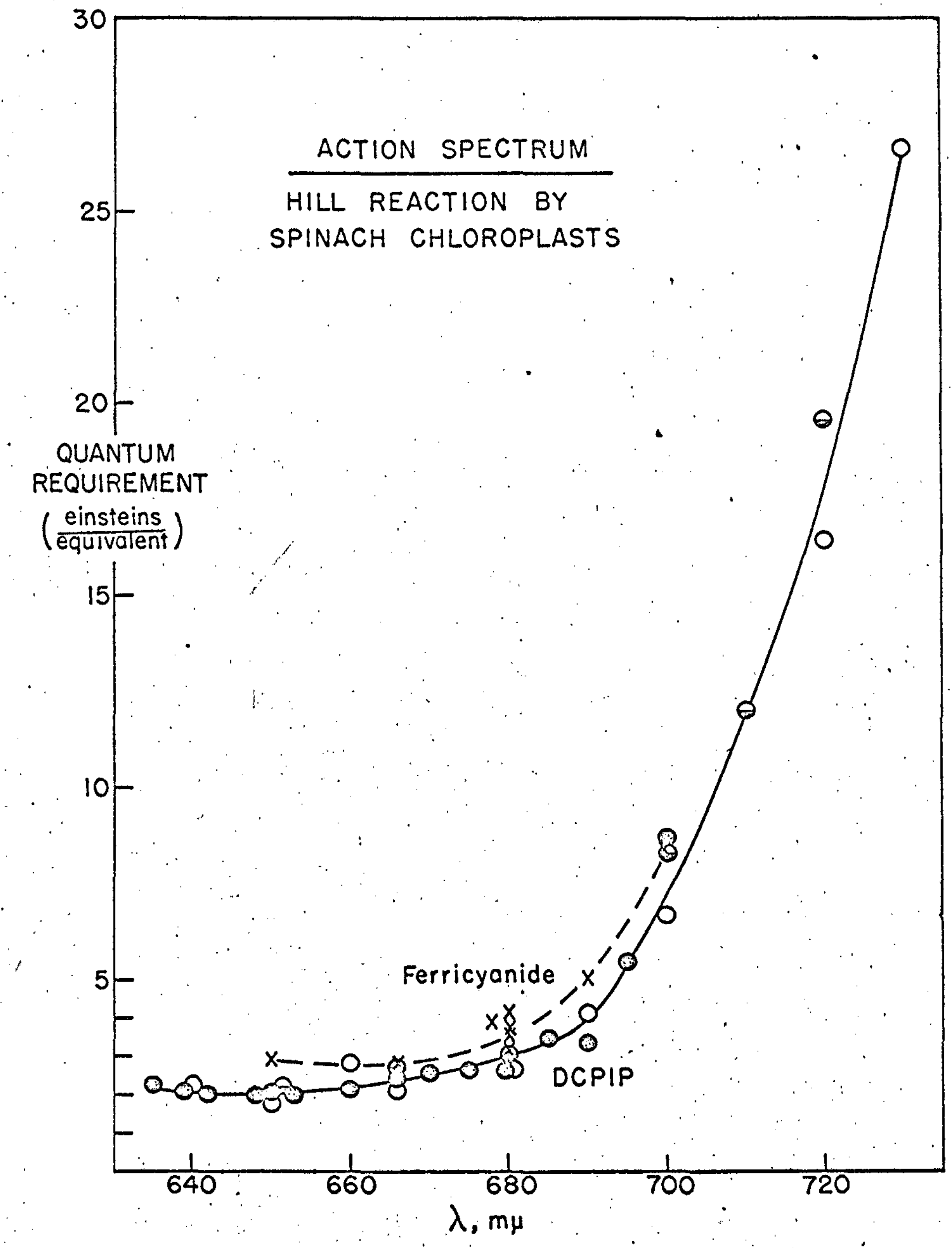
FERRICYANIDE HILL REACTION BY SPINACH CHLOROPLASTS AT 678 mμ
A_{678} = 0.57
QUANTUM REQUIREMENT (einsteins/equivalent)
20
15
10
5
0
1.0
2.0
3.0
INCIDENT INTENSITY (nanoeinsteins-cm^{-2}-sec^{-1})
NaCl
$CH_3NH_2 \cdot HCl$

MUB-5637



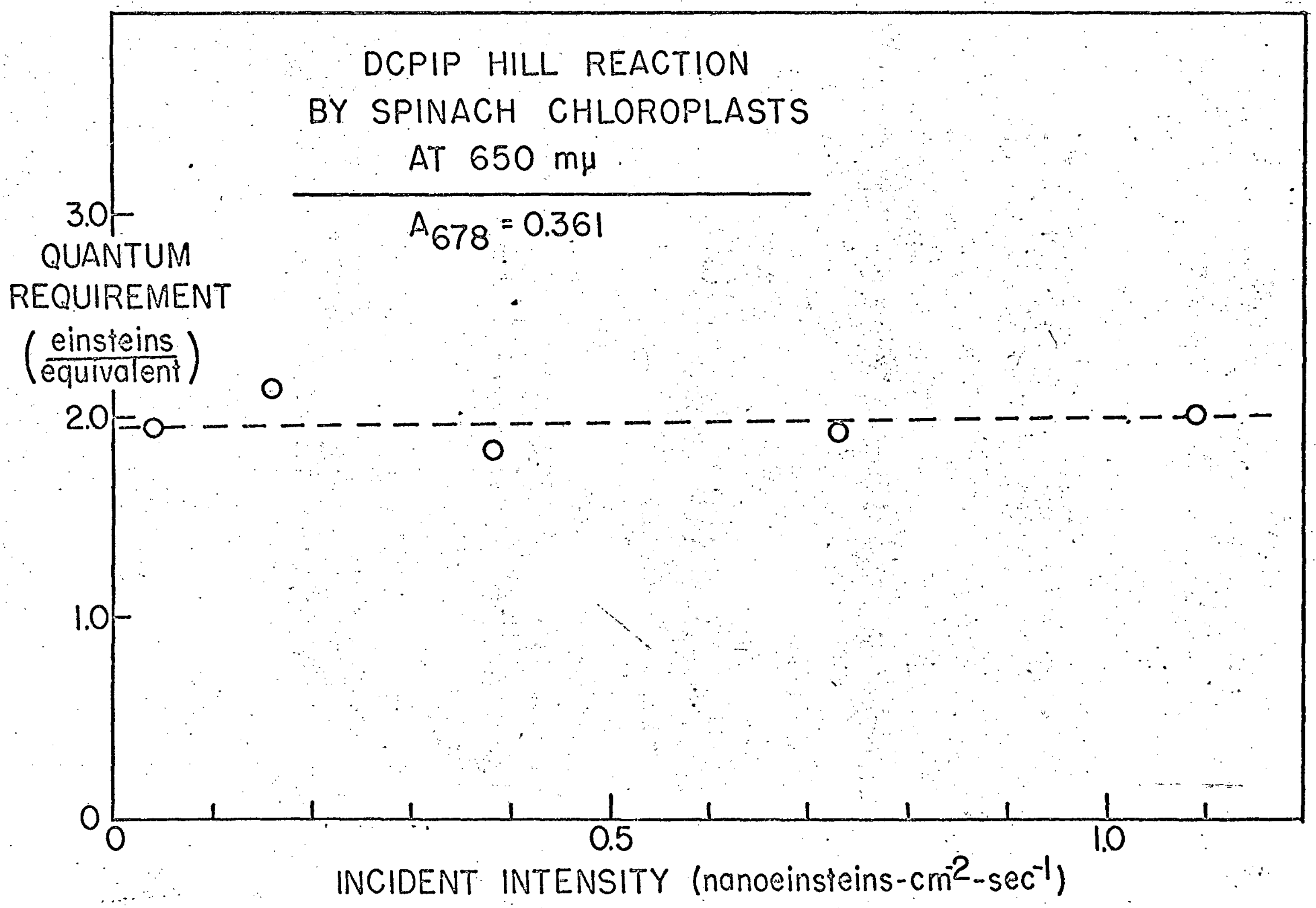
MUB-5636

Fig. 2


ACTION SPECTRUM
HILL REACTION BY SPINACH CHLOROPLASTS
QUANTUM REQUIREMENT (einsteins/equivalent)
30
25
20
15
10
5
Ferricyanide
DCPIP
640
660
680
700
720
λ, mμ

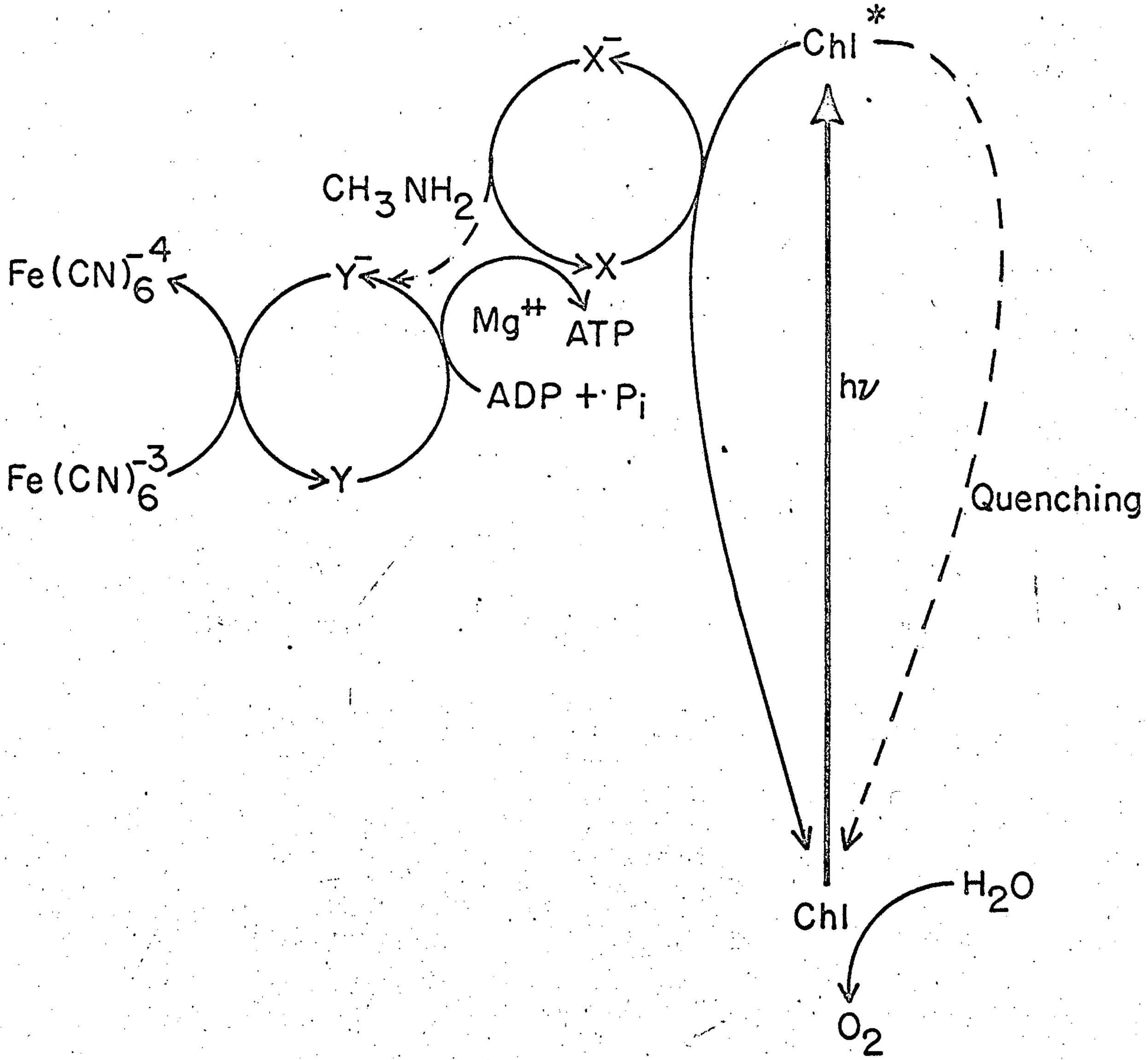
MUB-5639

ig. 3

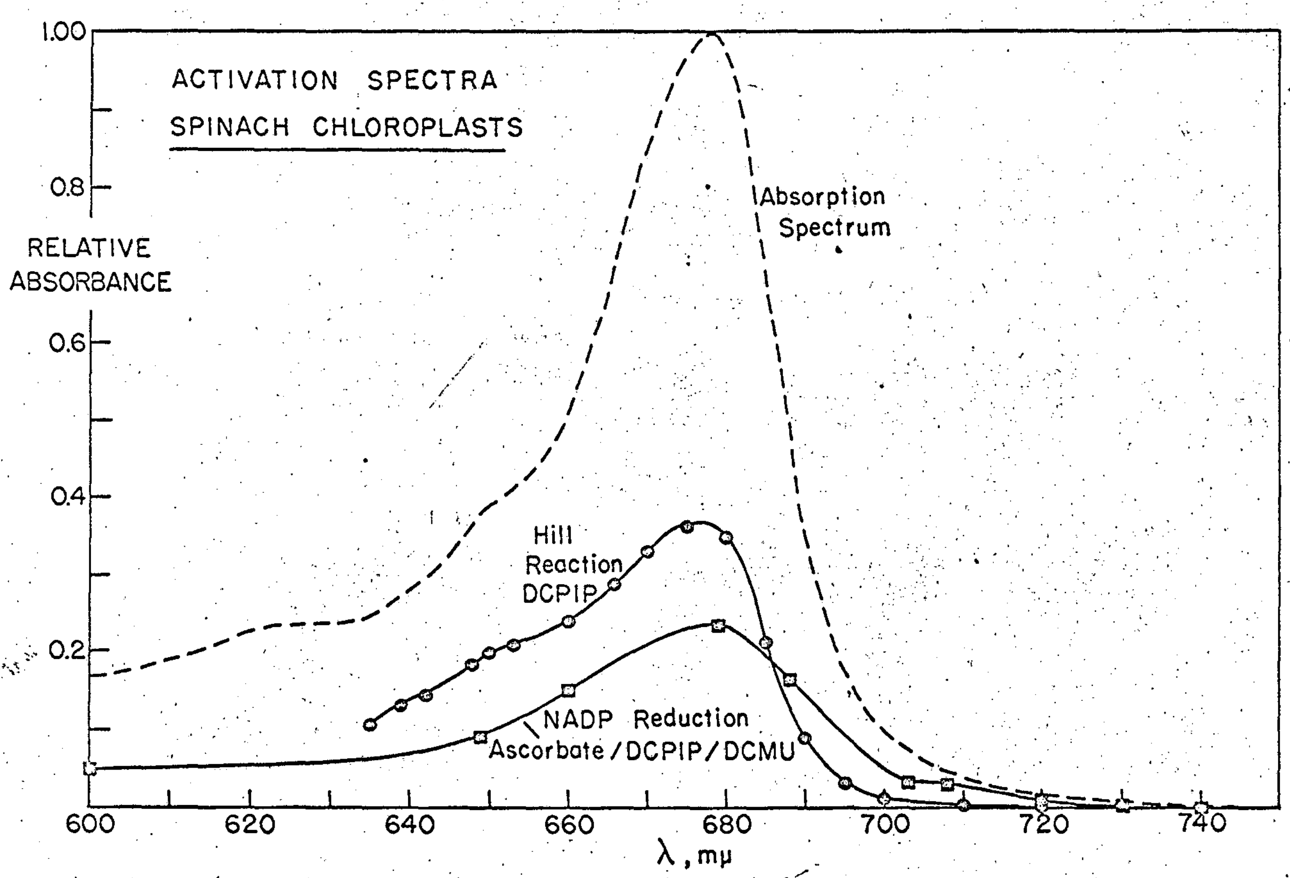


MUB-5638

Fig. 4


Chl*
X⁻
X
CH3NH2
Y⁻
Y
Fe(CN)6-4
Fe(CN)6-3
Mg++
ATP
ADP + Pi
hν
Quenching
Chl
H2O
O2

MUB-5950


ACTIVATION SPECTRA
SPINACH CHLOROPLASTS
RELATIVE ABSORBANCE
1.00
0.8
0.6
0.4
0.2
Absorption Spectrum
Hill Reaction DCPIP
NADP Reduction Ascorbate / DCPIP / DCMU
600
620
640
660
680
700
720
740
λ, mμ

MUB-5640

n.6