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Authors
Sauer, Kenneth
Biggins, John.

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University of California

Ernest O. Lawrence Radiation Laboratory

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Kenneth Sauer and John Biggins

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Kenneth Sauer and John Biggins

Department of Chemistry and Lawrence Radiation Laboratory,

University of California, Berkeley, California, U.S.A.

SUMMARY

The photoreduction of NADP by chloroplasts is observed to occur

with a requirement of 1 to 3 quanta per electron transferred to NADP

at wavelengths from 550 to 730 mμ in a DCMU-poisoned system when re-

duced DCPIP is present as electron donor. In the normal reaction,

with water as donor, a similar quantum requirement is observed from

550 to 680 mμ, but this rises to approximately 9 quanta per electron

at wavelengths longer than 700 mμ. The results are interpreted as

reflecting the participation of two pigment systems. Some characteristics

of the absorption spectra of the two systems are discussed.

The quantum requirements at nearly all wavelengths studied were

found to increase linearly with increasing incident light intensity for

both reactions. We propose that an activated intermediate of the photo-

synthetic unit, with an estimated lifetime of 0.05 sec in the normal

reduction of NADP by chloroplasts and 0.15 sec in the DCMU-poisoned

system, is responsible for this intensity dependence.

Small quantasome aggregates prepared from spinach chloroplasts are

shown to be nearly half as efficient as intact chloroplasts in the photo-

reduction of NADP using reduced DCPIP. A method is described whereby

the absorption spectrum of these quantasome suspensions can be used to

determine the true absorption spectrum of chloroplasts at wavelengths

where light scattering is predominant.

INTRODUCTION

The normal course of photosynthetic electron transport in higher plant chloroplasts leads to the reduction of NADP to $NADPH_2$ with water, as substrate, becoming oxidized to molecular oxygen. Coupled with this process is the non-cyclic phosphorylation of ADP to ATP. DCMU is a potent inhibitor of ~~~~~~~~~~~~~~~ in photosynthesis[1], but in preparations of isolated chloroplasts this inhibition can be relieved, in part, by the addition of the couple DCPIP plus ascorbate[2]. Under these circumstances the reduced DCPIP takes the place of water as the electron donor; NADP can then be reduced and ADP phosphorylated by chloroplasts in the light[3]. Oxygen is not liberated so long as DCMU is present; however, In the absence of DCMU, the oxidized form of DCPIP serves as an oxidant for the chloroplast Hill reaction, leading to the photooxidation of water to molecular oxygen. These observations led Losada, et al.[3] to the conclusions that (i) there are two light reactions involved in NADP reduction where water serves as the electron donor, (ii) only one of these is involved in the photoreduction of NADP in the presence of added DCMU and reduced DCPIP, and (iii) the other light reaction alone is responsible for the Hill reaction with oxidized DCPIP.

Evidence has been presented to demonstrate that these two light reactions arise from absorption by different pigment systems in chloroplasts[4]. Several recent studies have appeared describing action spectra and absolute quantum requirements of NADP reduction by chloroplasts for the normal system (water as donor)[5-7], and for the poisoned system (ascorbate/DCPIP as donor)[5]. There is a considerable measure of discordance among the various results. The present study is an attempt to

resolve some of the difficulties through the use of a different experimental approach. In particular, we have continuously monitored the concentration of $NADPH_2$ throughout the course of the reaction in order to correct for the reverse oxidation of $NADPH_2$. In addition, we have used a novel technique for determining the true absorption of chloroplasts at long wavelength where light scattering normally makes an overwhelming contribution.

MATERIALS AND METHODS

Preparation of chloroplasts and quantasomes

Chloroplasts were prepared by minor modifications of the method of Hoch and Martin[5]. Sixty gm fresh spinach leaves were thoroughly washed and refrigerated after the removal of the mid-ribs. They were blended in 100 ml of ice-cold 0.4 $\underline{M}$ sucrose, 0.05 $\underline{M}$ Tris chloride (pH 8.0), and 0.01 $\underline{M}$ NaCl for 10 sec. The homogenate was strained through eight layers of cheesecloth and centrifuged at 1000 g for one min. The supernatant was centrifuged at 1000 g for 7 min and the pellet resuspended in 50 ml blending medium. Whole chloroplasts were sedimented by recentrifugation at 1000 g for 7 min.

Quantasome aggregates were prepared by lysing the whole chloroplasts in 40 ml 10^{-2} $\underline{M}$ tris-Cl, pH 8. The suspension was agitated gently for 10 min and centrifuged at 10000 g, 10 min. The green sediment was resuspended in 15 ml 10^{-2} $\underline{M}$ tris-Cl, pH 8, and sonicated for 90 sec by means of a Raytheon 100W, 9 Kc magnetostriction oscillator operated at full voltage. The sonicate was then ultracentrifuged at 105000 g for 20 min and the supernatant retained as our preparation of quantasome aggregates. Typically these particles

contain an average of 8 quantasomes as seen in the electron microscope.

Enzyme preparations

PPNR was prepared from spinach according to the procedure of San Pietro and Lang[8] through the 75% acetone precipitation step. The protein was redissolved, centrifuged, dialyzed against 5×10^{-3} M Tris chloride (pH 8.0), and further purified through the first DEAE-cellulose column treatment of Hill and Bendall's method[9]. This partially-purified preparation contained chloroplast ferredoxin[10] and ferredoxin-NADP reductase[11]. The preparation was assayed in the photoreduction of NADP by chloroplasts using white light and thereafter used in saturating concentrations in the quantum requirement determinations.

Reagents

NADP and Trizma base were obtained from the Sigma Chemical Co., St. Louis; ADP from the Pabst Brewing Co., Milwaukee; DCMU from duPont de Nemours, Wilmington; and DCPIP from K and K Laboratories, Jamaica, New York.

Reaction mixtures

The reaction mixtures contained the following in umoles/ml: potassium phosphate, pH 7.5, 50; NADP, 0.5; ADP, 1.0; $MgCl_2$, 7.5 and a saturating amount of PPNR. In DCMU-poisoned preparations the above reaction mixture was used with the following in umoles/ml: DCMU, 0.01; sodium ascorbate, 5.0; and DCPIP, 0.04.

Apparatus

Experiments were carried out using a Cary Model 14 spectrophotometer modified in such a way that the absorbance of the reaction mixture at 339 mu could be monitored continuously while the sample was being irradiated from the side with longer wavelength light[12]. A diagram of the

apparatus is shown in Fig. 1. A Cary Model 1462 Scattered Transmission Accessory was modified so that the exciting light could be directed through a set of filters in the side wall to irradiate the sample cuvette in the spectrophotometer beam from one side. Light from a tungsten projection lamp (General Electric DFK, 1000 watt), powered with a Variac, was collimated using a spherical mirror and passed through 4 cm of water and a Corning 1-69 infrared-absorbing glass. One or more cut-off filters (CF 1) and an interference filter (IF 1) were placed in a port in the side of the Scattered Transmission Accessory and a lens inside served to concentrate the beam incident on the sample cuvette. An opaque barrier prevented exciting light from reaching the reference cuvette, which contained a portion of the same reaction mixture as that in the sample cuvette. All samples were irradiated at room temperature.

For a second set of experiments a grating monochromator (Bausch and Lomb, 500 mm) was used to provide actinic light in place of the arrangement involving interference filters described above. Suitable cut-off filters were used with the monochromator to reduce stray light at shorter wavelengths. Results obtained using the two activation systems were in good agreement at all wavelengths studied. With the interference filters the maximum intensity available was more than ten times greater than from the monochromator at an equivalent bandwidth, however.

The cuvettes used were constructed with four clear sides and a rectangular internal cross-section 3 mm x 10 mm. The spectrophotometer measuring beam traversed the long dimension, as shown in Fig. 1. It was necessary to place a set of complementary filters, CF 2 and IF 2, in front of the photomultiplier (Dumont 7664) in order to prevent stray

exciting light or chloroplast fluorescence from interfering with the absorption measurements. For this purpose, an interference filter (Baird Associates), transmitting in its third order in the ultraviolet, was coupled with a Corning ultraviolet-transmitting filter (7-60).

The excitation wavelength interference filters (IF 1) were of the blocked, narrow-band type (Baird Associates, Type B-1) with half bandwidths about 10 mμ. The supplementary cut-off filters were selected to reduce the transmitted intensity of shorter wavelengths to a very low level. The Corning supplementary filters used at each wavelength studied were: (i) 3-68 at 550 and 600 mμ; (ii) 2-58 at 649, 660, and 679 mμ; (iii) 2-64 at 688 mμ; (iv) 2-64 plus 4-77 at 703, 708, and 720 mμ; and (v) 2-64 plus 7-59 at 730 mμ.

Light intensity measurements

Measurements of light intensity were based on secondary standard lamps obtained from the National Bureau of Standards, Washington, D. C. A Reeder thermopile (C. M. Reeder Co., Detroit), calibrated using the standard lamp, was used to determine the wavelength dependence of the sensitivity of a silicon solar cell (Hoffman, Type 120 CG), using the Bausch and Lomb 500 mm grating monochromator and a tungsten source. Incident intensities for the illumination experiments were measured at the site where the sample cuvette is normally placed in the apparatus. Corrections were applied to the results for the measured reflection loss (5%) at incidence on the cuvette sides.

RESULTS

Absorption spectrum of chloroplasts

An essential prerequisite to the calculation of quantum requirements of photochemical reactions is an accurate measure of the number of quanta

absorbed. In the case of chloroplast suspensions this is difficult, especially at wavelengths longer than 690 mµ, owing to the substantial light scattering exhibited. Use of the measured transmission of the sample in the calculations may lead to large errors on this account.

The technique we used for estimating absorbed intensities is based on a study of the spectral properties of chloroplast and quantasome preparations[13]. Figure 2 presents a part of the absorption spectrum for a single preparation of spinach chloroplasts measured using three different techniques: direct absorption using a Cary 14 Spectrophotometer with a normal cell compartment, the opal glass technique of Shibata, Benson, and Calvin[14], and the scattered-transmission method with a red sensitive photomultiplier (Dumont 6911). The latter two spectra are nearly identical except that the opal glass technique is not reliable beyond 700 mµ[13]. A sample of quantasomes prepared from another portion of the same chloroplast preparation was also measured using the scattered-transmission method. The long wavelength tail of the red absorption band of a concentrated suspension of quantasomes is shown in Fig. 3 along with the spectrum in the same region of a concentrated suspension of the chloroplasts. The latter has been normalized so that the absorbances at 678 mµ are each 10.0. The effect of light scattering on the chloroplast spectrum at these long wavelengths is such that direct measurements, even using the scattered-transmission technique, would not give a good measure of the true absorption of the preparation.

The spectrum of the small quantasome aggregates can be satisfactorily corrected for light scattering. The correction is small in this case, and it can be executed accurately based on turbidity measurements from 760 to 900 mµ, where there is no absorption. Some measured

optical density values from 760 to 900 mμ are plotted versus the reciprocal fourth power of the wavelength in Fig. 4. The points fall on a good straight line passing through the origin, as is expected for light scattering from small particles (Rayleigh scattering). Extrapolation of this line to shorter wavelengths permits the calculation of the contribution of scattering to the measured spectrum at any wavelength and, by difference, the true absorption spectrum of the quantasome preparation (dashed curve in Fig. 3). The fact that the scattering correction is small in the case of the quantasome preparation permits the computation of a reliable absorption spectrum by this method. This is less true for chloroplast suspensions, where the particles are large and the turbidity of the preparations is greater than the true absorbance at wavelengths longer than 700 mμ.

We now make the assumption that the true absorption spectrum of chloroplasts is identical to that of quantasomes prepared from them, after normalization based on the measured values at the maximum at 678 mμ. Some arguments supporting the validity of this assumption are presented by Sauer and Park[13]. Thus, from a single scattered-transmission measurement of chloroplast absorption at 678 mμ, where the contribution from light scattering is relatively minimal, and from the normalized quantasome absorption spectrum, we compute the absorption of the chloroplast suspensions at wavelengths where the direct measurement is impossible by any technique short of using an ideal integrating sphere.

Photoreduction of NADP by chloroplasts

Our initial method of studying the photoreduction of NADP by

chloroplasts was similar to that of Black, et al.[7] and Hoch and Martin[5]. Samples were illuminated for a fixed time period, the chloroplasts were removed by centrifugation, and the 339 mμ absorbances of the supernatants recorded. In our hands this technique proved unreliable, owing to the very active first-order reoxidation of $NADPH_2$. We subsequently showed that catalytic activity for this reoxidation was associated with our PPNR preparations and with the chloroplast stroma, and that it could not be removed by short term ultracentrifugation of the samples. We found it necessary, therefore, to adopt the continuous spectrophotometric measurement of $NADPH_2$ concentration. In this way true initial velocities for the photoreduction uncomplicated by the reoxidation could be obtained.

A typical trace of the time course of the photoproduction of $NADPH_2$ is shown in Fig. 5. The following characteristic features of the trace were generally observed in determinations summarized in this report: (i) A stable level of absorbance at 339 mμ in the dark, (ii) an initial rapid rise during which about 0.2% of the available NADP is reduced, (iii) a period during which a slower, but constant, rate of formation of $NADPH_2$ occurs, (iv) a gradual decrease in this reaction velocity after about 5% reduction of the initial NADP, (v) a brief rapid decrease in absorbance upon cessation of illumination followed by (vi) a slower decrease of the absorbance which continued for a long period of time.

Stage (ii) was not observed if a second successive period of illumination was given to the same sample, even after a dark interval of several minutes.

The steady initial velocity chosen for the reaction was the limiting

slope of the nearly linear portion [stage (iii)] following the transient
initial rapid rise. The location of this limiting tangent could, in
general, be determined unambiguously from a region in which there was no
pronounced curvature of the trace. After this linear portion, the reoxi-
dation becomes sufficiently great even in the light to cause a distinct
decrease in the net rate of $NADPH_2$ formation.

In order to make certain that other changes in absorbance at 339 mµ
were not influencing the magnitude of our results, we scanned a number
of the difference spectra from 300 to 550 mµ before and after periods of
illumination. The spectra consisted invariably of a simple absorption
peak at 339 mµ, and there was no evidence of significant bleaching of
the photosynthetic pigments or changes in turbidity of the illuminated
sample. In separate experiments we determined that the presence of
chloroplasts at the highest concentrations used did not significantly
affect the extinction coefficient of $NADPH_2$ at 339 mµ in the difference
spectra measured using the scattered-transmission technique.

Quantum requirements and action spectra

Quantum requirements for NADP reduction for each experiment were
calculated from the steady initial reaction velocities and the calculated
absorbed intensities. Experiments were carried out alternately for the
normal and the DCMU-poisoned systems with otherwise identical reaction
conditions. At each wavelength the incident intensity was varied over
at least a ten-fold range by adjusting the lamp voltage.

Figures 5 and 7 show examples of the dependence of the quantum
requirement on incident intensity. At all wavelengths studied and for

both reaction systems the quantum requirement increases linearly with increasing intensity. The positive intercepts, determined from extrapolations to zero intensity, are plotted as a function of actinic wavelength in Fig. 8. These data are mean values resulting from up to five separate experiments at each wavelength. Some 40 chloroplast preparations and three PPNR preparations are involved. These quantum requirements are also presented in Table I, along with some values in the same units which we have calculated from data and curves given in the recent literature.

Both the normal and the DCMU-poisoned reactions have quantum requirements in the range 1.5 to 3.0 einsteins/equivalent of NADP reduced between 648 and 679 mμ. At wavelengths longer than 679 mμ the normal reaction shows a pronounced increase in quantum requirement characteristic of the first Emerson effect[15], whereas the requirement for the poisoned reaction appears to decrease to about one-half its value at shorter wavelengths. We took particular care to insure that the normal reaction does indeed proceed at 720 and 730 mμ with requirements of no more than about 9 quanta per electron. No effective stray light of wavelengths shorter than 700 mμ was present in the actinic beam, as demonstrated by the introduction of additional supplementary cut-off filters. By closing the spectrophotometer slit for a period of time during the progress of experiments at these long actinic wavelengths, we showed that the 339 mμ analyzing beam was ineffective in sensitizing the reduction of NADP. Furthermore, the reduction of NADP in the normal system at wavelengths greater than 700 mμ was completely inhibited by the addition of DCMU.

Dependence on light intensity

The quantum requirement for NADP reduction by chloroplasts increases linearly with increasing incident light intensity for both reactions studied and at all wavelengths from 550 to 730 mµ, except for the normal reaction at the longest wavelengths, as noted below. This behavior has been reported previously by Hoch and Martin[5], and a similar dependence was observed for the Hill reaction using ferricyanide[16,17]. Lumry and Rieske postulated several mechanisms for the interpretation of these results[18]. The following mechanism is one of the simpler ones they proposed and it appears to be sufficient to explain our observations:

$$C \xrightarrow{\phi I} C^* \tag{1}$$

$$C^* \xrightarrow{(1-p)} C \tag{2}$$

$$C^* + T \xrightarrow{p} C + T^* \tag{3}$$

$$T^* + R \xrightarrow{k_1'} T + P \tag{4}$$

In this scheme light intensity I absorbed by pigment molecules C (presumably chlorophyll in the wavelength range studied) excites them to reactive species C^* with a primary quantum efficiency ϕ. At very low light intensities some fraction p of C^* transfers its excitation to a trapping site or reactive intermediate, T, according to reaction (3). The remainder of C^* is deactivated according to reaction (2) with an intrinsic probability (1-p) and will not lead to the formation of products. The excited trapping sites T^*, once formed, inevitably lead to the conversion of reactants (NADP and water or ascorbate) to products ($NADPH_2$ and oxygen or dehydroascorbate) by a series of reactions summarized

in equation (4). At higher light intensities the steady-state concentration of T will be reduced from the value T_o present in the dark or at low limiting light intensities, and the probability for reaction (3) will decrease from p to $p(T/T_o)$.

Using the steady-state approximation for the concentration of the excited species C^* and T^*, (see Appendix for details), one readily obtains for the reaction velocity the result

$$V = \frac{k_1\, p\, \phi\, T_o\, I}{k_1\, T_o + p\, \phi\, I} \tag{5}$$

where

$$V = \frac{dp}{dt} = k_1'\, RT^* \doteq k_1\, T^*$$

The pseudo first-order rate constant, k_1, will be constant only during the early stages of the reaction before the concentration of reactant R has decreased appreciably. The quantum requirement, Q, is then given by

$$Q = \frac{I}{V} = \frac{1}{p\,\phi} + \frac{I}{k_1\, T_o} = Q_o + \frac{I}{k_D} \tag{6}$$

Equation (6) predicts a linear relationship between the quantum requirement and the absorbed intensity with a positive slope. The intercept, Q_o, is the intrinsic quantum requirement for the overall reaction at zero intensity, and the slope, $1/k_D$, is a measure of the rate parameter for reaction (4).

The slopes of plots of quantum requirement of NADP reduction versus absorbed light intensity for both the normal and the DCMU-poisoned system appear to be independent of wavelength; however, the data for the normal system at wavelengths beyond 708 mμ scatter too much to give reliable values (e.g., see Fig. 6). Average values for the pseudo zero-order rate constant k_D were calculated from all results regardless of

of wavelength. The values obtained were $1.6_4 \pm 0.7 \times 10^{-7}$ equivalents-liter^{-1}-sec^{-1} for the DCMU-poisoned system and $4.8 \pm 1.7 \times 10^{-7}$ for the normal system. This parameter characterizes the rate of reaction (4) in the mechanism presented above and presumably includes a dependence on the concentration of one or more endogenous chloroplast cofactors. It is significant that this parameter indicates that reaction (4) is three times faster in the normal system than in the DCMU-poisoned system.

A study was made to determine whether the concentration of the DCPIP/ascorbate couple is limiting for the DCMU-poisoned system. For this purpose it is sufficient to study the dependence of quantum yield of NADP reduction on DCPIP concentration. Figure 9 summarizes the results of such an experiment carried out using 678 mμ illumination at two different incident intensities. Sufficient chloroplasts were present to give an absorbance of 0.80 (1 cm path). Concentrations of all other components were the same as those normally used, with the exception of DCPIP, which was varied. At the higher light intensity a typical saturation curve is obtained. If indophenol is included specifically as a limiting reagent (first-order) in reaction (4) it can readily be shown that the quantum requirement, Q, should be linearly related to the reciprocal of DCPIP concentration. The data are in good agreement with such a prediction. For a plot of quantum yield versus DCPIP concentration the theory predicts a hyperbolic relationship. The curves shown in Fig. 8 are drawn on this basis for comparison with the data. The saturation values of quantum yield with increasing dye concentration, obtained from reciprocal plots, are 0.263 and 0.378 at the higher and lower intensities, respectively. The concentration of DCPIP normally used throughout this study (0.04 μmoles/ml) gives over 90% of the saturation quantum yield at

each intensity. This experiment shows that no increase in DCPIP concentration will increase the quantum efficiency of the photoreduction to anywhere near the level attainable by decreasing the incident light intensity. The DCPIP concentration (and, by inference, the ascorbate concentration) is therefore not a limiting factor in the kinetic scheme under the conditions studied.

Photoreduction of NADP by quantasomes

In addition to the ability of quantasome preparations to carry out the Hill reaction and to support CO_2 fixation[19], we have now observed a relatively high level of activity for NADP reduction in the presence of added PPNR. Best results were obtained using the ascorbate/DCPIP couple as reductant in the DCMU-poisoned reaction, and in the absence of ADP and $HgCl_2$. Under these conditions the quantum requirements at zero incident intensity were 6.2 at 649 and 678 mµ and 4.0 at 703 and 720 mµ. The intensity parameter k_D could be measured reliably only at 649 and 678 mµ and values of 1.3×10^{-7} equivalents-liter^{-1}-sec^{-1} were obtained in each case. These results show that the quantasome preparations are almost half as active as chloroplasts in their ability to photoreduce NADP. We feel that the demonstration of such a high level of activity increases the plausibility of the important assumption underlying our quantum requirement calculations for the chloroplast system--namely, that the true absorption spectra of chloroplasts and quantasomes are essentially identical in the long wavelength region.

DISCUSSION

Three recent publications present action spectra for the absolute quantum requirement of NADP reduction by chloroplasts with water as

reductant[5-7]. In addition, results for the DCMU-poisoned system using ascorbate/DCPIP as reductant are presented by Hoch and Martin[5]. Several values giving the wavelength dependence for the poisoned system have been reported on a relative basis by Arnon, et al.[4] Some pertinent results extracted from these studies are put in the common units and summarized along with our results in Table I. There is a substantial lack of agreement among the various workers. For example, for the normal reaction at about 700 mμ, Black, et al.[6] observe a quantum requirement of 14 einsteins/equivalent; Hoch and Martin[4] report 2.9 to 3.3, and Govindjee, et al.[5] report 6.3, and we observe 4.0.

Black, et al. used absorption values measured by the opal glass technique. It would appear from the previous discussion of the large contribution of scattered light to such measurements that the large quantum requirements they report for the normal reaction at long wavelengths may be appreciably overestimated on this basis. This factor may also account for their rise in quantum requirement around 550 mμ where the chloroplast scattering is, again, relatively great. Our studies show a quantum requirement at 550 mμ which is slightly lower, if anything, than those at longer wavelengths.

In general, our results agree rather well with those of Hoch and Martin[5] for both the normal and poisoned systems. Their study is based on absorption measurements using an integrating sphere spectrophotometer, which should markedly reduce the light scattering interference at long wavelengths. Although they were aware of the dark reoxidation of $NADPH_2$, it is not apparent from their paper that any correction for it was attempted. This factor could be expected to contribute to the discrepancies

which remain between their data and ours. These differences are greatest for the DCMU-poisoned system, where we find the reoxidation to be more pronounced. This would have the effect of making their quantum requirements appear to be too large. They are some 50% greater than our corresponding values.

The values obtained by Govindjee, et al.[6] are substantially greater throughout this wavelength region for the normal system. This discrepancy is puzzling since their measurements were carried out using the same techniques and with the same reaction mixture as were those reported by Hoch and Martin.

At wavelengths less than 680 mμ our measurements indicate quantum requirementsfor both the normal and the poisoned reactions of 1.5 to 3 einsteins/equivalent and not strongly wavelength dependent. Hoch and Martin and Black, et al. report little change in the quantum requirements from 649 to 678 mμ. The relative quantum requirements obtained by Arnon, et al.[4] for the DCMU-poisoned system exhibit a maximum at 661 mμ and lower values at both longer and shorter wavelengths. This behavior is different from each of the other previous studies and from ours. Arnon, et al. did not extrapolate their measurements to zero light intensity, and insufficient details of their experiments are reported in the publication for us to attempt to resolve the discrepancies.

Mechanism involving two pigment systems

Our observations of the dependence of quantum requirement for NADP reduction on wavelength for these two reaction systems can be accounted for nicely in terms of the two-pigment system mechanism proposed for higher plant photosynthetic energy conversion[21-23]. We follow the notation

of Duysens, et al.[21] in designating the long wavelength absorbing pigments as system I and the shorter wavelength pigments as system II. There is much evidence to support a current formulation of the mechanism of photosynthetic energy conversion in which two photo-absorbing steps act in series to give the reduction of NADP using water as reductant and leading to the evolution of molecular oxygen. In this mechanism pigment system I mediates the transfer of electrons from an electron transport intermediate (cytochrome f ?) to NADP via ferredoxin, and pigment system II mediates the transfer of electrons from water to an oxidized intermediate (plastoquinone ?) leading to oxygen production. The reduced plastoquinone would then react through an electron transport chain with oxidized cytochrome f in a dark reaction which can couple to a phosphorylation step. Both pigment systems must be activated for the normal reaction to proceed efficiently. With added DCMU the second light reaction is blocked and electrons provided by cofactors such as reduced DCPIP can be transferred to NADP using system I alone.

The mechanism described above would require a minimum of two quanta absorbed per electron transferred to NADP for the normal reaction and one quantum per electron for the poisoned system. At wavelengths where one of the pigment systems absorbs relatively weakly, higher quantum requirements would be expected.

Our results can be accounted for in the framework of this mechanism by assuming that, at wavelengths longer than 700 mμ, pigment system I accounts for approximately 80% of the total absorption and system II accounts for 20%. At wavelengths shorter than 680 mμ each pigment system is responsible for about half the total absorption. Furthermore, we

assume that electronic excitation energy cannot be transferred as such
from one pigment system to the other. These assumptions lead to the
following conclusions: (i) for the normal reaction at short wave-
lengths the optimum quantum requirement of 2 would be obtained since
both pigment systems are being activated at equal rates; (ii) at longer
wavelengths the normal reaction would have a quantum requirement four
times greater, since the low absorbance of pigment system II at these
wavelengths limits the overall efficiency with which electrons can be
obtained from water; (iii) for the DCMU-poisoned reaction a quantum
requirement of 2 is observed at short wavelengths, since only half the
quanta absorbed are used to excite system I; and (iv) at longer wavelengths,
where system I accounts for 80% of the absorption, this quantum require-
ment drops nearly to 1. These conclusions are in good agreement with
the data of Table I and, if the hypotheses are correct, they lead to the
observation that at low limiting light intensities these photoreductions
of NADP by chloroplasts are operating very near optimum values.

It would be tempting to interpret the initial rapid transient
increase in absorbance at 339 mµ (stage ii, Fig. 5) as representing an
even more efficient production of NADPH$_2$ during the very first stages
of the reaction. Such a conclusion is not yet warranted, however. The
effect is so small that we cannot convincingly demonstrate that this
earliest absorption change does indeed represent NADPH$_2$ formation or
even reflects a band which exhibits a maximum at 339 mµ. The effect
is somewhat variable and is frequently absent or barely observable at
the lowest light intensities. It may, therefore, reflect the initial
approach to the steady-state condition, during which the concentration
of T is decreasing from its initial value T_0 and during which a faster

reaction velocity would be expected. This simple explanation will not account for the rapid transient decrease in absorbance at 339 mμ (stage vi) when the light is turned off, however. The two effects together could be accounted for by postulating a small activated (bound) fraction of NADP which gets converted to $NADPH_2$ very efficiently at the onset of illumination and is regenerated at the expense of bound $NADPH_2$ when the light is turned off. Further studies are called for in order to elucidate the cause of these interesting transients.

Kinetic limitations at high light intensities

An analysis of the dependence of the quantum requirement for NADP reduction on the incident light intensity suggests that at higher intensities an increasing fraction of the absorbed radiation is diverted from the photochemical process. The simplest explanation implicates an intermediate or trapping site present in relatively low concentration in the system. Electronic excitation energy resulting from the absorbed light is transferred to this intermediate or trap and results in its activation. Apparently the return of this species to its unexcited state is a relatively slow reaction in comparison with the rate of absorption of quanta at high intensities, and a low steady-state concentration of unexcited intermediates results. Under these circumstances the excess absorbed quanta are dissipated, presumably largely by a thermal process, and a high quantum requirement for the photoreduction is observed. The kinetics are essentially independent of the wavelength of exciting light, with the possible exception of the normal reaction at long wavelengths. The limiting factor is apparently endogenous to the chloroplasts, since both PPNR and DCPIP (when present)

were demonstrated to be at concentrations near saturation levels.

A reasonable explanation of this effect can be made in terms of the photosynthetic unit. Park and Biggins[24] give evidence that the quantasomes observed in spinach chloroplasts are the morphological expression of the photosynthetic units. If we assume that there is only one trapping site per photosynthetic unit and that its pigment composition is that reported by Park and Biggins, we can estimate the decay time of the excited traps from our quantum requirement versus intensity data--specifically, from the values of k_D.

An absorbed intensity of 4×10^{-7} einsteins/sec-liter is sufficient to double the quantum requirement at 678 mμ for NADP reduction by reduced DCPIP in a suspension of spinach chloroplasts containing about 5×10^{-6} moles/liter of chlorophyll a. In accordance with the mechanism proposed for the intensity dependence, we propose that under these conditions at steady state only half the photosynthetic units are present in their unexcited states. Park and Biggins[24] estimate that each quantasome or photosynthetic unit contains 160 chlorophyll a molecules. The above suspension of chloroplasts will contain 3.1×10^{-8} moles of quantasomes/liter, on this basis. Half the absorbed intensity, or 2×10^{-7} einsteins/sec-liter, is actually effective in exciting the traps and, under these conditions, each trap is excited on the average 6.5 times per second. A 50% steady-state level of excited traps is maintained under conditions where the rate of decay of excited traps is roughly equal to their rate of formation. Hence, the decay time of excited traps must be of the order of 0.15 sec for the DCMU-poisoned system.

For the normal reaction, with water as electron donor, the light intensity required to double the quantum requirement of NADP reduction is 12×10^{-7} einsteins/sec-liter. The decay of excited units for this reaction is therefore about 0.05 sec. This compares favorably with values of 0.03 sec for the time between successive effective quantum absorption acts determined in intermittent illumination studies by Emerson and Arnold[25]. Because the mechanisms of the two reactions we have studied are certainly different, we cannot know whether the rate-limiting step occurs at the same point in each case. It does seem that the turnover time in isolated chloroplasts is three times faster when electrons are provided from water via pigment system II than when they derive from reduced DCPIP. Since DCPIP is present in saturating amounts, the slower rate-limiting step in the latter case implies the participation of an endogenous cofactor which is not common to the pathway where water acts as reductant. The dependence of quantum requirement on absorbed intensity for NADP reduction by reduced DCPIP is very similar for quantasome and for chloroplast preparations. Hence, the lifetime of the excited intermediate must be nearly identical for the two systems. This is a further indication that the intermediate is endogenous to the chloroplast or quantasome structure.

<div align="center">ACKNOWLEDGEMENTS</div>

via reaction (3) as opposed to reaction (2). It is this competition, plus the assumption that some reactive intermediate T is appreciably depleted at higher reaction rates, that leads to the prediction of a linear increase in quantum requirement with increasing light intensity.

ABBREVIATIONS USED

NADP   nicotinamide adenosine dinucleotide phosphate

$NADPH_2$ "   "   "   " , reduced form

ADP   adenosine 5'-diphosphate

DCMU   3-(3,4-dichlorophenyl)-1,1-dimethylurea

FPNR   photosynthetic pyridine nucleotide reductase

Tris   tris(hydroxymethyl) aminomethane

DCPIP   2,6-dichlorophenolindophenol

REFERENCES

[1] N. I. Bishop, Biochim. Biophys. Acta 27 (1958) 205.

[2] L. P. Vernon and W. S. Zaugg, J. Biol. Chem. 235 (1960) 2728.

[3] M. Losada, F. R. Whatley, and D. I. Arnon, Nature 190 (1961) 606.

[4] D. I. Arnon, M. Losada, F. R. Whatley, H. Y. Tsujimoto, D. O. Hall, and A. A. Horton, Proc. Nat. Acad. Sci., Washington, 47 (1961) 1314.

[5] G. Hoch and I. Martin, Arch. Biochem. Biophys. 102 (1963) 430.

[6] R. Govindjee, Govindjee, and G. Hoch, Plant Physiol. 39 (1963) 10.

[7] C. C. Black, C. A. Fewson, M. Gibbs, and S. A. Gordon, J. Biol. Chem. 238 (1963) 3802.

[8] A. San Pietro and H. M. Lang, J. Biol. Chem. 231 (1958) 211.

[9] R. Hill and F. Bendall, Nature 187 (1960) 417.

[10] K. Tagawa and D. I. Arnon, Nature 195 (1962) 537.

[11] M. Shin, K. Tagawa, and D. I. Arnon, Biochem. Z. 338 (1963) 84.

[12] I. D. Kuntz, Jr., P. A. Loach, and M. Calvin, Biophys. J. 4 (1964) 227.

[13] K. Sauer and R. B. Park, Biochim. Biophys. Acta 79 (1964) 476.

[14] K. Shibata, A. A. Benson, and M. Calvin, Biochim. Biophys. Acta 15 (1954) 461.

[15] R. Emerson and C. M. Lewis, Amer. J. Bot. 30 (1943) 165.

[16] R. Lumry, R. E. Wayrynen, and J. D. Spikes, Arch. Biochem. Biophys. 67 (1957) 453.

[17] J. S. Rieske, R. Lumry, and J. D. Spikes, Plant Physiol. 34 (1959) 293.

[18] R. Lumry and J. S. Rieske, Plant Physiol. 34 (1959) 301.

[19] R. B. Park and N. G. Pon, J. Mol. Biol. 3 (1961) 1.

[20] R. Emerson, R. Chalmers, and C. Cederstrand, Proc. Nat. Acad. Sci., Washington, 43 (1957) 133.

21 L. N. M. Duysens, J. Amesz, and B. M. Kamp, Nature 190 (1961) 510.

22 B. Kok and G. Hoch, in W. D. McElroy and B. Glass, Light and Life, Johns Hopkins Press, Baltimore, 1961, p. 397.

23 H. T. Witt, A. Müller, and B. Rumberg, Nature 191 (1961) 194.

24 R. B. Park and J. Biggins, Science 144 (1964) 1009.

25 R. Emerson and W. Arnold, J. Gen. Physiol. 15 (1932) 391.

TABLE I

Quantum requirement for NADP reduction by spinach chloroplasts at wavelengths from 550 to 740 mµ--comparison with literature values. Q_0 is the zero intensity quantum requirement in einsteins/equivalent at wavelength λ (mµ).

Normal System (H_2O Donor)								
Black, Fewson, Gibbs & Gordon[7]		Hoch & Martin[5]			Govindjee, Govindjee & Hoch[6]		This study	
λ	Q_0	λ	Q_0 Chlorophyll 10µg/ml	50µg/ml	λ	Q_0	λ	Q_0
550	5.0						550	1.6
600	4.5						600	1.75
650	4.2	649	2.0				649	2.2
660	3.9	658	2.4				660	2.7
675	3.8	678	2.3		678	4.1	679	2.5
690	4.3	693	2.0	3.0	693	4.0	688	3.75
700	14.	700	2.9	3.3	700	6.3	703	4.1
		710	6.2	5.5	714	10.0	708	6.9
		720		9.1	721	9.8	720	7.8
		730		8.7	730	12.5	730	9.6
		740		8.3	740	17.		

DCMU-poisoned System (Ascorbate/DCPIP Donor)					
Hoch & Martin[5]			This study		
λ	Q_0 Chlorophyll 10µg/ml	50µg/ml	λ	Q_0 Chloroplasts	Q_0 Quantasomes
			550	2.0	
			600	2.4	
649	3.7		649	2.8	6.2
668	4.3		660	2.2	
678	4.8		679	2.8	6.2
693	2.0	4.8	688	1.9	
700	1.8	2.7	703	1.5	4.0
710	1.6	2.1	708	0.9	
720		2.4	720	1.2	4.0
730		2.5	730	1.7	
740		3.1			

LEGENDS FOR FIGURES

Fig. 1. Apparatus; sample compartment of the Cary Model 1462
Scattered-Transmission Accessory, showing the modifications
for illumination of the sample cuvette. Filters IF 1 (or
monochromator) and CF 1 select the actinic wavelengths from
a tungsten lamp. Filters IF 2 and CF 2 prevent actinic
light and chloroplast fluorescence from interfering with
the photomultiplier.

Fig. 2. Absorption spectra of spinach chloroplasts from 550 to
800 mμ. Upper curve (solid): normal transmission spectrum
using Cary Model 14 Spectrophotometer with normal cell com-
partment and RTV R-136 photomultiplier (see Ref. 13). Lower
curve (solid): scattered-transmission spectrum using Cary
Model 1462 Scattered-Transmission Accessory with Dumont 6911
photomultiplier. Dashed curve: opal glass spectrum, using
Cary 14 with normal cell compartment and R-136 photomultiplier;
matched opal glass diffusing plates in sample and reference
beams; superimposed on scattered-transmission spectrum out to
700 mμ. All three measurements were made on the same chloro-
plast suspension.

Fig. 3. Absorption spectra of spinach chloroplasts and quantasomes at
long wavelengths using Cary 14 Spectrophotometer with Model
1462 Scattered-Transmission Accessory and Dumont 6911 photo-
multiplier. Curves are normalized to an absorbance of 10.0
at 678 mμ. Lower curve (dashed): computed quantasome absorp-
tion corrected for light scattering.

LEGENDS FOR FIGURES (Continued)

Fig. 4.   Rayleigh scattering of spinach quantasomes from 760 to 900 mμ.
Turbidity of quantasomes (middle curve of Fig. 3) versus
inverse fourth power of wavelength.

Fig. 5.   A trace of the time course of the photoreduction of NADP by
isolated chloroplasts excited at 720 mμ. (DCMU/DCPIP/ascorbate
system). A molar extinction coefficient of 6.2×10^3 1-mole^{-1}-cm^{-1}
for NADPH$_2$ at 339 mμ was used.
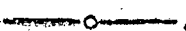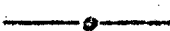
Figs. 6 and 7.   The relationship between the quantum requirement for
NADP reduction and incident intensity. ————o————, the normal
reaction where H$_2$O is electron donor and ————•————, ascorbate/
DCPIP as electron donor in the DCMU-poisoned reaction.

Fig. 8.   The action spectrum for NADP reduction by isolated chloroplasts
for the normal (————o————) and DCMU-poisoned (————•————)
systems. The quantum requirements are values obtained from
extrapolations to zero intensity.

Fig. 9.   The effect of the concentration of DCPIP on the quantum yield
of NADP reduction by chloroplasts excited at 678 mμ. The
standard reaction mixture was used with sufficient chloroplasts
(1 cm path).
to give an absorbance of 0.80 at 678 mμ. Incident intensities
used were 0.20 (filled circles) and 0.57 (open circles) nano-
einsteins/cm^2-sec.

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Fig. 1

MUB-2802

Fig. 2

Fig. 3

MUB-3258

Fig. 4

PHOTOREDUCTION OF NADP BY SPINACH CHLOROPLASTS
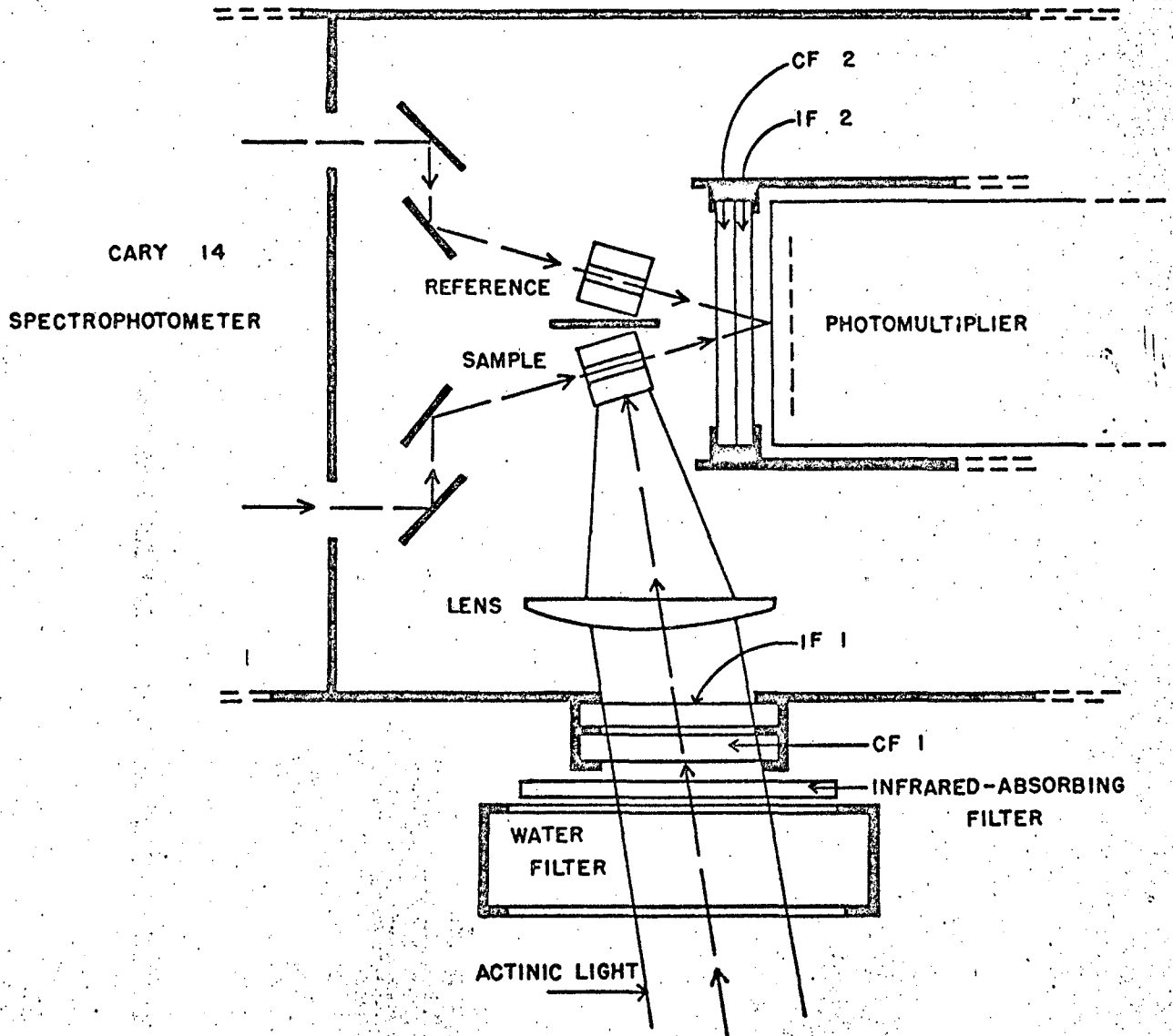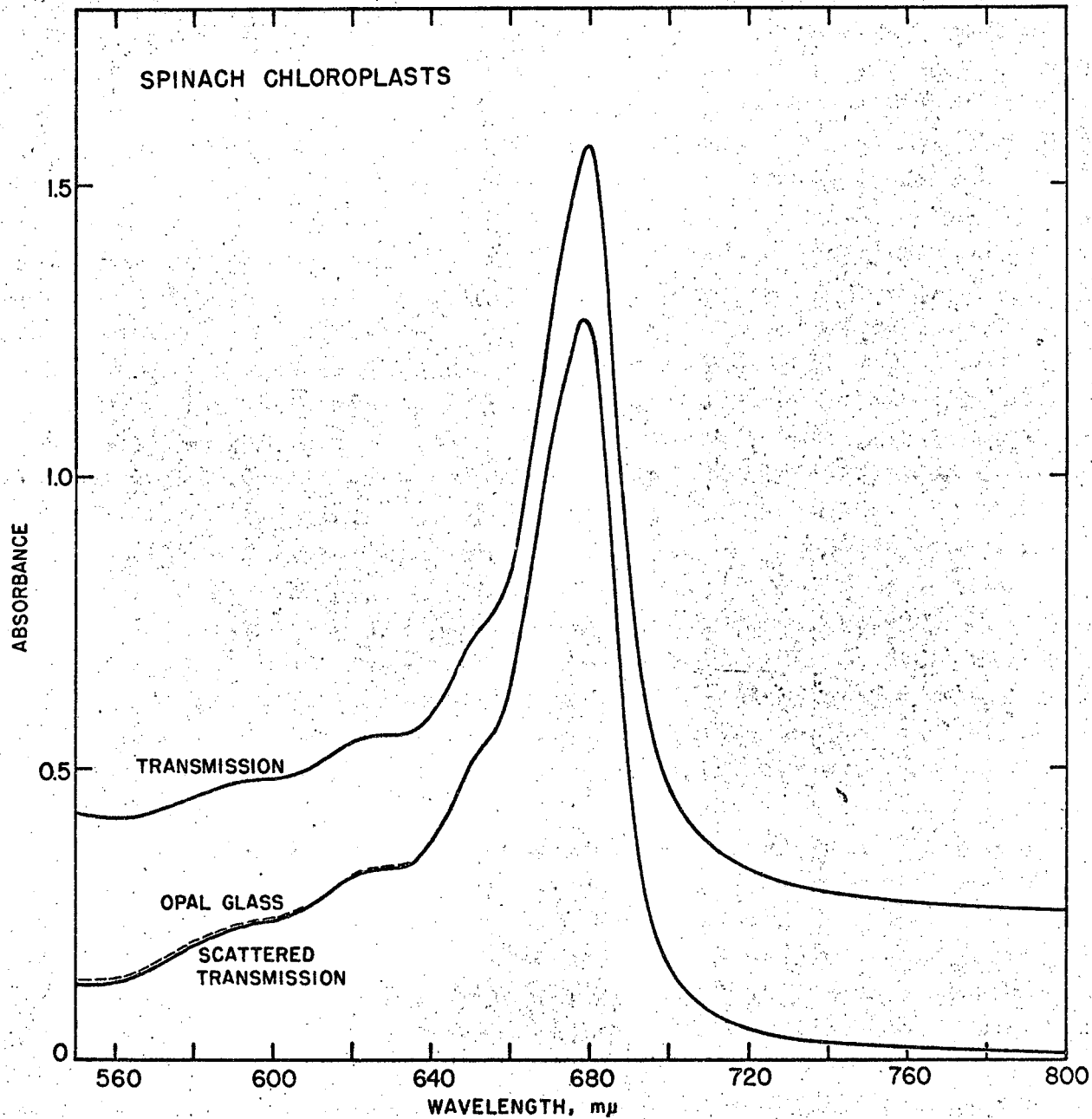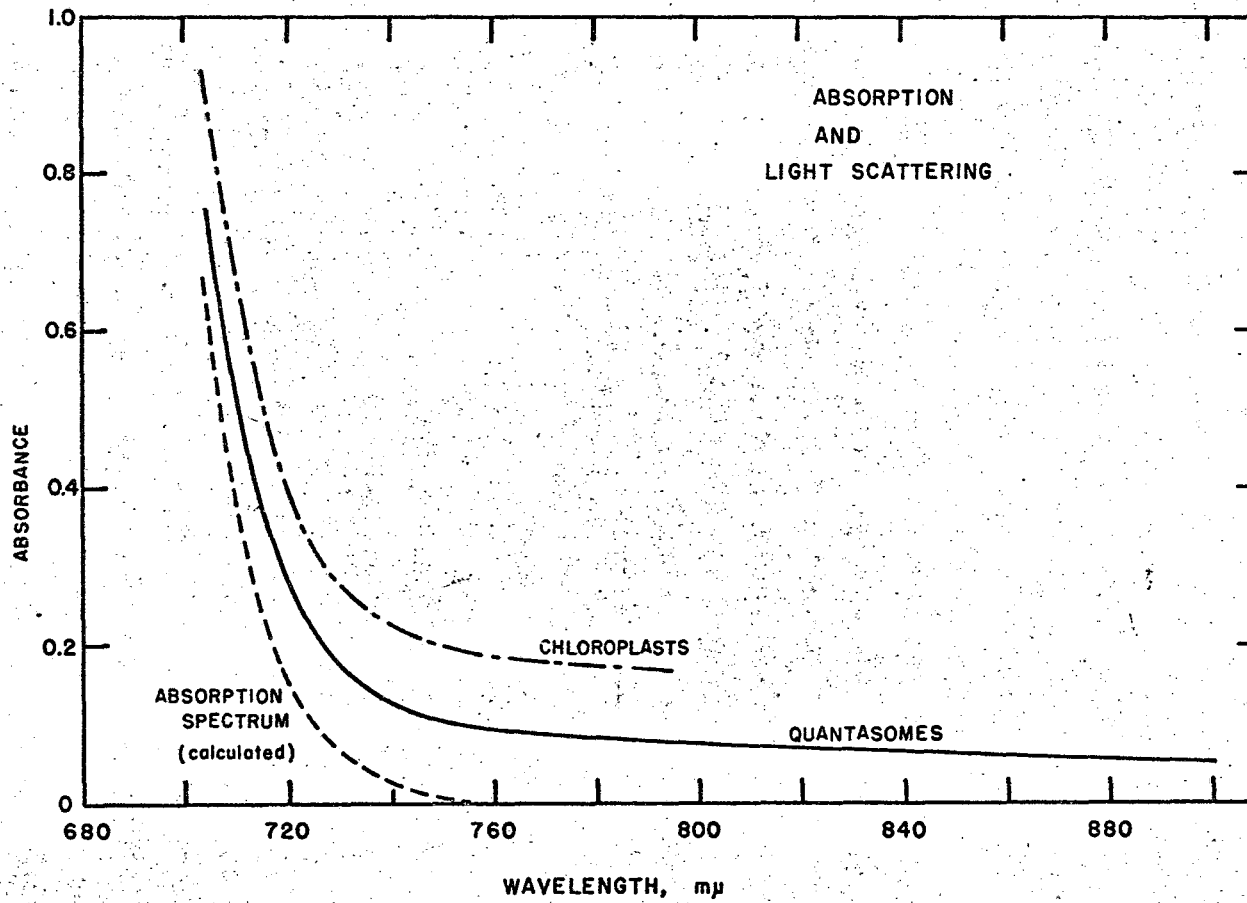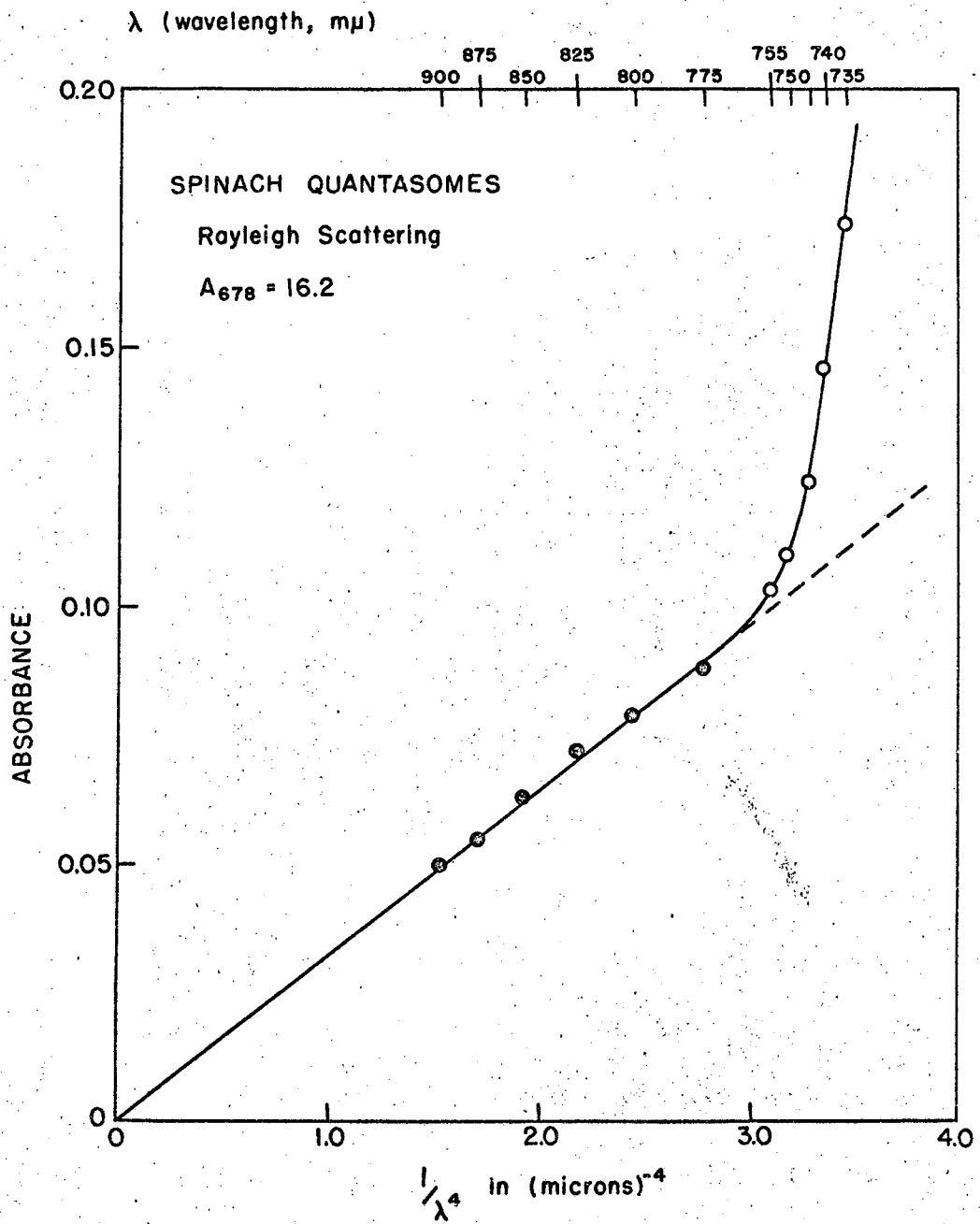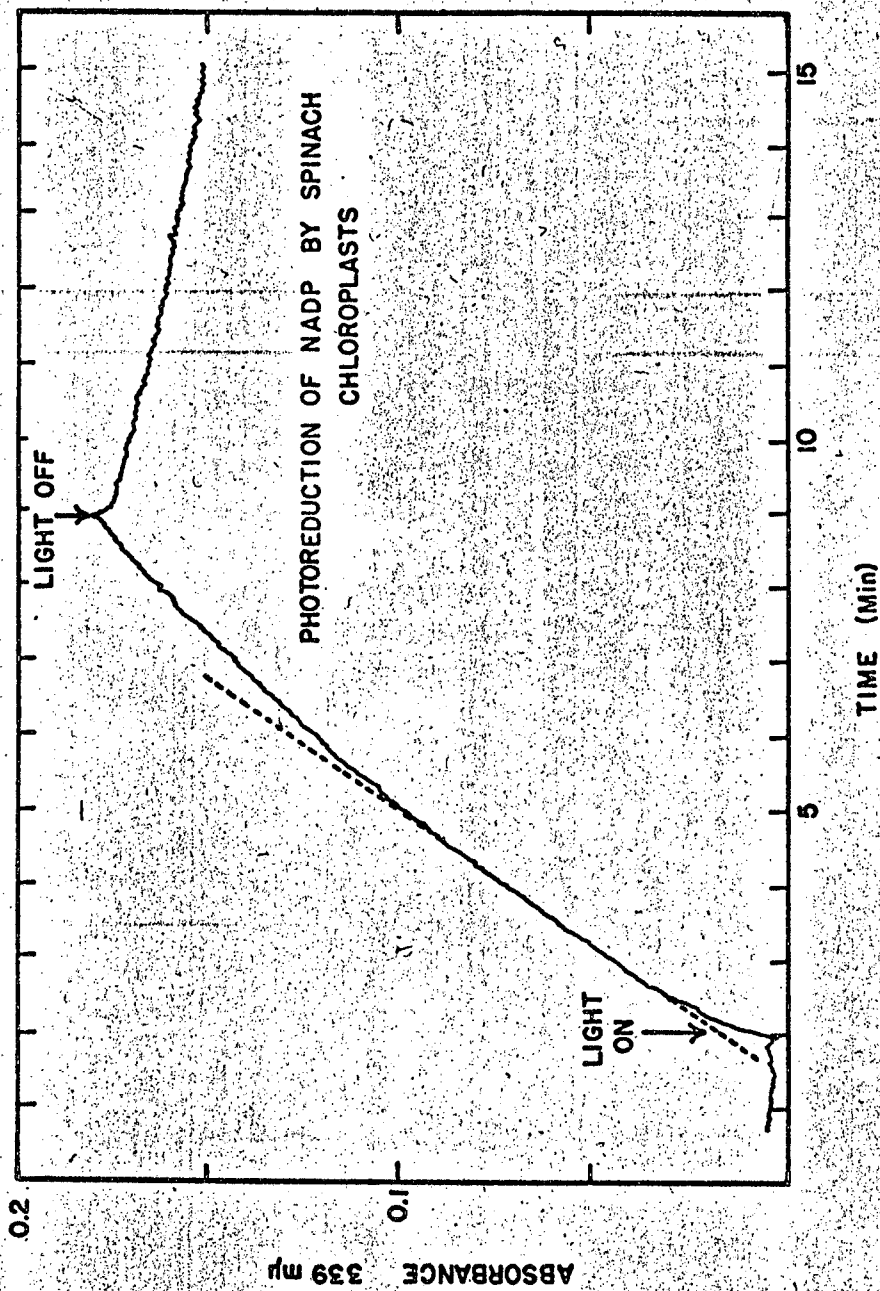
Fig. 5

MUB-2803

Fig. 7

MUB-2806

Fig. 8

MUB-3085

NADP REDUCTION BY CHLOROPLASTS
Dependence on DCPIP Concentration

$I_o = 0.20 \frac{\text{nanoeinsteins}}{\text{cm}^2 - \text{sec}}$

$I_o = 0.57 \frac{\text{nanoeinsteins}}{\text{cm}^2 - \text{sec}}$

QUANTUM YIELD $\left(\frac{\text{equivalents}}{\text{einstein}}\right)$

DCPIP Concentration (μ moles/ml)

MUB-3257

Fig. 9