Changes in Light-Absorption and Light-Scattering Properties of Spinach Chloroplasts upon Illumination: Relationship to Photophosphorylation*

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Spinach chloroplast suspensions exhibit light-dependent absorption changes (small-angle light scattering) amounting to 0.2–0.4 absorption units for 20 μg chlorophyll per ml of reaction mixture. Redox compounds such as quinones, flavins, and dyes stimulate the reaction up to 10-fold. The classical inhibitors of oxygen evolution (dichloromethylphenylurea, *o*-phenanthroline, etc.) inhibit the reaction, as do the common uncouplers of photophosphorylation (NH_4Cl and *m*-chlorocarbonylcyanide phenylhydrazone). Illumination also produced 90° light-scattering changes which correspond kinetically to the absorption changes and respond similarly to inhibitors. The observed changes appear to be a manifestation of a conformational change in the chloroplast structure related energetically to electron transfer and probably to early steps in phosphorylation. The presence of the phosphate-acceptor system diminishes the conformational change (as measured by the absorption change) suggesting that the unknown high-energy intermediate responsible for the conformational change is also utilized for adenosine triphosphate formation. Action spectra indicate that the stimulated conformational change is mediated by the long-wavelength pigment system in aged chloroplasts, while fresh chloroplasts utilize light absorbed at shorter wavelengths. The reaction is maximal between *p*H 5 and 6. The temperature coefficients (Q_{10}) for the initial forward and back reaction rates were found to be 3.7 and 2.9, respectively. The conformational change is more resistant to aging than either oxygen evolution or photophosphorylation, providing further evidence that it is probably not associated directly with the terminal step(s) in adenosine triphosphate formation in which phosphate is transferred to adenosine diphosphate, but is related to high-energy intermediates formed in a prior reaction. The inhibition pattern also bears this out. Quinacrine has a slightly stimulatory effect at a concentration of 2×10^{-5} M which inhibits adenosine triphosphate formation.

In an attempt to show NADP photoreduction by chloroplasts with trimethyl-1,4-benzoquinol ($TMQH_2$)[1] as the electron donor, it was observed that large absorbancy increases (0.2–0.4 absorbancy units/20 μg chlorophyll per ml at 340 mμ) occurred upon illuminating a system containing only chloroplasts, buffer, and $TMQH_2$. This result was surprising, since the amount of endogenous nucleotides in aqueous chloroplast preparations is sufficient to give only a fraction of the absorbance change observed (Krogmann, 1958). It was then observed that absorbancy increases occurred generally throughout the visible and near ultraviolet spectral range. The nature of these absorbancy changes is the substance of this report.

The observations outlined are of interest for three reasons: (A) it has been generally conceded that illumination does not produce large absorbancy changes in chloroplast suspensions (Kok and Hoch, 1961); (B) the occurrence of such large absorbancy changes may alter the interpretation of results obtained by spectrophotometric assay for the photoreduction by chloroplasts of NADP, DPIP, ferricyanide, etc.; and (C) the reactions appear to be energetically related to the photophosphorylation process.

Recently Itoh *et al.* (1963) reported on the light-dependent shrinkage of whole chloroplasts, measuring the rectilinear attenuation of parallel radiation at 750 mμ as affected by actinic illumination. They also determined volume distribution, packed volume, and the axial ratio of chloroplasts, proposing that illumination causes chloroplasts to undergo a considerable conformational change which is sensitive to ATP, NH_4Cl (an uncoupler of photophosphorylation), and *o*-phenanthroline (an inhibitor of the Hill reaction). Packer (1963a) has shown that chloroplast suspensions exhibit a light-induced increase in scattered light, indicating a conformational change of the chloroplast structure which is correlated with the process of photophosphorylation.

Conformational changes of mitochondria have been correlated with oxidative phosphorylation (Lehninger, 1962). As yet there is no understanding of the mechanisms by which such phenomena occur.

METHODS

Spinach chloroplast fragments were prepared by isolating intact chloroplasts in 0.35 M NaCl and 0.02 M Tris-HCl, *p*H 7.6, and suspending them in the same buffer diluted 10-fold. Chlorophyll was measured by the method of Arnon (1949).

The light absorption of chloroplast suspensions was recorded with a Beckman Model DB spectrophotometer, modified to allow illumination of the sample cuvet while the signal was traced on a strip chart (Zaugg, 1963.) The effect of light scattered from the actinic beam (filtered through a Corning 2304 red filter and 5 cm of water) was eliminated by placing a blue plastic filter between the cuvets and the photomultiplier tube entrance slit.

The light-minus-dark difference spectrum from 240 to 730 mμ was recorded with a Cary Model 14R spectrophotometer fitted with a tungsten actinic light source. The actinic beam was filtered through a Corning 2304 red filter and 5 cm of water. Action spectra were determined with a Perkin-Elmer Model

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[1] Abbreviations used in this work: TMQ, trimethyl-1,4-benzoquinone; $TMQH_2$, trimethyl-1,4-benzoquinol; DPIP, dichlorophenolindophenol; *m*-Cl-CCP, *m*-chlorocarbonylcyanide phenylhydrazone; quinacrine, 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino-butylamino)acridine; FAD, flavin-adenine dinucleotide.

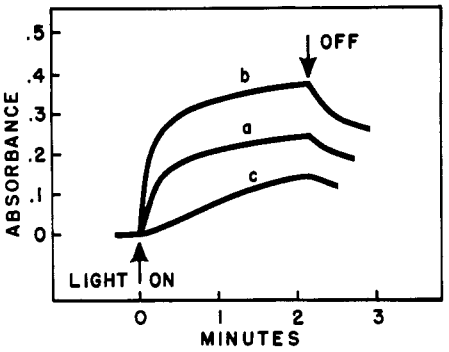

FIG. 1.—Light- and $TMQH_2$-stimulated absorbancy change at (a) 340 mμ, 24 μg chlorophyll per ml (chloroplast fragments); 0.033 M Tris-HCl, *p*H 7.1; 1.0 mM $TMQH_2$; (b) 510 mμ, 23 μg chlorophyll; 0.033 M Tris-HCl, *p*H 6.0; 1.1 mM $TMQH_2$; (c) control, 510 mμ, 23 μg chlorophyll; 0.033 M Tris-HCl, *p*H 6.0.

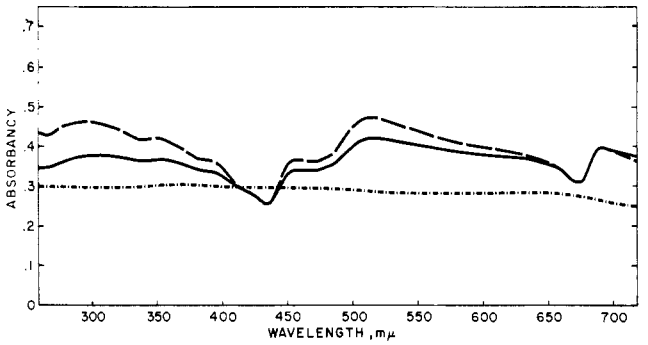

FIG. 2.—Light-minus-dark difference spectra of the light-induced absorption-increase reaction. Reaction mixture contained chloroplasts equivalent to 20 μg chlorophyll per ml plus 0.033 M Tris, *p*H 6.0. -·-·-, base line obtained prior to illumination; – – – –, 4-minute illumination prior to scan and continuous during scan; ———, dark scan 2 minutes after illuminated scan.

350 spectrophotometer for the light-induced absorbancy increase at 510 mμ elicited by $TMQH_2$. The actinic beam was filtered through appropriate interference filters (Baird Atomic, Inc.) and the voltage of the light source was adjusted to give equal incident energy on the cuvet (as measured by a silicon photodiode manufactured by Solar Systems, Inc.). Rates were measured at 640 and 670 mμ at several light intensities to ensure that the chosen level was in the region below saturation.

Light-scattering measurements were made with a Brice-Phoenix light-scattering photomultiplier Model 1000. The mercury lamp source was directly incident on the cuvet. The 90° scattered light was filtered through a 540-mμ interference filter before the photomultiplier entrance slit. Changes in the photomultiplier signal relating to both scattered and transmitted light were measured with a galvanometer.

Light intensity of a research illuminator Model LKR (Unitron Instrument Co.) used for the light-intensity studies were measured with a G.E. radiation meter type DW-60 Model 8DW60YZ, through a Corning 2304 red filter and an infrared filter.

Photophosphorylation was followed by the ^{32}P-assay method of Avron (1960) as described by Black *et al.* (1963). The photoreduction of DPIP by chloroplasts was measured by following the decrease in absorbance at 570 mμ of a solution containing 20–25 μg chlorophyll/ml, Tris-acetate buffer, *p*H 7.5, and 0.12 μmole DPIP in 3.0 ml. Quantasomes were prepared according to the method of Park and Pon (1961) except that Tris buffer was used rather than a phosphate buffer.

RESULTS

Kinetics and Wavelength Dependence of Absorption Change.—Chloroplast suspensions containing 20–25 μg chlorophyll per ml, 0.066 M Tris, *p*H 7.0, and 1.0 mM $TMQH_2$ exhibit a rapid increase in absorbancy at 340 mμ upon illumination with red light, as shown in Figure 1a. Typical reactions at 510 mμ obtained in the presence and absence of $TMQH_2$ are also presented, showing the marked stimulation caused by $TMQH_2$ (in this case, as with other additions, no absorbancy changes occur in the dark which can be related to the particular compound added). In Tris buffer the back reaction observed following illumination does not completely reverse the absorption change. The initial rate of the forward reaction is about the same in potassium phosphate buffer, but the extent of the absorbancy increase is only one-half that in Tris and the back reaction completely reverses the absorption change. The rise and decay times for the absorption change to reach one-half the steady-state value in Tris buffer are approximately 6 and 20 seconds, respectively (when $TMQH_2$ is used to elicit the rapid reaction), similar to the values reported by Packer (1936b) for 90° light-scattering changes. The rise time for one-half the absorption change in the absence of cofactor is about 3 minutes. Similar light-induced absorption changes of chloroplast suspensions have recently been reported (Jagendorf and Hind, 1963).

Difference spectra (light-minus-dark) from 250 mμ to 720 mμ show a general increase in absorbance with decreases superimposed at 680–690 mμ and 430–500 mμ (Fig. 2). The general absorption increase is to a large extent nullified when opal glass is placed between the cuvet and the photomultiplier entrance slit, indicating that the absorption increase is due mostly to a change in light scattering (Shibata *et al.*, 1954). The dashed curve of Figure 2 was obtained under conditions of continuous illumination. The solid curve is the dark scan taken 2 minutes after illumination. The perturbations of the curves at 680 mμ and 435 mμ remain undiminished for several minutes in the dark, ruling out fluorescence or delayed light emission. There is an exact correspondence between the position of these negative peaks and the peaks of the absorption spectrum of a chloroplast suspension.

The negative peak at 680 mμ could be caused by a "bleaching" of about 3% of the total chlorophyll a present. Experiments performed to test for permanent bleaching of the chlorophyll a (by extracting chloroplast suspensions with 80% acetone after the reaction had occurred) showed no noticeable differences. Therefore the observed curve shape in the 680–690 mμ is best explained as owing to the effect of an absorption peak on the light-scattering properties of the particles (Latimer, 1963). The large-particle theory as discussed by Latimer predicts the light-scattering curve in the region of an absorption peak will be S-shaped for smaller-sized particles.

Changes in 90° Light Scattering.—Light-scattering changes were measured under conditions favorable for the rapid light-absorption change. It was found that 90° scattered light increased with the same kinetics as the absorption increase at 510 mμ. The rapid light-scattering changes are dependent on the presence of an added redox agent (Table I). Either TMQ or $TMQH_2$ gives the light-scattering increase upon illumination. These data do not agree entirely with the earlier work of Packer (1963a), who implies

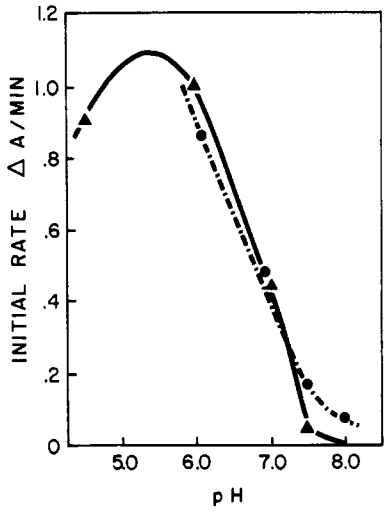

FIG. 3.—pH dependence for $TMQH_2$-stimulated light-induced absorption change at 340 mμ. Reaction mixture contained chloroplasts equivalent to 25 μg chlorophyll per ml, 0.033 M Tris-acetate O, or 0.033 M phosphate ▲, and 1.0 mM $TMQH_2$.

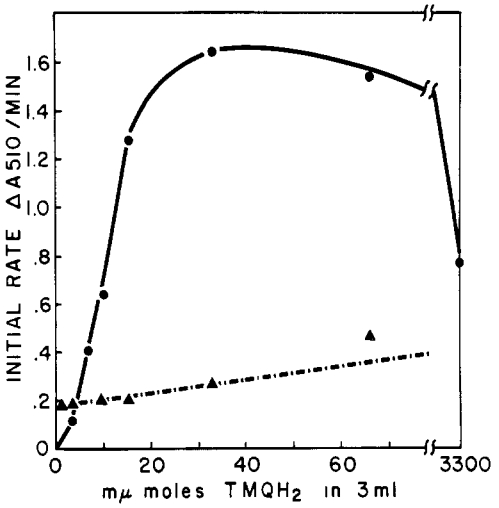

FIG. 4.—Effect of $TMQH_2$ concentration upon the light-induced absorbance change at 510 mμ. Reaction mixture contained 25 μg chlorophyll per ml, 0.033 M Tris-acetate, pH 6.0, and various amounts of $TMQH_2$. Initial rate, ●; extent of reaction for first 2 minutes, ▲.

TABLE I

LIGHT-SCATTERING CHANGES OF SPINACH CHLOROPLAST SUSPENSIONS[a]

Sample	Additions	Change in Scattering Intensity	Change in Transmitted Intensity
1	None	1.0	−1.0
2	3 mM ADP, 3.3 mM $MgCl_2$, 3.3 mM P_i	3.0	−3.0
3	No. 2 + 0.06 mM $TMQH_2$	21.0	−11.0
4	0.06 mM $TMQH_2$	22.0	−11.0
5	No. 4 + 3×10^{-5} M α-tocopherylphosphate	0	−3.0
6	0.06 mM TMQ	19.0	−6.0
7	2.0 units ferredoxin, 3.0 mM ADP, 3.3 mM $MgCl_2$, 3.3 mM P_i	3.5	−0.5
8	No. 7 + 0.5 mM NADP	17.0	−6.0
9	2.0 units ferredoxin + 0.5 mM NADP	16.0	−6.0

[a] Reaction mixture contained chloroplasts equivalent to 80 μg chlorophyll, 0.033 M Tris-acetate, pH 6.0, and other additions as noted in a total volume of 3 ml. Scattering changes were taken as the difference in scattered-light intensity before and after 1 minute of illumination by the mercury lamp, and are reported as arbitrary units on the galvanometer.

that the presence of a phosphate-acceptor system is necessary in order to observe the changes in light scatter. This is not the case under our conditions, where light-scattering changes accompany electron-transfer reactions in the absence of the phosphate-acceptor system. At pH 6.0 there is no effect of the phosphate-acceptor system on the light-scattering changes (Table I), while at pH 7.8 the scattering changes are diminished to one-half the control value when the acceptor system is present (see Fig. 6).

Most of the data presented below were obtained by measuring changes in absorption, which in this case is the same as small-angle light scattering. The two measuring techniques seem to be similar in sensitivity.

pH and Buffer Effects.—Figure 3 shows the pH dependence of the light-induced absorption change in Tris and phosphate buffers. The initial rate of the reaction is maximal in the pH range 5–6, becoming very low at pH 8.0. This pH dependence is similar to that for the formation of what Hind and Jagendorf (1963) describe as a high-energy intermediate in photophosphorylation. Light and $TMQH_2$-dependent light-scattering changes as functions of pH were also measured and were found at pH 8.0 to be 15% the rate obtained at pH 6.0.

With phosphate buffer the absorption increase is of smaller magnitude than in Tris and is completely reversed in the dark. The biphasic nature of reaction (see Fig. 1a) in Tris does not manifest itself in phosphate buffer. Mixtures of Tris and phosphate in the proportions shown in Figure 3 also result in a biphasic reaction. Sodium succinate buffer, pH 6.0, 0.033 M gives results similar to Tris buffer, while citrate and histidine buffers are quite inhibitory.

Effect of Redox Compounds.—The reaction is stimulated by the compounds listed in Table II, including quinones, flavins, dyes, and other redox compounds. Most of these compounds are capable of acting as electron acceptors for the oxygen-evolving mechanism of chloroplasts, and thus catalyze phosphorylation coupled to either cyclic or noncyclic electron flow. A wide range of redox potential is represented by this group of compounds. It is interesting that in some cases only the oxidized form of a compound gives the reaction, i.e., ferricyanide, whereas either reduced or oxidized quinones yield equivalent reactions. Those compounds whose reduced forms are inactive are also not able to catalyze "cyclic photophosphorylation," and thus the reduced form would not support light-induced electron flow in the chloroplast.

The effect of $TMQH_2$ concentration on the initial rate of the absorption change at 510 mμ is shown in Figure 4. It is seen that the reaction rate saturates at about 10^{-5} M $TMQH_2$. A similar saturation curve was obtained with ferricyanide. The extent of the initial, rapid phase is much less at these low concentrations, and it begins to saturate at about 10^{-3} M $TMQH_2$.

Effect of Inhibitors.—In view of the fact that the fast reaction is dependent upon Hill oxidants, a study was made of the effect of inhibitors of the Hill reaction on the absorption-change reaction. It is seen in Table III that the classical inhibitors of oxygen evolution such as dichloromethylphenylurea, o-phenanthroline, and 2-n-heptyl-4-hydroxyquinoline-N-oxide (which pre-

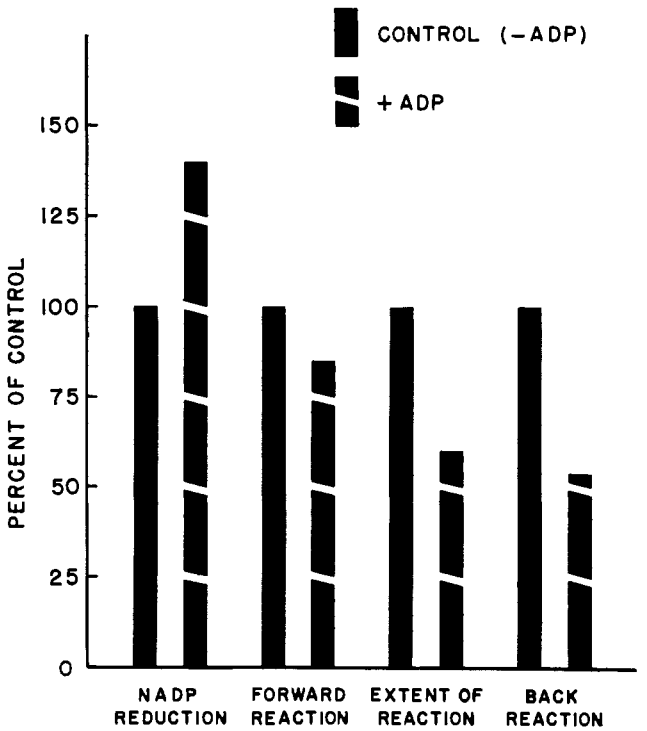

FIG. 5.—Effect of phosphate acceptor system on light-induced absorption-change reaction. Reaction mixtures contained 35 μg per ml chlorophyll, 10 units ferredoxin, 0.30 mM NADP, 3.4 mM $MgCl_2$, 13.3 mM HK_2PO_4, 0.05 M *p*H 7.8 Tris, and 0.33 mM ADP where added. Total volume 3.0 ml. Absorption-change reactions measured at 520 mμ: forward reaction initial rate, ΔA/min; extent for the first minute; back reaction initial rate, $-\Delta A$/min. NADP reduction measured by ΔA 345 mμ per 2 minutes. Values are averages of three readings. Control taken as rates obtained without ADP.

TABLE II

ACTIVATION OF LIGHT-INDUCED ABSORBANCY CHANGES OF SPINACH CHLOROPLAST SUSPENSIONS BY REDOX AGENTS

Compound Added	Rate with Compound/ Rate of Control	Concentrations (μmoles/ml)
A. *None* (control)	1	
B. *Quinones*		
Trimethyl-1,4-benzohydroquinone ($TMQH_2$)	30	1.
Trimethyl-1,4-benzoquinone (TMQ)	30	0.7
2,5-Dichloro-1,4-benzoquinone	6	0.4
1,4-Benzoquinone	12	2.2
Menadione	12	0.07
C. *Flavins*		
FAD	21	0.07
FMN	20	0.07
D. *Miscellaneous*		
Phenazine methosulfate	27	0.03
Pyocyanine	27	0.03
Tetrazolium blue	30	0.07
Methylene blue	6	0.01
Ferredoxin + NADP	10	3 units and 0.5 mM
Ascorbate	2	6.6
Ascorbate-DPIP	3	6.6 and 0.033
Phosphodoxin[a]	10	
Ferricyanide	10	0.03

[a] Black *et al.* (1963).

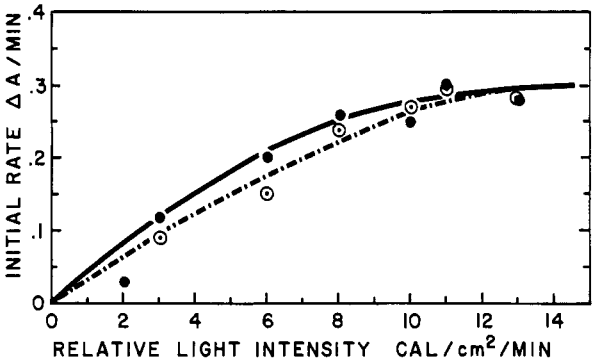

FIG. 6.—Effect of light intensity upon the $TMQH_2$-stimulated absorbance change and the DPIP Hill reaction. For the absorbance-change reaction at 510 mμ, •, reaction mixture contained 26 μg chlorophyll per ml, 0.033 M Tris-acetate, *p*H 6.0, and 0.11 mM $TMQH_2$. For the DPIP Hill reaction followed at 570 mμ, ⊙, the reaction mixture contained 0.066 M Tris-acetate, *p*H 7.5, 26 μg per ml chlorophyll, and 0.04 mM DPIP.

TABLE III

INHIBITION OF LIGHT-INDUCED ABSORBANCY CHANGE IN SPINACH CHLOROPLAST SUSPENSIONS

Compound	Concentration (M)	Inhibition (%) $TMQH_2$	Inhibition (%) TMQ
Dichloromethylphenylurea	8.0×10^{-6}	82	96
o-Phenanthroline	5×10^{-4}	70	96
Phenylurethane	4×10^{-3}	80	90
2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide	1.7×10^{-5}	75	
2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide	7×10^{-5}	90	
Antimycin A	4.5×10^{-5}	0	
m-Cl-CCP	3.2×10^{-5}	97	
2,4-Dinitrophenol (DNP)	3×10^{-4}	60	
NH_4Cl	3×10^{-3}	31	
NH_4Cl	9×10^{-3}	83	
NH_2OH	4.8×10^{-4}	50	
NH_2OH	1.6×10^{-2}	90	
α-Tocopherylphosphate	8×10^{-5}	50	
α-Tocopherylphosphate	8×10^{-4}	90	
Triton X-100	0.1% (v/v)	98	

vent transfer of electrons from water to NADP) inhibit the reaction nearly completely if the oxidized cofactor (TMQ) is used. However, when $TMQH_2$ or the ascorbic acid–DPIP couple is employed the inhibition brought about by 8×10^{-6} M dichloromethylphenylurea is 80 and 70%, respectively. In the latter cases the $TMQH_2$ or $DPIPH_2$ can supply electrons to the chloroplast system and partially overcome the effect of dichloromethylphenylurea.

Heptyl quinoline *N*-oxide, *m*-Cl-CCP, and NH_4Cl were inhibitory to the TMQ-stimulated reaction, and also for light-scattering changes as reported by Packer (1963a). *m*-Cl-CCP and NH_4Cl have been shown to be true uncouplers of photophosphorylation (Bamberger *et al.*, 1963; Krogmann *et al.*, 1959).

Quinacrine (8×10^{-5} M) and antimycin A (4.5×10^{-5} M) have the effect of converting the biphasic curve (with Tris buffer) into a monophasic curve which rises rapidly to a plateau. The dark decay reaction is also faster than in the absence of the compound. These compounds stimulate the reaction to the extent that they eliminate the second, slower phase observed in Tris buffer.

The detergent Triton X-100 was completely inhibitory at 0.1% (v/v). α-Tocopherylphosphate, also a potent inhibitor, is believed to act in part like a detergent. It was found that the Hill reaction (with DPIP as the oxidant) was inhibited by α-tocopherylphosphate in a similar manner. Detergent inhibition of the Hill reaction has been previously reported (Krogmann and Jagendorf, 1959).

The following compounds showed no inhibition, at a concentration of 10^{-4} M, of the light-induced absorption changes: bathocuproine (a copper-binding agent), bathophenanthroline (an iron-binding agent), KCN, NaF, iodoacetamide, *p*-mercuriphenylsulfonic acid, and *N*-ethylmaleamide.

The data presented in Table III show that the reactions responsible for the light-induced absorption changes are inhibited by a number of compounds, which generally fall into three groups in terms of their mode of action on the spinach chloroplasts. The first group involves those which serve as inhibitors of photosynthetic electron transfer, and includes dichloromethylphenylurea, *o*-phenanthroline, phenylurethane, and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide. The second group, including *m*-Cl-CCP, DNP, and ammonium ion, are uncoupling agents in the photophosphorylation process. The detergents α-tocopherylphosphate and Triton X-100 both serve to disrupt the chloroplast structure. The inhibition produced by all three groups of compounds can be related to their effect on the formation of high energy intermediates preceding ATP formation, and these data further support the concept that the light-scattering changes and absorption changes caused by illumination of chloroplasts are the result of conformational changes brought about by high-energy intermediates formed in the chloroplast.

Effect of Phosphate-Acceptor System.—If the light-induced absorption-change reaction indicates events relevant to phosphorylation, then the presence of the phosphorylation components (ADP, P_i, and Mg^{2+}) should affect the reaction. This possibility was tested by following both the absorption change reactions at 520 mμ and NADP photoreduction. For the latter reaction the system of Keister *et al.* (1961) was used in which ferredoxin was present in excess. A comparison of the reactions obtained with the complete phosphorylating system to the system lacking ADP reveals that conditions favorable for ATP synthesis diminish all aspects of the absorption change reaction while stimulating NADP reduction (Fig. 5). The diminution of the reaction responsible for absorbancy change may be explained in terms of a decrease in the high-energy intermediates leading ultimately to ATP formation when ADP is present. The rate of NADP reduction increases when coupled to ATP formation in the expected manner, as reported by Davenport (1959). The substitution of ATP for ADP gave kinetics similar to those of the minus ADP treatment, indicating that the above noted effect is not due to a nonspecific nucleotide influence.

Further evidence that the conformational changes (as measured by the absorption increase) are a manifestation of the accumulation of intermediates in the process of photophosphorylation was obtained from experiments in which ATP formation and the absorption change at 520 mμ were measured simultaneously. Table IV shows that TMQ-catalyzed photophosphorylation depresses the conformational change 72% if ATP synthesis occurs; but in the presence of quinacrine there is an increase in the conformational change, consistent with the hypothesis that a high-energy intermediate(s) is trapped.

TABLE IV

COMPARISON OF THE LIGHT-INDUCED CONFORMATIONAL CHANGE WITH CONCOMITANT TMQ-CATALYZED PHOTOPHOSPHORYLATION[a]

Additions	ATP (μmoles/hr per mg chlorophyll)	Inhibition[b] (%)	ΔA_{520} mμ (per min)	Inhibition[c] (%)
Control, dark	0.73		0	100
Control	27.4	0	0.13	72
Control, minus (Mg^{2+}, P_i, ADP)			0.46	0
2×10^{-5} M Quinacrine	11.2	61	0.70	−152
5×10^{-5} M Quinacrine	0.78	100	0.92	−200

[a] Reaction mixtures contained the following (2 ml total volume): 86 μg chlorophyll-equivalent chloroplasts, 0.05 M Tris, *p*H 7.8, 6.8 μmoles $MgCl_2$, 2.0 μmoles ADP, 2 μmoles K_2HPO_4 containing 2.54×10^5 cpm ^{32}P, 1.32 μmoles TMQ, and other additions as stated. Illumination was through a red filter (Corning 2304) for 2 minutes, during which time the absorbance at 520 mμ was monitored continuously.
[b] Relative to the value obtained with illuminated control.
[c] Relative to the value obtained with the illuminated control without Mg^{2+}, P_i, ADP.

Similar experiments were conducted at *p*H 6.0 where the formation of what we consider to be high-energy intermediate(s) is maximal. The phosphate-acceptor system did not have the same effect on the absorption reaction at *p*H 6.0. The only measurable effect was that Mg^{2+} stimulated the initial forward rate and rate of the back reaction. The lack of an ADP effect at this *p*H is reasonable since ATP formation is largely abolished at *p*H 6.0. These data also indicate that the absorption change reaction reflects conformational changes related to early events in the energy-conserving process, distinct from the terminal event of ATP formation. Data presented below corroborate this point.

Light-Intensity and Temperature Effects.—The response of the light-induced absorption change to light intensity was compared to that obtained for the DPIP Hill reaction with the same preparation. It is seen in Figure 6 that the reactions are similar in their response to increasing light intensity, becoming saturated at high intensity as expected for reactions which are rate limited by enzymatic "dark" reactions.

The initial rate of the reaction increased as the temperature was raised in the range of 16–28°, showing a Q_{10} value of 3.7 for the forward reaction and 2.9 for the back reaction.

Effects of Aging.—If chloroplasts are allowed to age at 0–4° they gradually lose the ability to perform the Hill reaction and photophosphorylation. The light-induced absorption-change reaction which occurs in such aged chloroplasts (devoid of Hill-reaction activity with DPIP) is absolutely dependent on the presence of the reduced form of an added cofactor. $TMQH_2$, the ascorbic acid–DPIP couple, vitamin K_5 (2-methyl-4-amino-1-naphthol), and photoreduced phenazine methosulfate elicit the reaction in chloroplasts; whereas TMQ, ferricyanide, FAD, menadione (vitamin K_3), or other oxidized cofactors are not active (Table V). This ability of aged chloroplasts to give the absorption-change reaction, if provided with a reduced cofactor, suggests that the long-wavelength system is operative, since it has been shown that NADP reduction by ascorbic acid–DPIP is resistant to aging (Vernon and Zaugg, 1960) and is mediated by system I (in the Duysens and Amesz (1962) terminology).

The inhibitor dichloromethylphenylurea gives only

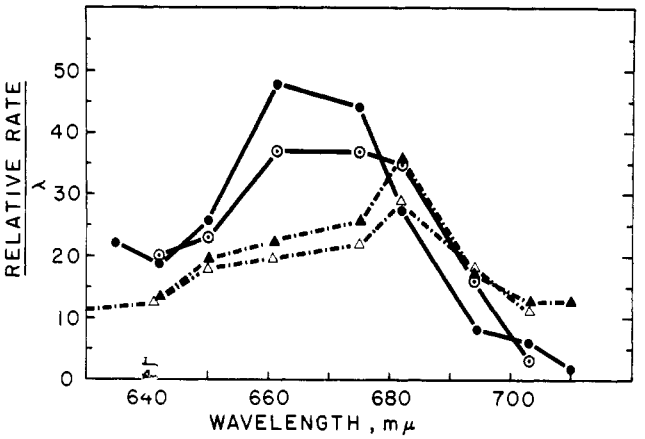

FIG. 7.—Action spectrum of light-induced absorbancy increase. Reaction mixtures contained: -●-, fresh chloroplasts with 4.7×10^{-5} M chlorophyll, 0.033 M Tris-acetate, *p*H 6.0, and 0.11 mM $TMQH_2$; -⊙-, fresh chloroplasts, 8×10^{-6} M chlorophyll, 0.066 M Tris-acetate, *p*H 6.0, 0.11 mM TMQ; -▲-, 8-day-old chloroplasts, 2.2×10^{-6} M chlorophyll, 0.066 M Tris-acetate, *p*H 6.0, 0.3 mM DPIP, 7 mM ascorbic acid; -△-, 8-day-old chloroplasts, 2.2×10^{-6} M chlorophyll, 0.066 M Tris, *p*H 6.0, 0.66 mM $TMQH_2$. Instrumentation as described under Methods. The ordinate is in terms of initial rate divided by the wavelength to give a "quantized" action spectrum.

TABLE V

ABSORPTION CHANGES IN AGED CHLOROPLASTS[a]

Sample	Additions	Initial Rate (ΔA_{510}/min)
a	0.66 μM/ml $TMQH_2$	0.29
b	0.66 μM/ml TMQ	0.05
c	0.013 μM/ml phenazine methosulfate	0.36
d	0.17 μM/ml FAD	0.05
e	0.17 μM/ml Menadione	0.05
f	0.17 μM/ml Ferricyanide	0.04
g	7 μM/ml ascorbic acid, 0.3 μM/ml DPIP	0.21
	7 μM/ml ascorbic acid, 0.3 μM/ml DPIP + 1.2×10^{-5} M dichloromethylphenylurea	0.15 (25% inhibition)

[a] Chloroplasts aged 7 days at about 4°. Reaction mixtures contained 25 μg chlorophyll per ml, 0.066 M Tris-acetate, *p*H 6.0, and other additions as listed.

21% inhibition to the ascorbic acid–DPIP–dependent reaction in aged particles (Table V) compared to 70% inhibition of the same reaction in fresh particles. This is reasonable in view of the fact that with fresh particles both the ascorbic acid–DPIP couple and the endogenous water-oxidizing system (inhibited by dichloromethylphenylurea) are sources of electrons. The uncoupler *m*-Cl-CCP was completely inhibitory in both aged and fresh chloroplasts. Difference spectra of aged chloroplasts (with $TMQH_2$ added) are similar to those shown in Figure 2 except that the magnitude of the change is less.

Chloroplasts aged 5 days were checked for 90° light-scattering changes in the presence of $TMQH_2$ and showed about 30% as much as the fresh material (the same as for the absorption-increase reaction). We could not obtain a restoration of scattering changes in aged chloroplasts by adding ATP as previously reported by Packer (1963a). Addition of ATP plus the phosphorylation components and $TMQH_2$ was no different than $TMQH_2$ alone. The aged chloroplasts were not capable of photophosphorylation with phenazine methosulfate as the cofactor.

Action Spectra for Absorption Change.—The data presented above indicate that the light-induced absorption change is closely associated with the long-wavelength pigment system (system I) for aged chloroplasts. Action spectra with fresh and aged particles were measured in the range 630–720 mμ. With fresh chloroplasts both TMQ and $TMQH_2$ were used, yielding essentially similar action spectra (Fig. 7). In aged chloroplasts which had lost the ability to give the light-induced absorption change with oxidized cofactors (TMQ, ferricyanide, etc.), $TMQH_2$ and the ascorbic acid–DPIP were used to elicit the response. There is a striking difference in the action spectra obtained with fresh and aged materials. The fresh particles show much more reaction in the 660-mμ range (chlorophyll b absorption). The aged chloroplasts' action peak is around 680 mμ (chlorophyll-a-absorption peak), indicating the operation of the long-wavelength system. These data indicate that in fresh chloroplasts both light-absorbing systems are operative, while with aged chloroplasts the long-wavelength system is the only one active. This agrees with the type of cofactor required in each case: with fresh chloroplasts the oxidized forms are active, since they are capable of being reduced in a Hill reaction, but with aged chloroplasts only the long-wavelength system operates and the cofactor must be added in the reduced form.

Oxygen Effects.—A glucose–glucose oxidase plus catalase system in conjunction with four evacuation and argon-replacement steps was used to produce anaerobicity. That the glucose oxidase system was operative was shown by observing a drop in *p*H (due to gluconic acid production) in a control sample containing both enzymes, glucose, and no buffer. The reaction under investigation was not affected by removal of oxygen from the system.

Absorption-Change Reaction in Quantasomes.—Quantasomes prepared from spinach chloroplasts by sonication retained 50% of their activity for photoreducing DPIP (relative to the sonicated chloroplasts before centrifugal fractionation) and 36% of the $TMQH_2$-stimulated increase in absorption at 510 mμ. If the view is taken that the light-absorption (small-angle light-scattering) changes reflect events leading to ATP formation, this is somewhat surprising, since there is no evidence that quantasomes have the capacity to photophosphorylate. However, these data indicate that quantasomes retain some of the initial steps leading to ATP formation.

DISCUSSION

The results of these experiments indicate that under conditions of electron transfer in chloroplasts there is a conformational change induced which may be measured either by changes in 90° scattered light or by changes in absorption (small-angle scattered light). The 90° scattered-light changes were first reported by Packer (1963a). However, Packer's experiments were performed with the complete system for photophosphorylation always present. From this he concluded that the light-scattering changes are coupled to terminal events in ATP formation. We have shown that the large changes in light scattering occur in the absence of the phosphorylating components ADP, P_i and Mg^{2+}, requiring only a redox compound capable of allowing photosynthetic electron transfer to proceed. In fact, the less ATP formed in our system, the greater is the conformational change. Thus we feel the light-

TABLE VI

DIFFERENTIAL EFFECTS OF VARIOUS INHIBITORS ON THE LIGHT-INDUCED ABSORPTION CHANGES

	Electron Transfer (% inhibition)	Conformational Change (% inhibition)	ATP Formation (% inhibition)
Dichloromethylphenylurea 10^{-6} M	100[a]	>95	100[a]
o-Phenanthroline 10^{-4} M	100[a]	>95	100[a]
m-Cl-CCP 10^{-5} M	<10[b]	>95	100[b]
NH_4^+ 5×10^{-3} M	0[c]	80	100[c]
Antimycin A 5×10^{-5} M	<10[b,d]	0	>80[d]
Quinacrine 8×10^{-5} M	0[e]	0	>95[d]

[a] Krogmann and Vennesland (1959). [b] Bamberger *et al.* (1963). [c] Krogmann *et al.* (1959). [d] Baltscheffsky (1960). [e] Dr. W. S. Zaugg, personal communication.

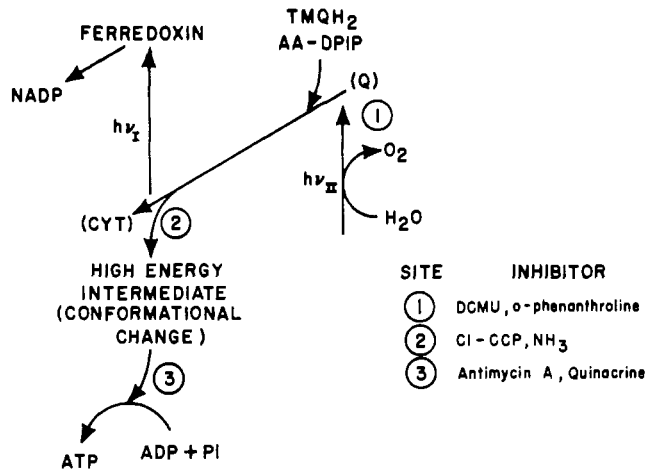

FIG. 8.—Relationship between photosynthetic electron transfer and the high-energy intermediate(s) responsible for the observed conformational changes. The accompanying table lists the inhibitors which act at the three sites indicated.

scattering increase is due to the formation of high energy intermediates which may or may not lead to ATP formation depending upon the presence of the phosphate-acceptor system. The compound responsible for the reaction we have observed is probably the same as studied by Hind and Jagendorf (1963), since the *p*H response is very similar for both reactions.

The data presented above show that those treatments which inhibit or prevent photosynthetic electron-transport reactions also inhibit the conformational changes studied. Adding inhibitors of oxygen evolution, heating the chloroplast, or aging the chloroplast serves to inhibit the reaction. With fresh chloroplasts which have the ability to photoreduce the added cofactors in a Hill reaction, either the oxidized or reduced form of the cofactor (TMQ, phenazine methosulfate) catalyzes the reaction. With aged chloroplasts, or in the presence of dichloromethylphenylurea, the oxidized cofactors become much less effective, which reflects the inability of the chloroplast to reduce them photochemically. In such cases the reaction is catalyzed by compounds such as $TMQH_2$ or the ascorbate-DPIP couple which can feed electrons into the long-wavelength system and thus catalyze cyclic electron transport. The action spectra for fresh and aged chloroplasts agrees with this interpretation, since the action spectrum shifts to longer wavelengths with aged chloroplasts which must use an alternate electron donor.

All the agents which stimulate the reaction also stimulate photophosphorylation, most likely by increasing the rate of electron flow through a phosphorylating site. The effect of these agents in the present case could be due to either an intrinsic effect upon the reaction rate (increasing the rate of light-induced electron flow) or due to their changing the effective concentration of some component of the reaction system. The former explanation appears to be the correct one since the extent of the reaction is not greatly different in the absence or presence of the activators (see Fig. 1), and the half-times for the reactions are different in the presence of the activators. A plot of the data from several representative traces of the stimulated reaction shows the reaction to follow apparent first-order kinetics. However, in a system as complex as this it is difficult to perform a meaningful kinetic analysis of the reaction.

The inhibition pattern observed for this reaction is very interesting (Table III). When this is compared with the effect of the same inhibitors upon both photosynthetic electron transfer and ATP formation the interesting pattern shown in Table VI develops. Those compounds which are able to inhibit photosynthetic electron transfer also inhibit the conformational change and ATP formation. The two inhibitors *m*-Cl-CCP and ammonium ion do not inhibit electron transfer but do inhibit both the conformational change and ATP formation. Antimycin A at relatively high levels and quinacrine inhibit neither photosynthetic electron transfer nor conformational change, but do inhibit the ATP formation. The last four compounds mentioned are considered as uncoupling agents in the photophosphorylation process. However, their mode of action is distinguished when the conformational change is compared to ATP formation, and indicates that *m*-Cl-CCP and ammonium ion are inhibiting at an earlier step than are antimycin A and quinacrine.

The above interpretation of the inhibition pattern is incorporated into Figure 8, which is a schematic representation of the photosynthetic reactions and possible sites of action of these various inhibitors. This diagram represents the inhibitors of photosynthetic electron transfer as acting early in the transfer of electrons from water in the short-wavelength light reaction. The subsequent transfer of electrons from the first acceptor quinone to cytochrome allows the formation of high energy intermediates which are inhibited by the compounds *m*-Cl-CCP and ammonium ion. Coupling of ADP and inorganic phosphate to the high-energy intermediate(s) then produces ATP and this latter reaction would be inhibited by quinacrine and antimycin A.

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Enzymatic Synthesis of β-Aminoglutaramic Acid (β-Glutamine) by Glutamine Synthetase: Evidence for the Utilization of β-Aminoglutarylphosphate*

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Glutamine synthetase from sheep brain catalyzes the synthesis of β-aminoglutaramic acid (β-glutamine) from β-aminoglutarate (β-glutamate) and ammonia; when hydroxylamine is substituted for ammonia, the monohydroxamate of β-glutamate is formed. When the enzyme was incubated with adenosine diphosphate and chemically synthesized β-aminoglutarylphosphate, adenosine triphosphate was formed. The data are consistent with previous findings that indicate intermediate formation of enzyme-bound γ-carboxyl-activated glutamic acid in glutamine synthesis, and they support the hypothesis that enzyme-bound glutamylphosphate is formed in the reactions catalyzed by glutamine synthetase.

Recent investigations in this laboratory on the mechanism of the enzymatic synthesis of glutamine by glutamine synthetase led to the conclusion that this reaction involves enzyme-bound γ-carboxyl-activated glutamic acid; the available data are consistent with the intermediate formation of γ-glutamylphosphate (Krishnaswamy *et al.*, 1960, 1962; Meister *et al.*, 1962). However, studies with chemically synthesized γ-glutamylphosphate and attempts to isolate the presumed intermediate from the enzyme have thus far been hindered by the extreme lability of γ-glutamylphosphate and its considerable tendency to cyclize to yield pyrrolidone carboxylate. Both D-glutamate and L-glutamate are substrates for several of the reactions catalyzed by glutamine synthetase (Levintow and Meister, 1953; Meister, 1962), and it is therefore evident that the amino group of the substrate need not be in a specific position for enzymatic activity. The present studies were carried out in an attempt to learn whether the enzyme would act on a substrate in which the amino group was attached to the third possible position of the glutamate carbon chain, i.e., β-glutamic acid.[1] The answer to this question is of special interest because the β-aminoglutarylphosphate intermediate that might be postulated in the enzymatic synthesis of β-glutamine would be expected to be relatively stable as compared to γ-glutamylphosphate, and might therefore be useful in experiments on the mechanism of this reaction.

The present communication describes the enzymatic synthesis of β-glutamine and the corresponding hydroxamate from β-glutamate. We have also found that glutamine synthetase catalyzes the synthesis of adenosine triphosphate from adenosine diphosphate and chemically synthesized β-aminoglutarylphosphate.

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[1] In this paper, the trivial terms β-glutamic acid and β-glutamine are used for β-aminoglutaric acid and β-aminoglutaramic acid, respectively.

Experimental

Materials.—Glutamine synthetase was isolated from sheep brain as described previously (Pamiljans *et al.*, 1962). Adenosine [8-^{14}C]diphosphate was purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y. Inorganic [^{32}P] phosphate was obtained from Atomic Energy of Canada, Ltd., Ottawa. Sodium phosphoenolpyruvate was a product of the Sigma Chemical Co., St. Louis, Mo., and crystalline pyruvate kinase was obtained from California Corp. for Biochemical Research. Diethyl glutaconate was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. The other compounds used in these studies were obtained as previously described (Pamiljans *et al.*, 1962; Krishnaswamy *et al.*, 1962).