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THE EFFECT OF ULTRAVIOLET LIGHT ON PHOTOPHOSPHORYLATION AND THE HILL REACTION

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SUMMARY

1. Photophosphorylation and Hill-reaction activity of swiss-chard chloroplasts or chloroplast fragments were decreased by irradiation with short-wavelength ultraviolet light.

2. Photophosphorylation was more resistant to irradiation than the Hill reaction.

3. The presence of ascorbate during irradiation prevented the decrease in Hill reaction and photophosphorylative activity. Protection was more pronounced for the Hill reaction than for photophosphorylative activity. Compounds containing SH-groups could not replace ascorbate; it is suggested that ascorbate protects mostly by reducing the effective ultraviolet intensity, and in addition by exerting a specific effect on the chloroplasts themselves.

4. By ultraviolet irradiation, it was possible to obtain chloroplast preparations which had lost the ability to perform the Hill reaction, but still possessed high photophosphorylative activity. Such preparations may be useful in further research.

5. The endogeneous plastoquinone of chloroplasts was shown to be destroyed by irradiation.

6. The activity of irradiated chloroplasts was stimulated by the addition of plastoquinone. However, a similar stimulation was obtained on addition of plastoquinone to non-irradiated chloroplasts.

INTRODUCTION

Short-wavelength ultraviolet light (2537 Å) has been shown to strongly inhibit the Hill reaction of isolated chloroplasts[1]. However, the chlorophyll of the chloroplasts showed no changes in absorption after irradiation, nor was the activity of catalase, polyphenol oxidase or cytochrome oxidase seriously decreased. The component whose absorption was responsible for the inhibitory effect have not yet been identified.

In studies of the effect of far- and near-ultraviolet irradiation on mitochondria[2,3] and bacterial systems[4], evidence has been obtained for the participation of naphthoquinones (vitamin K-like compounds) in the phosphorylative pathway of oxidative phosphorylation.

A few years ago, a benzoquinone called plastoquinone, with maximum absorption at about 255 mμ, was isolated from plant material[5] and shown to be localized in the chloroplasts. It was found to restore Hill-reaction activity[6] and phosphorylative activity[7] of chloroplasts which had previously been extracted with organic solvents.

This report deals with the effect of ultraviolet light on photophosphorylation. The inhibition of the Hill reaction by ultraviolet irradiation was confirmed, and an attempt was made to correlate the effects of ultraviolet radiation with the presence of plastoquinone in chloroplasts.

METHODS

Once-washed chloroplasts from swiss-chard leaves were prepared as previously described[8]. In order to prepare ascorbate-free chloroplast preparations the following modifications were introduced: The pellet obtained in the first centrifugation at 1000 × *g* was suspended in chilled washing medium (0.04 M sucrose, 0.05 M Tris–HCl, 0.01 N NaCl, pH 7.8). The final pellet was suspended in an appropriate volume of washing medium or 0.1 M sucrose solution.

Irradiation procedure

(*a*) *For small volumes*: 0.5 ml once-washed chloroplast suspensions containing approx. 200 μg chlorophyll per ml were placed in 2-mm internal-width Silica cells and irradiated with "Mineralight-Model R-51" ultraviolet lamp (Ultra-Violet Products, Inc. San Gabriel, Calif., U.S.A.) at 1 cm distance from the lamp in a cold room (4°) for the time indicated. When the protection provided by ascorbate against irradiation was tested, a second 2-mm Silica cell containing water or a solution of ascorbate was placed between the cell containing the chloroplasts and the ultraviolet lamp.

(*b*) *For large volumes*: 2.5 ml of once-washed chloroplast suspensions containing approx. 200 μg chlorophyll per ml were placed in open Petri dishes, 2 cm in dia. The Petri dishes were inserted in the holes of an aluminum plate fixed in an ice bucket packed with crushed ice to keep the suspension cold during irradiation. The ice bucket was placed on an Eberbach reciprocating shaker which was operated at around 100 rev./min. The ultraviolet lamp was placed at a distance of about 4 cm from the suspension and the samples irradiated for the time indicated.

Washing procedure

To wash out compounds present with the chloroplasts during irradiation two alternative treatments were used.

(*a*) *Repeated washing*: 35 ml of the washing medium were added to the irradiated once-washed chloroplast suspensions which were then centrifuged at 20000 × *g* for 5 min. The pellet obtained was resuspended and centrifuged as above and the final pellet suspended in a small volume of the washing medium.

(*b*) *Preparation of chloroplast fragments*: The irradiated once-washed chloroplast suspensions were centrifuged at 20000 × *g* for 5 min. They were then fragmented by suspension in 10^{-3} M Tris buffer as previously described[8]. The final pellet was suspended in a small volume of washing medium.

Lyophilization procedure

After irradiation, 1 ml of once-washed chloroplasts in 0.1 M sucrose medium was deposited on the inner wall of a 50-ml centrifuge glass tube, by rotating the tube in liquid air, and immediately attached to a lyophilizer. Pressures of 10^{-2} mm Hg or less were maintained throughout a drying period of about 3 h. The samples were then stored overnight over P_2O_5 in darkness at $-15°$ *in vacuo*.

Plastoquinone extraction and addition procedures

25 ml of spectral-grade isooctane were added to each tube containing the dry chloroplastic material, ground with a glass plunger several times during a 15-min period at room temperature followed by centrifugation at 500 × *g* for 3 min. The extracts were decanted and 2 ml of spectral-grade isooctane containing the desired amount of plastoquinone added. The pellet was ground and the solvent evaporated with a rotating flash evaporator connected to an oil vacuum pump through a liquid-air trap.

Extracted chloroplasts were prepared by the same procedure except that 2 ml of spectral-grade isooctane were added in the final step. To non-extracted chloroplasts only the final 2.0 ml of isooctane were added without previous extraction.

Crystalline plastoquinone was prepared from isolated chloroplasts of swiss chard according to the procedure described by CRANE[5].

Photophosphorylative activity was assayed as previously described[9], and Hill-reaction activity with dichlorophenolindophenol as oxidant according to JAGENDORF[10]. Chlorophyll was determined according to ARNON[11].

RESULTS

Irradiation effects on photophosphorylation and the Hill reaction

The effect of ultraviolet irradiation on photophosphorylation and the Hill reaction of swiss-chard chloroplasts is shown in Fig. 1. It is evident that the Hill reaction was more sensitive to ultraviolet light than the phosphorylative activity. The inhibition of photophosphorylation was not due to enhanced ATPase activity since no increase in ATP hydrolysis was found in the light or in the dark after irradiation causing 50% inactivation of photophosphorylation. On the contrary, the small ATPase activity normally found[12] was also reduced by ultraviolet irradiation.

The difference in sensitivity to ultraviolet irradiation of photophosphorylation and the Hill reaction did not significantly change when lower intensities of ultraviolet light were used. The extent of inactivation attained varied with different preparations but the pattern of inactivation illustrated in Fig. 1 remained unchanged. The

inactivation time required to cause 50% inactivation of different chloroplast preparations was around 10 min for the Hill reaction and 30 min or more for photophosphorylation. However, chloroplast fragments were much more sensitive to ultraviolet irradiation. 50% inactivation was attained after only 1–2 min for the Hill reaction and after 5–10 min for photophosphorylation.

Effect of ascorbic acid

In the above experiments ascorbate was omitted from the medium used for washing and suspending the chloroplasts because of its absorption in the ultraviolet region and its oxidation–reduction properties. Fig. 2 illustrates the inactivation observed when the chloroplast suspension contained ascorbate during irradiation. Photophosphorylative activity was protected to a small extent. By contrast the Hill reaction was more sensitive to irradiation in the absence of ascorbate than in its presence. However, it was essential that the ascorbate be present during irradiation in order to prevent rapid inactivation. The addition of ascorbate immediately after irradiation did not restore the activity lost.

The protective action of ascorbate could have been due (a) to its reductive capacity, (b) to a lowering of the effective ultraviolet light reaching the chloroplasts (*i.e.* screening) due to the high absorption of ascorbate in the ultraviolet, and (c) to a direct effect on the chloroplasts, causing them to become more resistant to irradiation.

The first possibility was tested by replacing ascorbate with compounds containing SH-groups. Table I illustrates the results obtained with cysteine, cysteamine and glutathione. These compounds did not provide protection.

TABLE I

PROTECTION OF CHLOROPLASTS AGAINST INACTIVATION BY ULTRAVIOLET LIGHT WITH REDUCING COMPOUNDS

Once-washed chloroplasts containing 150 μg chlorophyll per ml and freshly prepared, neutralized solutions of the various compounds indicated in a final concentration of $2 \cdot 10^{-2}$ M, were irradiated for 10 min as described under METHODS (*a*). Assays as described under FIG. 1, except that the added compounds were washed out as described under METHODS and the thrice-washed chloroplasts obtained were added to reaction mixtures to give a concentration of 20–30 μg chlorophyll per flask. Non-irradiated chloroplasts (control) values: for photophosphorylation between 300–400 μmoles ATP formed per mg chlorophyll per h; for Hill reaction between 60–130 μequiv of dichlorophenolindophenol reduced per mg chlorophyll per h.

Compound present during irradiation	*% control activity*	
	Photophosphorylation	*Hill reaction*
None	53	27
Ascorbate	111	104
Cysteine	59	37
Cysteamine	25	10
Glutathione	44	30

If the protection by ascorbate was due only to screening, similar protection should have been obtained whether ascorbate was present in the irradiated sample together with the chloroplasts (internally), or between the sample and the light source

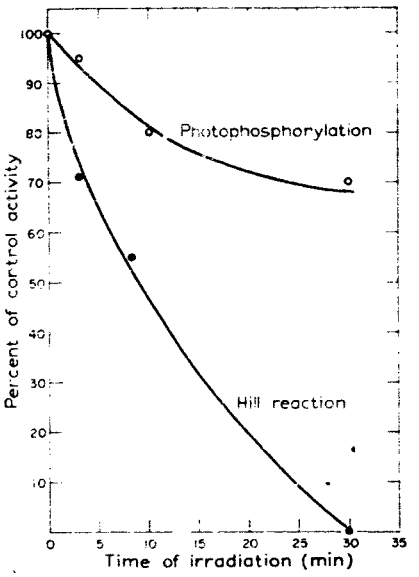

Fig. 1. Inhibition of photophosphorylation and Hill-reaction activities by irradiating chloroplasts with ultraviolet light (2537 Å) in the absence of ascorbate. Once-washed chloroplasts containing 136 μg chlorophyll per ml were irradiated as described under METHODS (*a*). The reaction mixture for the photophosphorylation assay contained the following components in μmoles: Tris–HCl (pH 7.8), 45; NaCl, 60; $MgCl_2$, 12; sodium ascorbate, 30; ADP (pH 7.8), 4; sodium–potassium phosphate (pH 7.8) 12 (containing $2 \cdot 10^7$ counts/min of ^{32}P); phenazine methosulphate, 0.09 and chloroplast fragments prepared as described under METHODS, containing between 14–19 μg chlorophyll in a total volume of 3 ml. Reaction time, 2 min; light intensity, 120000 lux; temperature, 15°; gas phase, air. Perchloric acid was added to a final concentration of 3%, the reaction mixtures centrifuged, and the supernatant analyzed for its $[^{32}P]$ATP content as already described[9]. The reaction mixture for the Hill-reaction assay contained, in μmoles: dichlorophenolindophenol, 0.075; sodium–potassium phosphate (pH 7.8), 100; and chloroplast fragments containing between 14–19 μg chlorophyll in a total volume of 3 ml. Reaction time, periods of 15 sec; light intensity, 60000 lux; temperature, 22°; gas phase, air. Absorption at 610 mμ was measured before and after exposure to light in a Unicam SP-600 spectrophotometer, and rates calculated from the initial linear part of the curve. 100 on the ordinate corresponds to a photophosphorylative activity of 526 μmoles ATP formed per mg chlorophyll per h, and to a Hill-reaction activity of 304 μequiv of dichlorophenolindophenol reduced per mg chlorophyll per h.

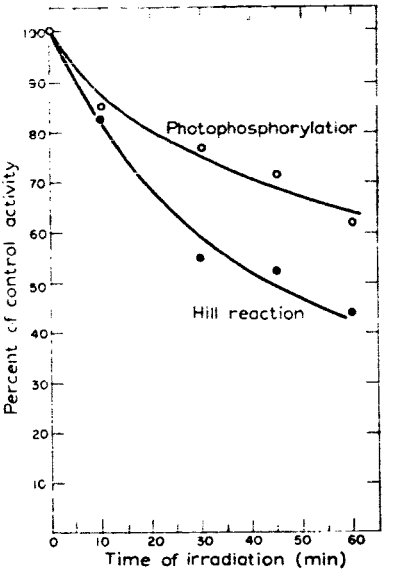

Fig. 2. Inhibition of photophosphorylation and Hill-reaction activities by irradiating chloroplasts with ultraviolet light (2537 Å) in the presence of ascorbate. Once-washed chloroplasts containing 127 μg chlorophyll per ml and sodium ascorbate at a concentration of $2 \cdot 10^{-2}$ M were irradiated as described under METHODS (*a*). Assays as described under Fig. 1, except that chloroplast fragments containing between 10–13 μg chlorophyll were added to each flask. Control values: for photophosphorylation 860 μmoles ATP formed per mg chlorophyll per h; and for Hill reaction 190 μequiv of dichlorophenolindophenol reduced per mg chlorophyll per h.

(externally). As can be seen from Table II it appears that a large portion of the protection provided by ascorbate could be obtained externally at a concentration equal to that used internally. However, it is difficult to calculate the internal concentration of ascorbate which will provide protection, only by ultraviolet absorption, to an extent equal to that obtained with a given concentration tested externally. It is obvious that the radiation reaching the chloroplasts is higher for a given internal ascorbate concentration than when the same concentration of ascorbate is used

TABLE II

COMPARISON OF THE PROTECTION PROVIDED BY ASCORBATE WHEN PRESENT TOGETHER WITH OR EXTERNAL TO THE CHLOROPLASTS

Once-washed chloroplasts containing 164–175 μg chlorophyll per ml and 0.02 M sodium ascorbate where indicated, were irradiated for 15 min as described under METHODS (*a*). Assays as described under Fig. 1 except that thrice-washed chloroplasts containing 16–22 μg chlorophyll were added to each flask; non-irradiated chloroplasts (control) values: for photophosphorylation 510–750 μmoles ATP formed per mg chlorophyll per h; for Hill reaction between 110–120 μequiv of dichlorophenolindophenol reduced per mg chlorophyll per h.

Compounds present during irradiation		*% control activity*	
Internally	*Externally*	*Photophosphorylation*	*Hill reaction*
None	Water	52	0
None	Ascorbate	85	87
Ascorbate	Water	90	98

externally. Therefore, the fact that even when the same concentration of ascorbate was used internally and externally, internal protection was more efficient, could be explained by postulating that ascorbate exerts a direct effect on the chloroplasts themselves over and above protection by absorption of ultraviolet light.

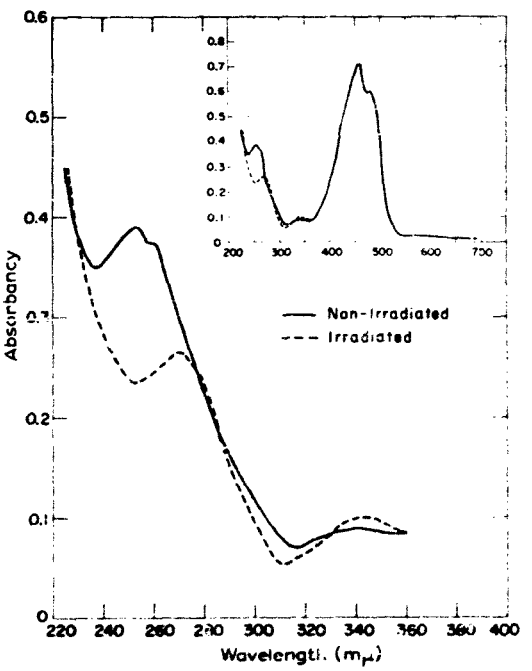

Fig. 3. Absorption spectrum of crude petroleum ether extracts of non-irradiated and irradiated chloroplasts. Once-washed chloroplasts containing 156 μg chlorophyll per ml were irradiated for 60 min as described under METHODS (*a*). After irradiation the samples were centrifuged at 20000 × *g* for 10 min. The pellets obtained were washed twice with 35 ml of cold water and centrifuged for 10 min at 20000 × *g*. The final pellets were suspended in a small volume of cold water and lyophilized. The dry chloroplasts obtained were extracted with petroleum ether (30–40°) according to BISHOP[6]. The petroleum ether extracts obtained were evaporated to dryness under reduced pressure. The residues were re-dissolved in absolute ethanol and their absorption spectrum recorded in a Beckman DK spectrophotometer.

Correlation of inhibition by ultraviolet irradiation with plastoquinone

The absorption spectrum of petroleum ether extracts of non-irradiated dry chloroplasts is illustrated in Fig. 3. On addition of a few crystals of KBH_4 the peak at 254 mμ disappeared and simultaneously a new peak with lower absorbancy at

about 290 mμ appeared. This change corresponds to the reduction of plastoquinone as previously described[5]. Petroleum ether extracts of dry chloroplasts which had been irradiated for 60 min and were almost totally inhibited gave a different spectrum in the ultraviolet region (Fig. 3). Peak absorption was at about 270 mμ and remained unchanged after the addition of KBH_4 or subsequent addition of Ag_2O. These results were interpreted as indicating destruction of the plastoquinone present in the chloroplasts by irradiation with ultraviolet light.

Acting on the assumption that inhibition of the photoreactions of the chloroplasts was due chiefly to a destruction of their plastoquinone, we tried to reactivate the irradiated chloroplasts by addition of crystalline plastoquinone. Table III shows the results of a few typical experiments in which plastoquinone was added to irradiated or irradiated and extracted chloroplast preparations. Considerable variation occurred between experiments. However, it was observed in several experiments that the addition of plastoquinone restored part of the inhibition brought about by irradiation or extraction of the chloroplasts. In addition it can be seen in Table III that a considerable stimulation of non-treated dry chloroplast preparations had also occurred. This stimulation complicates the simple explanation that plastoquinone restored activity after irradiation. It is in agreement with the *per se* stimulation by plastoquinone recently reported by FRIEND AND REDFEARN[13]. The reactivation

TABLE III

RESTORATION OF ACTIVITY BY ADDITION OF PLASTOQUINONE TO IRRADIATED OR EXTRACTED CHLOROPLASTS

Once-washed chloroplasts in 0.1 M sucrose were irradiated as described under METHODS (*b*). Extraction and readdition procedures were as described in METHODS. Assays as described under Fig. 1 except that chlorophyll concentration was 30–50 μg in a total volume of 3 ml. The molar ratio of plastoquinone to chlorophyll was 0.2. *Expt. 1*: The chlorophyll concentration during irradiation was 340 μg/ml; irradiation time 30 min. The control value corresponds to 138 μmoles ATP formed per mg chlorophyll per h. *Expt. 2*: The chlorophyll concentration during irradiation was 285 μg/ml; irradiation time 30 min. Control value corresponds to 72 μmoles ATP formed per mg chlorophyll per h, and 100 μequiv of dichlorophenolindophenol reduced per mg chlorophyll per h. *Expt. 3*: Chlorophyll concentration during irradiation was 230 μg/ml; irradiation time 30 min. Control value corresponds to 113 μmoles ATP formed per mg chlorophyll per h and 110 μequiv of dichlorophenolindophenol reduced per mg chlorophyll per h.

Treatment	% *control activity*				
	Photophosphorylation			*Hill reaction*	
Expt. No.	*1*	*2*	*3*	*2**	*3**
Control (untreated)	(100)	(100)	(100)	(100)	(100)
Control plus plastoquinone	126	150	100	144	100
Extraction	59	—	—	—	—
Extraction plus plastoquinone	78	—	—	—	—
Irradiation	46	54	26	72*	40*
Irradiation plus plastoquinone	69	83	26	120*	40*
Irradiation and extraction	46	—	—	—	—
Irradiation, extraction plus plastoquinone	61	—	—	—	—

* In order to be able to measure Hill-reaction activity with irradiated samples time of irradiation was shortened to 5 min.

by addition of plastoquinone after irradiation seems to be related to the stimulation in the non-irradiated samples. When no such stimulation was observed, the irradiated samples were also unaffected.

CRANE[14] reported that the plastoquinone present in the chloroplasts could be oxidized by treatment with 0.03 M ferricyanide in the dark. Since the oxidized form of plastoquinone is the one which absorbs around 254 mμ, we checked the possibility of accelerating the destruction of the plastoquinone in the chloroplasts by its pre-oxidation. However, no faster rates of inactivation were attained by irradiation of chloroplast suspensions pre-treated with ferricyanide.

DISCUSSION

The inhibition of photosynthesis, photoreduction (with hydrogen as reductant) and the Hill reaction (with quinone as oxidant) by short-wavelength ultraviolet irradiation has been previously noted[1,15]. In this paper evidence is presented for the inclusion of the photophosphorylative activity of chloroplasts in this list. The latter activity, however, was more resistant to irradiation than the Hill reaction. By means of ultraviolet irradiation we could obtain chloroplasts which had lost their ability to perform the Hill reaction with dichlorophenolindophenol as oxidant, but still possessed a significant part of their phosphorylative activity. This separation may be useful as a tool for further research.

There is no doubt that a large portion of the protection by ascorbate was a result of its absorption of ultraviolet light. However, its higher efficiency when present with the chloroplasts, points to a specific action of ascorbate, in addition to the screening effect. The reason why the Hill reaction was protected to a larger extent than photophosphorylation is not clear. A possible explanation is that a factor which participates in the Hill reaction and is not needed or is not rate determining in the case of photophosphorylation, was strongly affected by ultraviolet light. Ascorbate may protect this factor from inactivation.

We attempted to relate the inactivation of photophosphorylation and the Hill reaction to the destruction of plastoquinone by ultraviolet irradiation. As found by BISHOP[16] a correlation could be shown between inactivation of the Hill reaction and the level of plastoquinone in the chloroplasts. However, we could not achieve a specific restoration of the irradiation-damage by addition of plastoquinone. It therefore appears that the inactivation by short-wavelength ultraviolet irradiation was due to destruction of other factors in addition to the plastoquinone.

A stimulation of non-treated chloroplasts by plastoquinone was found. The simple interpretation of extraction and readdition experiments[6,7] as well as the irradiation and readdition experiments reported herewith is complicated by this stimulation. It must be taken into consideration when assigning a possible role to plastoquinone in photophosphorylation or the Hill reaction.

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