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THE RESPONSE OF PHOTOSYNTHETIC PYRIDINE NUCLEOTIDE TO LIGHT AND SUBSTRATES

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SUMMARY

The reducing (TPN–TPNH) and oxidizing (cytochromes, ATP-formation) moieties of the photosynthetic cycle may be separately regulated but are connected via the *b*-cytochromes. The concentration of TPNH is normally 20–40 times higher than that of the single cytochromes. At illumination by white light from a projection lamp the steady state may be displaced to oxidation or reduction of TPNH owing to a number of factors, *e.g.* light intensity, O_2, N_2, CO_2, temperature, TPN-photoreductase, etc. The relative photosynthetic activity increases with rising dilution of the chloroplast suspension. Ferricyanide is more reliable than TPN for measuring the reducing activity at constant light. At illumination by electric flashes the concentration of TPN-photoreductase is not limiting. The reduction of TPN proceeds in two steps: (1) the extremely rapid reduction of an initial reductant which is probably structurally linked to chlorophyll *a*; (2) a reductase system operating at lower speed and transferring electrons from the initial reductant to TPN. The reaction velocities at flash light are several times higher than those observed at normal photosynthesis in constant light from a projector. Spinach chloroplasts and Chlorella show approximately identical activities.

INTRODUCTION

A layer of pigments (chlorophylls etc.) may be figured acting as a photoelectric generator, *viz.* emitting electrons from one side (= the negative pole) and accepting electrons from the opposite side (= the positive pole). If the poles are connected by a number of conducting systems, forming an outer circuit, the excited pigments may be continuously reduced again, thus avoiding bleaching of the pigments. This is the normal proceeding of the photosynthesis. If electrons are trapped somewhere in the cycle, serious disturbances may occur.

Recent investigations on the response of chloroplast cytochromes to light and substrates[1] have shown that the stream of electrons may be accelerated or retarded according to the chemical activity of those intermediate systems which are involved in photophosphorylation. It was also shown that the state of oxidation of the reducing and oxidizing moieties of the cycle, *viz.* TPN-linked enzymes and cytochromes respectively, is influenced by the addition of TPN or TPNH or by a change from

O_2 to N_2 (ref. 2). The state of oxidation–reduction of the photosynthetic cycle is thus liable to regulations in the dark, too.

The cyclic transference of electrons constitutes a closed circuit as long as the electrons are not trapped in products removed from the system. Formation of ATP from ADP and phosphate may go on without any loss or gain of electrons[3]. This process is primarily confined to the oxydative moiety (cytochromes) of the cycle[1,2]. Formation of TPNH from TPN on the other hand means consumption of protons from the surroundings. Removal of TPNH from the cycle, *e.g.* during synthesis of carbohydrates, thus implies a continuous supply of H^+ and a continuous trapping of the electrons coming from excited chlorophyll. Simultaneously the increase of electropositive charge at the excited pigments, noted as oxidation of the cytochromes, attracts OH^- ions, and deprives them of an electron under production of O_2. This photolysis of water is characteristic of the complete CO_2 assimilation but may else be induced by any artificial trapping of electrons as known from the Hill reaction.

An earlier part of this investigation has been devoted to the activities of the chloroplast cytochromes[1,2,4]. The present communication deals with the response of pyridine nucleotide to light. It was shown that TPN is at least three times more effective in photosynthesis than DPN. Even if it is likely to assume a transference between the two coenzymes, TPN is here assumed to be the primary substrate acceptor of photoelectrons.

EXPERIMENTAL TECHNIQUE

The spectrophotometers

Schemes of two of the recording spectrophotometers have been shown in earlier communications[5]. One of the leading principles of these instruments is the separate measurement of both reference and sample, an arrangement facilitating plotting of difference spectrograms and promoting ample control of the readings. A second

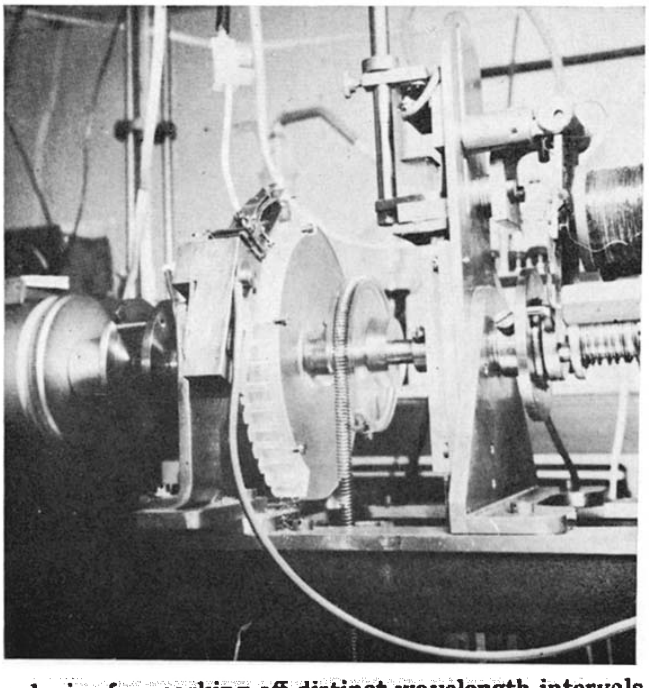

Fig. 1. The cam device for marking off distinct wavelength intervals (see Fig. 2).

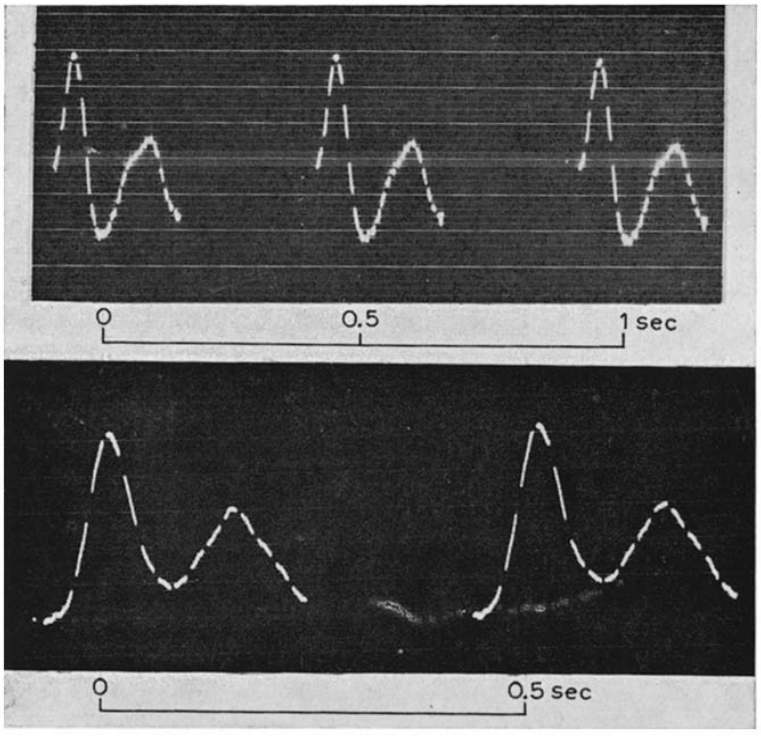

Fig. 2. Record of reduced cytochrome *c* (Sigma) at scanning in intervals of 0.5 sec and at wavelength intervals of 2 mμ (above) and 4 mμ (below). The height of the α-band in the difference spectrum $\varepsilon_{red} - \varepsilon_{ox}$ may be measured from the difference in transparency between 550 and 542 mμ.

principle is splitting up the continuous spectrum in a series of distinctly reproducible wavelength intervals (mostly 2 mμ, or else *ad libitum*), thus omitting inconveniences and errors at the quantitative valuation of a spectrum recorded as a continuous line (Fig. 1).

These instruments have been used for recording slow reactions, *e.g.* the effect of light in 10 min, or the dark effects with O_2 and N_2. For series of spectrograms in rapid succession (0.25–1 sec) a special scanning spectrophotometer was constructed. A series of wavelength intervals may be recorded at a rapid pen-recorder (for intervals

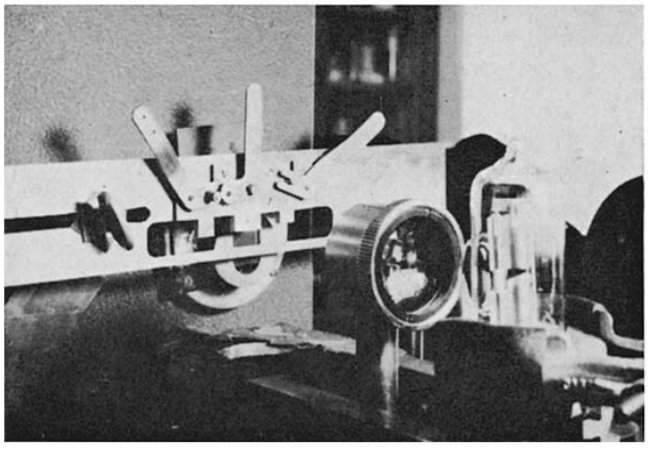

Fig. 3. A system of three shutters moving synchronously with the wavelength screw in front of the entrance slit. This is a device for eliminating large differences in transparency at different parts of the spectrum, *e.g.* at simultaneous recording of cytochromes and TPNH.

down to 6 sec) or an oscillograph (intervals down to 0.25 sec between the single spectrograms). The instrument turns the wavelength screw of a grating monochromator (Bausch and Lomb) to and along a series of preselected wavelengths. Two such instruments have been constructed, one with an electromagnetic, the other with a mechanically operating clutch which releases the grip of the uniformly moving axis on the wavelength screw and lets it by means of a spring device rapidly fall back to the start position (see Fig. 2). For eliminating large differences in transparency, *e.g.* in the chlorophyll spectrum, a frame carrying a series of adjustable shutters is synchronously sliding in front of the entrance slit (Fig. 3).

Samples, recipients and light sources

By courtesy of A. B. FINDUS in Bjuv and Dr. B. HYLMÖ spinach was cultivated during the whole year and sent to the laboratory. Grinding or homogenization were performed owing to a previously mentioned scheme (0.33 M NaCl–0.02 M Tris (pH 8–9), or alternately 0.6 M sucrose–Tris, as first medium; resuspension in NaCl–0.02 M Tris; all operations at 0°–4°).

Chlorella ellipsoidea (courtesy of Prof. H. TAMIYA, Tokyo) was cultivated in inorganic nutrient solution at 16°–18° Flat 1-l pyrex flasks were slowly shaken above a screen of strip lamps and aerated by CO_2-enriched air. For sampling the Chlorella suspension was passed through a filter of sintered glass, centrifugated and resuspended in fresh nutrient solution, or distilled water.

For illumination at long intervals (5–30 min) the samples were transferred to a large 2-mm wide cuvette (4 ml sample covering a surface of 20 cm^2), submersed in a large cuvette provided with a cooling device. Illumination was performed from a projector (2500 $W \cdot m^{-2}$). Spinach suspensions were cooled to 5°, Chlorella held at 18°. After the illumination the suspension was transferred to a 1-cm quartz cuvette for recording the difference spectrum between the original suspension, kept in darkness, and the illuminated one. For very concentrated suspensions (up to 0.2 mg chlorophyll/ml) 1- or 2-mm wide cuvettes were used.

For studying the response to light in periods less than 1 min the pre-cooled cuvettes were placed in a cooled metal frame in front of the entrance lens and the recording started just before the lamp (a 6 V 23-W Osram bulb or a H_2-lamp) was switched on (*cf.* ref. 1). For testing the thermal sensitivity of the response a special device was constructed, keeping the temperature of the cuvettes at a pre-selected figure.

For illumination by electric flashes an U-shaped Xenon lamp was placed a few millimeters in front of the cuvette, enabling the continuous light, used for the recording, to pass through the opening of the U. Flashes were as a rule administered in intervals of 4 sec, the flash turned on just in the interval between two records, *e.g.* at intervals of 1 sec one flash 0.5 sec before the record and recovery from the flash to be studied during the following 0.5, 1.5, 2.5 and 3.5 sec. The continuous light, which is indispensable for the recording, exerts of course some effect of its own, but experience shows that the response remains far below that of an optimal effect of a flash, notably if the ground light is screened off by filters, *e.g.* green for cytochromes and ultraviolet for TPNH.

Spectrophotometric determination of reduced pyridine nucleotide and added ferricyanide

Measurements of the extinction at 340 mμ without corrections are frequently

unreliable because a number of different spectral effects are induced in the region 300–370 mμ, noticeable primarily as a slope of the difference spectrum $\varepsilon_{light} - \varepsilon_{dark}$. Involved are here effects on chlorophyll, quinones, and ferredoxin[6], none of which, however, appear as narrow bands. The 340-mμ band may thus be fairly accurately measured from a baseline connecting 310 and 370 mμ. This procedure was testified by addition of TPNH to the samples. The original band at 340 mμ then increases according to the calculated values (see Fig. 4). Half of the band-height may be measured from a baseline between 320 and 360 mμ. The region between 350 and 370 mμ is, however, exposed to changes in the state of oxidation–reduction of flavoprotein, an enzyme probably present in chloroplasts (see below).

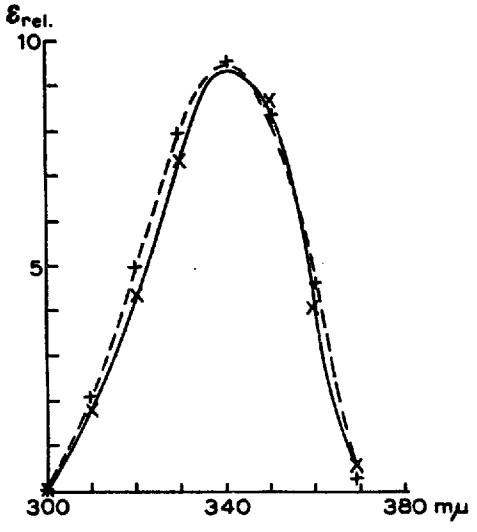

Fig. 4. The 340-mμ band of TPNH. ×—×, from a solution in phosphate (pH 8). +—+, from a suspension of spinach chloroplasts without addition. Both curves are drawn from a base-line connecting 300 and 370 mμ. They are practically identical.

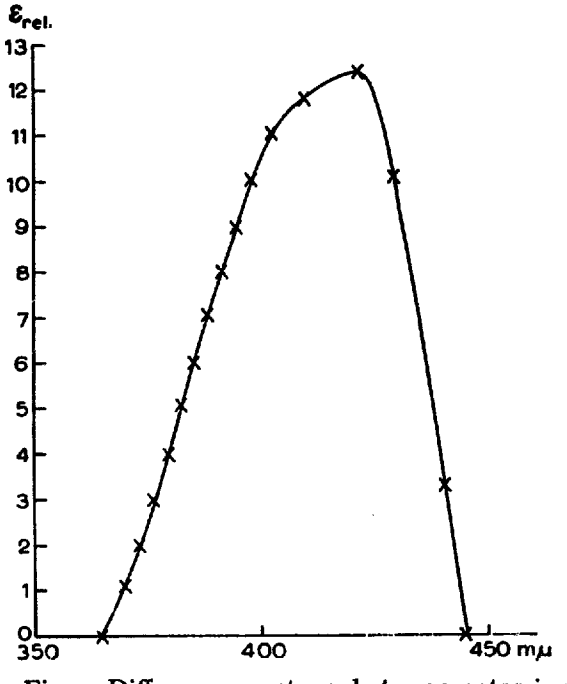

Fig. 5. Difference spectrum between potassium ferri- and ferrocyanide. Isosbestic points at 365 and 444 mμ. Peak at 422 mμ.

Measurements of the 340-mμ band are unfortunately complicated by combination of reduced pyridine nucleotide with a number of apoenzymes[7]. Best known among these are alcohol dehydrogenase (band at 325 mμ) and triosephosphate dehydrogenase (band at about 360 mμ) combining with DPNH. TPNH is known as the coenzyme of malate dehydrogenase, which is always present in chloroplasts[2]. The displacement of the peak at 340 mμ may be studied on flash spectrograms, too (see below). One advantage of absorption spectra is the fact that the total band-height is only little affected by the combinations. This is on the contrary a draw-back of fluorescence spectra[8]. According to the experience of this laboratory, absorption spectroscopy yields more reliable quantitative results than measurements of the fluorescence.

Ferricyanide was used for stimulating the O_2-production in the Warburg apparatus and for measurements of the power of light-induced reduction. Ferricyanide is

to be preferred to a number of organic Hill oxydants because it is not reoxidized by the evolved O_2. In spectrophotometric measurements of the peak at 422 mμ, corrections for changes in the background must be made. The isosbestic points between ferri- and ferrocyanide appear at 365 and 444 mμ (see Fig. 5). The molar extinction (log. I_0/I) at 422 mμ amounts to $7.38 \cdot 10^2$. Measurements may be made from a baseline connecting the isosbestic points. Because the ferrocyanide band is largely screened off by chlorophyll plus carotene the measurements must be made in the supernatent. It ought to be mentioned here, that ferricyanide shows absorption also at 340 mμ, a fact complicating simultaneous measurements of the reduced pyridine nucleotide band. In view of the frequently claimed disturbances owing to turbid suspensions it may be mentioned here that a linear relation between quantity and extinction was stated up to concentrations of at least 0.200 mg chlorophyll/ml. Higher concentrated suspensions were not used in this investigation. Quantities of TPNH added to the chloroplast suspensions show the same increase of the 340-mμ band as aqueous solutions of the same concentration.

EXPERIMENTAL RESULTS

Spinach chloroplasts

TPNH added to an aerated suspension of chloroplasts in the dark is not appreciably oxidized in the first few minutes, but 50–80 % oxidation of 1–2 mg TPNH in 4-ml suspension is mostly observed in 1 or 2 h. The oxidation is independent of a simultaneous addition of TPN and is accompanied by reduction of the chloroplast cytochromes[1]. Added TPNH is largely fixed in the chloroplast, only little remains in the medium. Measurements were performed both on green suspensions and on extracts in ethyl ether. Judging from the height of the band at 340 mμ the concentration of TPNH is fairly high. A fresh chloroplast suspension, kept in the dark, contained 55 μmoles chlorophyll and 9 μmoles reduced pyridine nucleotide (see Fig. 6). The ratio chlorophyll:reduced pyridine nucleotide amounted on the average to 6:1, the relation TPNH:cytochrome b_3 to 135:1 and the relation TPNH:total cytochrome to about 7:1 (*cf.* Table I). It is here not calculated with the quantities of TPN, the

TABLE I

QUANTITATIVE RELATIONS BETWEEN CHLOROPHYLL AND THOSE QUANTITIES OF PYRIDINE NUCLEOTIDE AND CYTOCHROMES WHICH ARE REACTING AT FLASH ILLUMINATION

Chloroplasts in 0.33 M NaCl–Tris (pH 8.2). Chlorophyll concentration 0.100 mg/ml.

	Cytochromes			*Reduced pyridine nucleotide*	*Chlorophyll*
	f	b_3	*b*		
A. Spinach					
μ moles/ml	0.0025	0.0055	0.0055	0.0200*–0.0350	0.111
Relative	1	2.6	2.2	80	420
B. Red clover					
μ moles/ml	0.0006	0.0013	0.0017	0.040	0.254
Relative	1	2.2	2.8	67	423

* Response to added 0.50 mg TPNH in the dark: oxidation by 0.20 mg = 40 %.

presence of which is shown by occasional reduction in light without extra addition of this substance. It may be inferred from the flash experiments (see below) that the quantity of TPN can amount to 20–80% of that of TPNH. As shown by the given relations only about 14% oxidation of the existing TPNH is thus sufficient

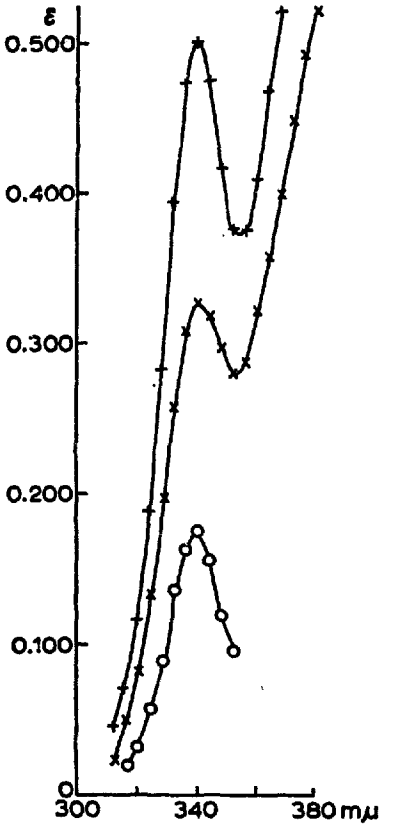

Fig. 6. Direct spectrogram of a suspension of spinach chloroplasts with distilled water as reference. Relation chlorophyll to reduced pyridine nucleotide 3 to 1. ×—×, without additions; +—+, 0.125 mg TPNH/ml added; O—O, difference between the upper two curves (+—+ *minus* ×—×).

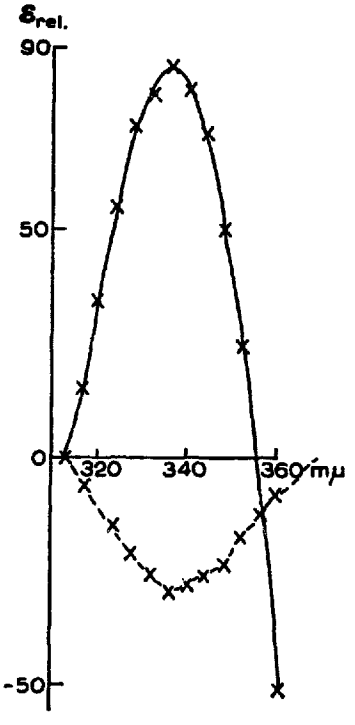

Fig. 7. Oxidation of TPNH by light. Spinach chloroplasts to which 2.5 mg TPNH and 1 mg TPN were added in the dark. ×—×, band of TPNH from the difference spectrum between sample plus TPNH and sample without addition; +—+, difference spectrum between sample plus TPNH in light during 10 min and the same sample in the dark.

for accomplishing full reduction of the cytochromes. Because of the autoxidability of cytochrome b_3 (*cf.* ref. 2) the actual figure is considerably higher and this may explain why in the presence of O_2 extraneous addition of TPNH is needed for full reduction. TPNH also keeps the chlorophyll in its dark (reduced) state. In the presence of added TPNH no bleaching of chlorophyll occurs in the light.

The response of the steady state TPN $\rightleftharpoons$ TPNH to light is dependent on various factors: on the up-hill side the light intensity and the activity of TPN-photoreductase (or ferredoxin), on the down-hill side consumption during chemosynthesis of carbohydrates, and the oxidation of the cytochrome system. Of importance is here also the autoxidability of cytochrome b_3 that via the TPNH-linked malate dehydrogenase participates in the oxidation of malate[2]. Because the reductase is soluble in water the remaining quantities are higher at homogenization in a small volume of

the medium and if washing is omitted. The response may thus vary according to the preparation.

The response to illumination by condensed light from a 250-W Osram lamp is during the first few minutes mostly an increased oxidation of reduced pyridine nucleotide, both with and without addition of TPNH (Fig. 7). This effect is frequently observed also in whole leaves and in Chlorella (see below). The oxidation is far more pronounced than in the dark, in particular on previous addition of TPNH (see Table II). At higher concentration of this substance the oxidation amounts to quantities comparable with the figures from ferricyanide reduction (see below and Table II)

TABLE II

OXIDATION OF REDUCED PYRIDINE NUCLEOTIDE BY LIGHT

Spinach chloroplasts in 0.33 M NaCl–Tris (pH 8.2). Kept in air at 5°. Projection lamp 250 W. Sample 4 ml, 20 cm^2.

Additions	*Effect of illumination during 10–15 min (μmoles/mg chlorophyll/h)*
	Oxidized TPNH
1. None	— 2.6
0.55 mg TPNH	— 5.6
2.4 mg TPNH	—20.0
	Reduced ferricyanide
2. 20 μM $K_3Fe(CN)_6$	100.0
20 μM + 2 mg TPNH	20.0

TABLE III

REDUCTION OF PYRIDINE NUCLEOTIDE IN LIGHT

Illumination by projector (250 W) at 5° during 10 min. Spinach chloroplasts in 0.33 M NaCl–Tris*.

Treatment	*μmoles reduced pyridine nucleotide/mg chlorophyll/h*
Unwashed chloroplasts	+0.35**, 1.9*** and 5.6§
Unwashed chloroplasts plus 1 mg TPN and 1 mg TPNH in 2 ml	+3.1 to +4.3
Crude homogenate (Bühler homogenizer)	+1.84 to +2.7

* The chlorophyll varied between 0.060 and 0.200 mg per ml. The power of reduction rises with the concentration of the suspension.

** Ground in a comparatively large quantity of the medium.

*** Glass homogenizer and little medium.

§ Glass homogenizer and little medium but kept in N_2.

and the production of O_2 in the Warburg apparatus (Fig. 11). The oxidation of TPNH at low reductase activity thus reflects the power of oxidation at the positive pole of the excited chlorophyll and its recovery via the cytochromes. The oxidation of TPNH in light shows that the electrons emitted from the negative pole of the chlorophyll are only incompletely caught by TPN. They are apparently accepted by other oxidants which are not so rapidly reacting with TPN as the TPN-reductase or they are accompanied by a bleaching of the chlorophyll. The experiments of SAN PIETRO[9]

and SAN PIETRO AND LANG[10] and of TURNER *et al.*[11,18] show that shortage in reductase is probably the principal cause of the primary TPNH oxidation[17,19].

Reduction of TPN in light was nevertheless observed in a number of homogenates (see Table III and Figs. 8 and 9) but the quantities remained far below the values attained at addition of reductase (*cf.* refs. 9–11). The response is also dependent on the state of oxidation–reduction before turning on the light. Addition of TPNH,

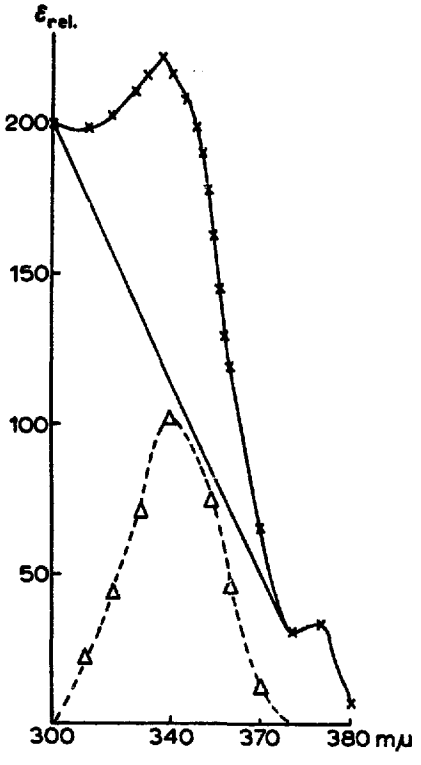

Fig. 8. A concentrated suspension of spinach chloroplasts, illuminated during 60 min. Spectrum of chloroplasts decolorized in ether, showing reduction of pyridine nucleotide. ×—×, the direct spectrum with distilled water as reference. Δ—Δ, 340-mμ band calculated from a base line between 300 and 380 mμ.

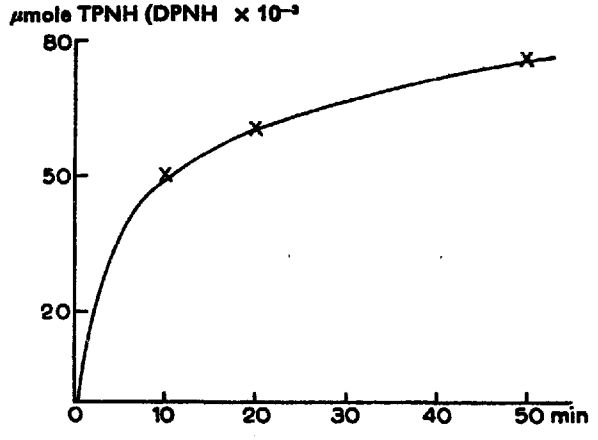

Fig. 9. Time course of the 340-mμ band after turning on illumination. Spinach was here homogenized in a small quantity of the medium, the suspension evacuated and kept in N_2. Slow scanning in intervals of 20 sec.

which according to what is said above keeps the reduction of the involved redox systems at a high level, thus promotes the reduction by light (Tables IV and V). In one experiment, in which added 0.6 mg TPNH/ml was oxidized to 85 % in the dark, 0.37 mg or more than 50 % were recovered at illumination during 20 min at 4°. A promoting effect is also observed at addition of ADP plus phosphate (Table VI).

Because so many single reactions are involved in the total cycle of electron transference, the time course frequently shows periodic fluctuations. The oxidation

TABLE IV

RESPONSE OF SPINACH CHLOROPLASTS TO LIGHT

Illumination by projector at 5°. Start content of reduced pyridine nucleotide = 0.035 μmole/ml. 0.031 mg chlorophyll/ml. Values in μmole/ml.

Treatment	*10 min light*	*25 min light*
Control	—0.002	—
Plus 2 mg TPN	—0.002	—
Plus 1 mg TPN and 2 mg TPNH	+0.014	+0.025*

* This figure corresponds to 4.3 μmoles/mg chlorophyll/h. Aging during 24 h (cold) turns the response into —0.45 μmole.

of cytochromes *f* and b_3 sets in instantaneously with a maximum in about 4–6 sec (see ref. 1), followed by a partial reduction, which is coordinated with oxidation of TPNH. The reaction between TPNH (probably via a flavoprotein) and cytochromes is thus slower than the transport of electrons in the cytochrome system.

The complexity of the steady state TPN $\rightleftharpoons$ TPNH makes the formation of TPNH less appropriate for measuring the reducing power of excited chlorophyll unless super-normal quantities of reductase are added. The reduction of ferricyanide is to be preferred for this purpose. It is only slightly (up to 5 %) reduced in the dark.

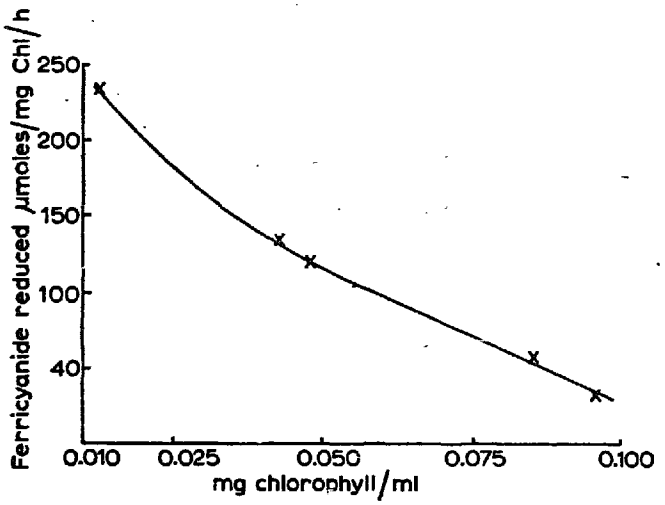

Fig. 10. Reduction of ferricyanide (20 μmoles in 4 ml) in light (condensed Osram light, 2500 $W \cdot m^{-2}$) in relation to the concentration of the suspension of spinach chloroplasts. Ferricyanide is slightly reduced in the dark (up to 5 %). The values imply the difference between samples in the dark and in light. Temperature 5°.

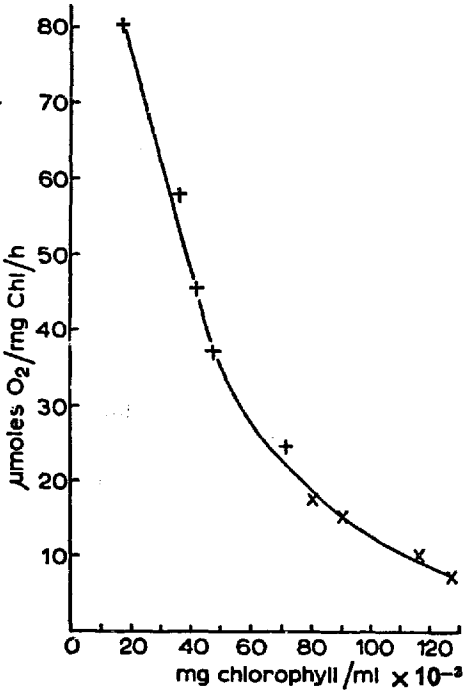

Fig. 11. O_2-production of spinach chloroplasts and *Chlorella ellipsoidea*, measured in the Warburg respirometer at 15°. The vessels, containing 2 ml suspension, were illuminated by a circular strip lamp at the bottom of the water basin in a distance from the flasks of 1–2 cm. The curve shows the inverse relation between the rate of photosynthesis and the density of the suspension. ×, spinach; +, chlorella.

A series of experiments with ferricyanide is shown in Fig. 10. The efficiency of the light is rapidly increased at dilution of the suspension, an observation also made by TURNER *et al.*[11] with TPN as oxidant. Theoretically 4 emitted electrons correspond to 1 molecule O_2 in the Hill reaction. A comparison between Figs. 10 and 11 shows that this relation is approximately reached at higher concentrations of the suspension. Ferricyanide and TPN are competing for electrons from excited chlorophyll. In one experiment, in which ferricyanide and TPN were simultaneously added, the reduction of the former was reduced to 25 % of the value shown in Fig. 10 (20 against 100 μmoles per mg chlorophyll/h, see Table II). This competition between the acceptors may explain why the reduction of ferricyanide, in particular at higher dilutions, remains at a somewhat lower value than the theoretical one.

The rapid increase of the light effect at dilution of the suspension is most probably caused by a more efficient illumination of the single cells or chloroplasts, *viz.* a decrease of the mutual screening of the particles. That very high light intensities are needed for maximum efficiency is shown by flash experiments.

The power of photoreduction of TPN decreases rapidly at ageing (Table IV). The fact that reduction of ferricyanide shows the same decline indicates the existence of a transferring factor operating very closely to the chlorophyll, a factor which is apparently not identical with TPN-photoreductase.

TABLE V

RESPONSE OF THE PEAKS AT 340 AND 350–356 mμ (DIFFERENCE SPECTRUM WITH DARK SUSPENSIONS AS REFERENCE) TO ILLUMINATION

Chlorophyll concentration 0.031 mg/ml (projector 10–15 min).

Light	*340 mμ*	*350–356 mμ*
A. Without additions		
10 min	−0.0011	+0.0020
40 min	−0.0025	+0.0030
85 min	−0.0103	+0.0058
B. Addition of 0.5 μmole ADP + 2 μmoles K_2HPO_4		
10 min	+0.0020	±0.0000
40 min	+0.0020	+0.0027
85 min	±0.0000	+0.0075
C. Addition of 0.5 μmole TPN		
10 min	−0.0005	±0.0000
40 min	−0.0053	+0.0053
85 min	−0.0030	+0.0030

TABLE VI

EFFECT OF FLASHES ON SPINACH CHLOROPLASTS

Average of 5 flashes in intervals of 4 sec (4 records). 1- or 2-mm wide quartz cuvettes. Continuous light green or hydrogen lamp. Values in μmoles/ml.

Conditions	*Cytochromes*		*Reduced pyridine nucleotide*	*Chlorophyll*
	f	*b*		
After *minus* before flash	−0.000 25	−0.000 40	+0.0180	0.100
Relative change	−1	−1.6	+72*	400
1.5 sec after flash *minus* 0.5 sec after flash	+0.000 25	+0.000 40	+0.0227	
Relative change	+1	+1.6	+91*	400

* These quantities are together corresponding to a reaction velocity that is 15–20 times higher than the maximum of O_2-production and reduction of ferricyanide (see Figs. 10 and 11) at continuous illumination with the projection lamp during 10–30 min.

The observations quoted in Figs. 8 and 9 are further elucidated by flash experiments, in which, owing to the extremely high intensity of each flash, it is possible to study a maximum response even in comparatively concentrated suspensions and also learn something of the reaction velocities (Figs. 12 and 13).

As shown in Table VI each flash brings the cytochromes *f* and b_3 (+*b*) to full oxidation. The oxidation lasts about 1 sec and is completely recovered in the following second (green light). Corresponding figures from the simultaneous effect at 340 mμ

are shown in Table VI (hydrogen lamp). Quantities of TPNH 70–90 times higher than those of cytochrome *f* and amounting to 20–80 % of the initial quantity are formed in 0.5 sec and disappear again in another second. Because the involved quantities of TPN–TPNH far exceed those of the cytochromes it must be concluded that the recovery is primarily a return to the steady state TPN $\rightleftharpoons$ TPNH existing during the

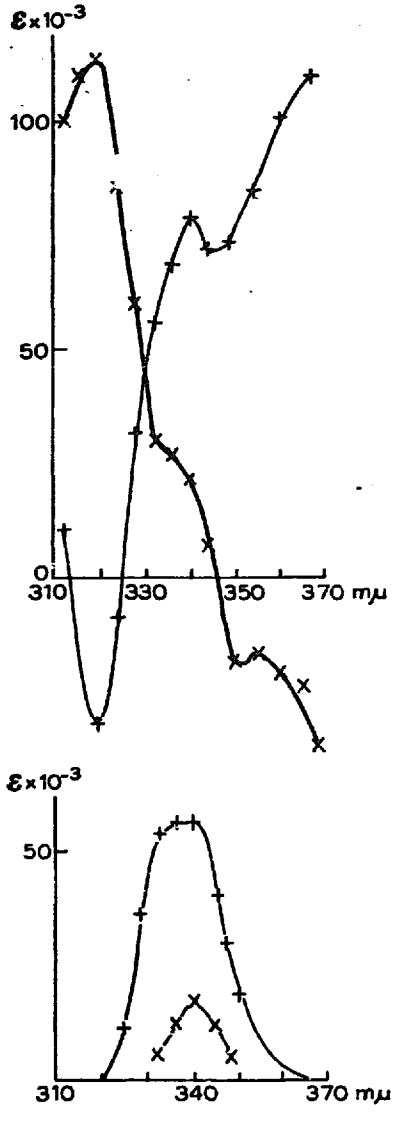

Fig. 12. Flash experiment with spinach chloroplasts. Above: ×—×, difference between after and before flash, showing the sudden increase in extinction at 340 mμ; +—+, difference between 1.5 sec and 0.5 sec after flash, showing that reduction still continues. Below: corresponding curves calculated from the base-line between 320 and 360 mμ.

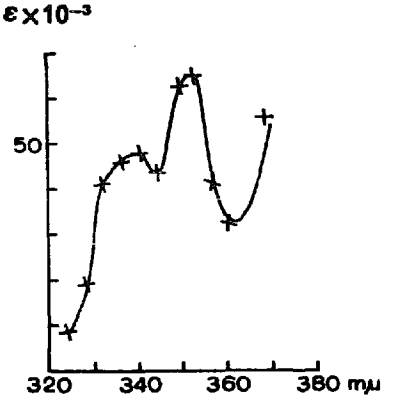

Fig. 13. Flash experiment with spinach chloroplasts. The curve shows the effect proceeding between 1.5 and 0.5 sec after the flash, *viz.* the still increasing reduction at 340 and 350 mμ.

continuous illumination. The velocities are, however, at least 100 % higher than at illumination with white light from the projector. We know that the velocity of electron transference through the cycle is regulated by a number of factors, *e.g.* the intensity of phosphorylation (see refs. 1, 3). But there are also other points of velocity control. In one experiment the recovery from a flash was studied in suspensions aerated by air, N_2, or CO_2. The recovery in air and N_2 amounted to only 57–58 % in 1 sec, whereas CO_2 promoted 90–100 % recovery. CO_2 accelerates the dark reactions of chemosynthesis, in which TPNH is oxidized and would be expected to accelerate the recovery of TPN. Addition of ATP had no effect on the recovery.

It may be emphasized here that in this experiment only 57–58 % TPN was recovered in 1 sec, whereas in Table VI the recovery was complete in this time.

Such a lasting effect was, as a matter of fact, frequently observed both in spinach and Chlorella (see below) and it depends obviously on a prolonged dark reaction following the flash. The experiments clearly show the existence of an initial light effect, in which the electrons emitted from the excited chlorophyll are charging an initial acceptor. This is an extremely rapid process, because the flash lasts only 0.3–0.5 msec. The initially created "power of reduction" is then, via reductases more slowly transferred to TPN. As shown in Table VI and Figs. 12 and 13 the reduction of TPN proceeds with a velocity of 0.004–0.006 μmole/ml in 0.5 sec. This time course may also explain the delay of recovery dealt with above. The velocity exceeds, however, even in this case the values observed at "normal" exposure during 10–15 min.

Chlorella ellipsoidea

Suspensions of Chlorella show an absorption spectrum in ultraviolet very similar to that of spinach chloroplasts. The peak at 340 mμ must also here be partly attributed to reduced pyridine nucleotide. The relative concentration of reduced pyridine nucleotide is on the average 3–5 times higher than in spinach, the quotient chlorophyll:reduced pyridine nucleotide amounting to about 2. As shown in Fig. 11 the specific power of photosynthesis, measured as O_2-production in the Warburg apparatus, is practically identical with that of spinach chloroplasts.

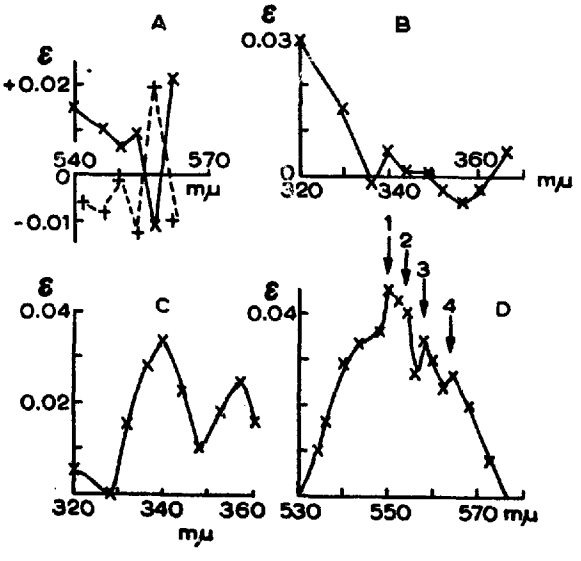

Fig. 14. Spectrograms of *Chlorella ellipsoidea*. A. Flash experiments: ×—×, difference between after and before flash (0.5 sec), showing response of cytochrome b_3 (558 mμ); +– – +, recovery in 1 sec after flash. B. Effect of a flash on samples aerated with CO_2. Peak at 340 mμ corresponding to reduced pyridine nucleotide. C. Peaks at 340 and 356 mμ, appearing as the difference between samples evacuated in N_2 and samples aerated by CO_2. D. Difference spectrum between suspensions evacuated in N_2 and aerated by O_2, showing Peaks 1, 2, 3 and 4, corresponding to the cytochromes *c*, *f*, b_3 and *b* (b_6) of higher plants.

All attempts to remove the chlorophyll from the Chlorella cells without damaging the other pigments have so far been insuccessful. The presence and concentration of cytochromes was consequently hitherto studied on green cells. Difference spectrograms between suspensions aerated with O_2 or N_2 show peaks at 550, 558, and 564–566 mμ, corresponding to the cytochromes *c*, b_3, and *b* of higher plants, whereas the peak at 554 mμ, corresponding to cytochrome *f* is less pronounced (Fig. 14). The calculated relation chlorophyll:cytochrome *c* amounts to about 40, a figure indicating a much higher concentration of cytochromes than usually found in higher organisms.

Flashes induce strong oxidation at 558 and 566 mμ (Fig. 14A), followed by rapid recovery. The recovery is mostly complete in 1 sec but sometimes extended to 2 or 3 sec.

Chlorella shows at reduction a negative band at about 560–570 mμ which may possibly correspond to a flavoprotein.

The response of the bands at 554 and 558 mμ (difference spectrum with 546 mμ as reference) to white light (same arrangement as previously used; *cf.* ref. 1) is shown in Fig. 15. The cytochromes are strongly oxidized in the first 1–2 sec, in 5–10 sec shifting over to a passing reduction and again returning to a more steady state. This is about the same as earlier observed on spinach chloroplasts.

The response of the reduced pyridine nucleotide band at 340 mμ, too, is very similar to that of isolated chloroplasts. The steady state in the dark may be strongly influenced by a change from an evacuated state in N_2 to aeration by CO_2, as shown in Fig. 14. CO_2 removes reduced pyridine nucleotide, obviously by stimulation of carbohydrate formation in the dark. In Fig. 14C 25 % of the quantity of reduced pyridine nucleotide stored in dark N_2 were removed by dark CO_2. At illumination the quantity of reduced pyridine nucleotide that is protected by an atmosphere of N_2 is rapidly decreasing, apparently because of the simultaneous oxidation of the cytochromes. The response of normally aerated Chlorella to light is, however, frequently a sudden increase of reduced pyridine nucleotide. The band at 340 mμ appearing in the first 4 sec may change into a band at about 344 mμ, probably owing to enzyme linkage. Suspensions aerated by N_2 preferably show an initial negative response at 340 mμ (Fig. 16).

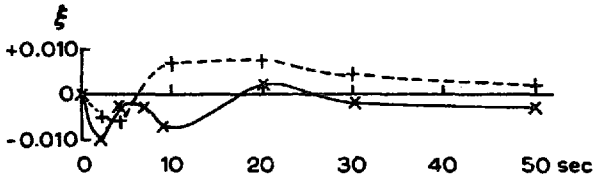

Fig. 15. Chlorella. Response of the peaks at 554 mμ (+ – – – +, reference 546 mμ) and at 558 mμ (×—×, reference 546 mμ) to illumination by condensed white light. Scanning in intervals of 4 sec.

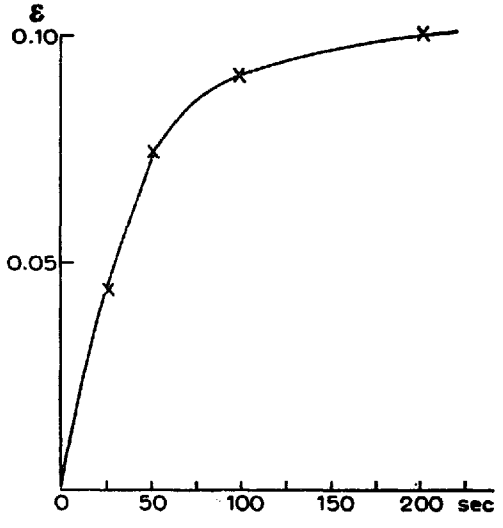

Fig. 16. Chlorella in N_2. Response of the peak at 340 mμ (reference 312 mμ) to light from the hydrogen lamp plus white light. Slow scanning (25-sec intervals).

The response of Chlorella to flashes is characterized mainly by a strong initial oxidation of cytochrome b_3 (558 mμ), recovered in 1–3 sec. The results from one experiment are shown in Table VII. The quantities of reduced pyridine nucleotide formed in 0.5 sec correspond to about 20 % of the already present quantities and the velocity of the reaction is several times higher than at continuous illumination by the projector. The corresponding negative effect of flashes on the cytochromes of Chlorella is shown in Table VII.

As shown in Table VII the formation of reduced pyridine nucleotide frequently continues 1–2.5 sec after the flash. This phenomenon was also observed in spinach chloroplasts. It was assumed that the flash extremely rapidly reduces an initial electron acceptor and keeps this "power of reduction" during a period of 1–2 sec. Reduction

of pyridine nucleotide constitutes a second step. This reaction proceeds much slower but at flash light nevertheless far more rapidly than at "normal" photosynthesis. The effect of flashes is without exception positive, *viz.* the up-hill reactions are always dominating. At lower or medium light intensities the response may be negative or positive, owing to the relatively larger influence of the down-hill reactions, *e.g.* substrate factors, and the temperature (*cf.* Table VIII).

TABLE VII

FLASH EXPERIMENTS WITH *Chlorella ellipsoidea* IN INORGANIC NUTRIENT SOLUTION

Temperature 19°. 1- or 2-mm wide quartz cuvettes. Average of 5 flashes.

	0.5 sec before flash	*After flash*		
		0.5 sec	*1.5 sec*	*2.5 sec*
μmoles reduced pyridine nucleotide/ml	0.0132	0.015 70	0.014 4	0.0132
Difference	±0	+0.002 50*	+0.001 2	±0.0000
μmoles oxidized (—) or reduced (+) cytochrome *b*/ml		—0.000 06	+0.000 06	

* This value corresponds to an about 6 times higher velocity than the maximum photosynthesis at 10–30-min continuous illumination by a strip lamp (distance from the Warburg vessels 1–2 cm). The back-reaction (disappearance of reduced pyridine nucleotide) runs about 4 times slower. Relation between reduced pyridine nucleotide and each cytochrome (assumed to be *c*, b_3, and *b*) about 40 to 1. Relation between chlorophyll and total reduced pyridine nucleotide about 2 to 1.

TABLE VIII

INFLUENCE OF THE TEMPERATURE ON THE STEADY STATE PYRIDINE NUCLEOTIDE ⇋ REDUCED PYRIDINE NUCLEOTIDE

Height of the 340-mμ band in the difference spectrum $\varepsilon_{light} - \varepsilon_{dark}$. *Chlorella ellipsoida.*

Light	*18°*	*5.5°*
10 min	—0.021	—0.013
20 min	—0.023	±0.000
35 min	—	+0.009

DISCUSSION

Normal photosynthesis is characterized by a steady state in which the electrons emitted from one side of the pigment complex are compensated by an equivalent quantity of electrons returning to another side of it. It is justified to speak of two poles of the pigment complex. The electrons expelled at the negative pole are reducing TPN, the electrons returning at the positive pole are oxidizing the cytochromes *f*, b_3, and *b* (b_6). The flash experiments prove the existence of an efficient path of electron transference connecting the reducing and oxidizing moieties. This is the cyclic process (*cf.* ref. 1). The "water bridge" (*cf.* ref. 1) of the cycle compensates for electrons and protons trapped in the "path of hydrogen" (*cf.* ref. 12) that via TPNH goes to carbohydrate formation. The electrons consumed in this process are at the oxidizing moiety of the cycle compensated by an equivalent deionization of OH^- to O_2 and H_2O.

At undisturbed circulation of electrons the photoactive pigments are maintaining their normal color. At local changes in the conductivity of the cycle the state of oxidation–reduction of the participating enzymes is raised or lowered and the chlorophyll pigments may be exposed to bleaching. Unless the photosynthetic apparatus is suffering from bleaching such experiments cannot last long. As shown in a previous investigation[1] variations in the conductivity of the oxidizing moiety (cytochromes) may be brought about by variations in the intensity of phosphorylation (upon addition of ADP or ATP) or in the supply of electrons from the reducing moiety (TPN–TPNH).

The present investigation is primarily focussed on the reactions regulating the steady state of oxidized and reduced pyridine nucleotide. Provided free access to protons (eventually by photolysis of water) the up-hill side of the state is dominated by the light intensity and the systems reducing TPN. The experiments show that extraneous oxidants, *e.g.* ferricyanide, may competitively trap electrons from the initial reductant (Table II). Because reduced ferricyanide is not, or only to a small extent, reoxidized by the aerated chloroplast this oxidant gives a better measure of the reducing power than TPN because of the involvement of TPNH in a number of down-hill reactions. Among these versatile down-hill reactions the regulation of the oxidation–reduction of the cytochrome chain is an indispensable complement to the reduction (see above) a circumstance which may, at absence of substrate, lead to an idling steady state. Other down-hill reactions are brought into action by substrates reacting with TPNH, *e.g.* in the dark chemosynthesis, in oxidation of malate *etc.* Such reactions may cause oxidation of reduced pyridine nucleotide in the dark or in light. The dominating reduction of TPN upon flash light is obviously caused by quantitative differences between the reducing and oxidizing moieties of the cycle.

The experiments, notably those with flashes, show the very rapid crossing of the water bridge, *viz.* photolytic activation of the paths of H^- and OH^- (the former ones together with electrons reducing TPN), noticed as a rapid reduction of cytochrome *f* immediately upon its extremely rapid initial oxidation (Table VI). But the capacity of the cytochrome system, as compared with the quantitative dominance of TPN, is apparently limited.

At more "normal" light intensities, shortage in reductase and/or interference of other oxidants the return of electrons through the cytochrome chain is sufficiently rapid to cause a passing wave of TPNH oxidation (Tables II, IV, V). The experiments with Chlorella teach us that this shortage in reducing power may exist independently of a washing out of reductase if quantities of TPNH (and DPNH) are simultaneously oxidized in the dark chemosynthesis.

The following conclusions may be inferred from the flash experiments: (1) The concentration of TPN-photoreductase is not a limiting factor at extremely high light intensities; (2) the reduction proceeds in two steps, an initial very rapid (< 0.3–0.5 msec) building up of a reducing power and a slower (1–2 sec) proceeding transference of this power to TPN. The velocity relation between the two reactions amounts to 100/1 or more.

Assuming identity between the TPN-photoreductase and ferredoxin (*cf.* ref. 6) this enzyme may operate next to TPN. Its concentration amounts according to TAGAWA AND ARNON only to 0.25 % of that of the chlorophyll, *viz.* about the same as cytochrome *f* and it is consequently very unlikely that this enzyme could act as

initial reductant, the capacity of which amounts to at least 10–20 times higher molar values. The flash experiments clearly show, too, that the reaction velocity is of a much higher order of magnitude. The initial reductant appears to attain about a concentration close to that of chlorophyll *a*. Because of the extremely rapid transfer of electrons it must furthermore be concluded that the primary reductant is structurally very closely linked to a pigment. The TPN-photoreductase is on the contrary, according to JACOBI AND PERNER[13], probably localized in the stromalamellae. The only substance known to approach similar concentrations as the initial reductant is plastoquinone[14,15]. It amounts to about 10–20% of the total chlorophyll. We know too little about the position of plastoquinone in the chain of oxidation–reduction. It seems more likely to assume that plastoquinone acts as a carrier substance that facilitates the ground conductivity of the system and the linkage to various intermediate energy-converting systems. The initial reductant could possibly be a protein in direct structural linkage to a pigment. A copper protein, "plastocyanin", was recently described[16]. Its position in the photosynthetic cycle is, however, still uncertain.

That spinach chloroplasts and Chlorella almost identically respond to light illustrates the universality of the photosynthetic mechanism. Judging from the observed bands, Chlorella contains more cytochromes than spinach chloroplasts and the possibility cannot be excluded that some of them are operating in the respiratory chain. It is, however, significant that *Chlorella ellipsoidea* shows a very low respiration as compared with a very effective photosynthesis. Both Chlorella and spinach chloroplasts frequently show a disappearance of reduced pyridine nucleotide in the first minutes of "normal" illumination, which in Chlorella later on is often turned over into a positive response. The speed of reduction of TPN is thus normally comparable to the speed of oxidation in the cytochrome chain. As mentioned above[2] the autoxidation of cytochrome b_3 contributes to keeping the system at a stage of slight oxidation in the dark. The activity of TPN in malate oxidation is of course one more circumstance to be considered at discussions of the steady state TPN $\rightleftharpoons$ TPNH. It was observed that malate oxidation is accelerated in light, most probably owing to the simultaneous local increase of the O_2-concentration. This interference of O_2 is noticed also in the effects of O_2 or N_2 on the light response.

Regarding the observed displacement of the band peak of reduced pyridine nucleotide from the main position at 340 mμ little can yet be said about the small shifts toward about 336 or 344 mμ. No specific time-course was observed here and the deviations may partly be caused by variations in the back-ground. Of larger displacements, which are obviously combined with a corresponding lowering of the main peak at 340 mμ, only one seems to be characteristic for the photosynthetic apparatus, namely the band at 350–356 mμ. As shown at an earlier date baker's yeast shows distinct bands at 325 and about 360 mμ (ref. 7), of which the former corresponds to alcohol dehydrogenase, the latter to triosephosphate dehydrogenase. No one of these bands appears in chloroplasts. As to the interpretation of the band at 350–356 mμ attention is called to the fact that it is formed very rapidly, the speed being well comparable with that of TPN reduction (see Fig. 13). TPN is possibly already attached to an enzyme and then reduced. Attention is called here to the rapid adsorption of added TPN–TPNH.

As to the relation between photosynthetic production of ATP and photosyn-

thetic reduction of TPN it may be inferred from our earlier results as well from the present investigation that this relation can never be stoichiometrical, because the two processes are linked to different parts of the cycle. It was shown that the overwhelming part of ATP is synthetisized in the cytochrome chain[2] and that very little may be attributed to the systems acting around TPN–TPNH, whereas the latter is placed near the negative pole of the cycle. Each one of these products may be varied owing to the prevailing situation of the steady states and the availability of substrates. It may be expected that optimal conditions yield an approximately constant relation TPNH/ATP. Optimal conditions may be realized at a smoothly running CO_2-assimilation, because the relative quantities of ATP and TPNH (or DPNH) consumed here are chemically well defined. At impaired CO_2-assimilation and under the more or less artificial conditions of short experiments the relations may, however, vary considerably. Only the reduction of TPN is directly related to the photolysis (O_2-evolution in the water bridge), the formation of ATP not (*cf.* refs. 1–3).

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