PHOTOREACTIONS OF HYDRAZINE COMPOUNDS WITH CHOROPLASTS AND SUBCHLOROPLAST PARTICLES FROM SPINACH

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STELLINGEN

1. De werking van CCCP in het fotosynthesemechanisme kan wellicht voor een belangrijk gedeelte worden toegeschreven aan de vorming van een positief (CCCP-) radicaal na oxydatie door fotosysteem 2.

   e.g. G. Renger, *Z. Naturforsch.*, 26 b (1971) 149

2. De conclusie van D. Miles *et al.* dat Hg^{2+} een DCMU ongevoelige electronen acceptor van fotosysteem 2 zou zijn, berust op onvoldoende gegevens.

   D. Miles, P. Bolen, S. Farag, R. Goodin, J. Lutz, A. Moustafa, B. Rodriguez, C. Weil, *Biochem. Biophys. Res. Commun.*, 50 (1973) 1113

3. De waterstofbrugvorming tussen DCMU en geoxydeerd plastochinon door Van Rensen voorgesteld als mogelijk mechanisme van de DCMU remming biedt daarvoor een onvoldoende verklaring.

   J.J.S. van Rensen, *proefschrift*, Wageningen (1971) 65.

   S. Izawa en N.E. Good, *Biochim. Biophys. Acta*, 102 (1965) 20

4. De empirische regels voor de structuur-activiteitsrelatie van verschillende groepen herbiciden bieden slechts beperkte informatie betreffende het moleculaire werkingsmechanisme van deze herbiciden.

   C. Hansch in H. Metzner, ed., *Progress in Photosynthesis Research*, vol. III (1969) 1685

   P.A. Gabbott in H. Metzner, ed., *Progress in Photosynthesis Research*, vol. III (1969) 1712

5. De methode van Huzisige en Yamamoto om de absorptieveranderingen ten gevolge van ferricyanidereductie te corrigeren voor pigmentbleking is incorrect.

   H. Huzisige en Y. Yamamoto, *Plant & Cell Physiol.*, 13 (1972) 481

6. Hiller *et al.* houden, bij hun verklaring van het feit dat cyt. b_{559} in aanwezigheid van CCCP door systeem 1 wordt geoxydeerd, geen rekening met de mogelijkheid dat CCCP de redoxpotentiaal van cyt. b_{559} beinvloedt.

R.G. Hiller, J.M. Anderson en N.K. Boardman, *Biochim. Biophys. Acta,* 245 (1971) 439

7. Aan de specificiteit van kalf-thymus histonen, die volgens Darden samen met een *Volvox* extract de differentiatie van mannelijke kolonien bij *Volvox aurens* M5 induceren, dient te worden getwijfeld.

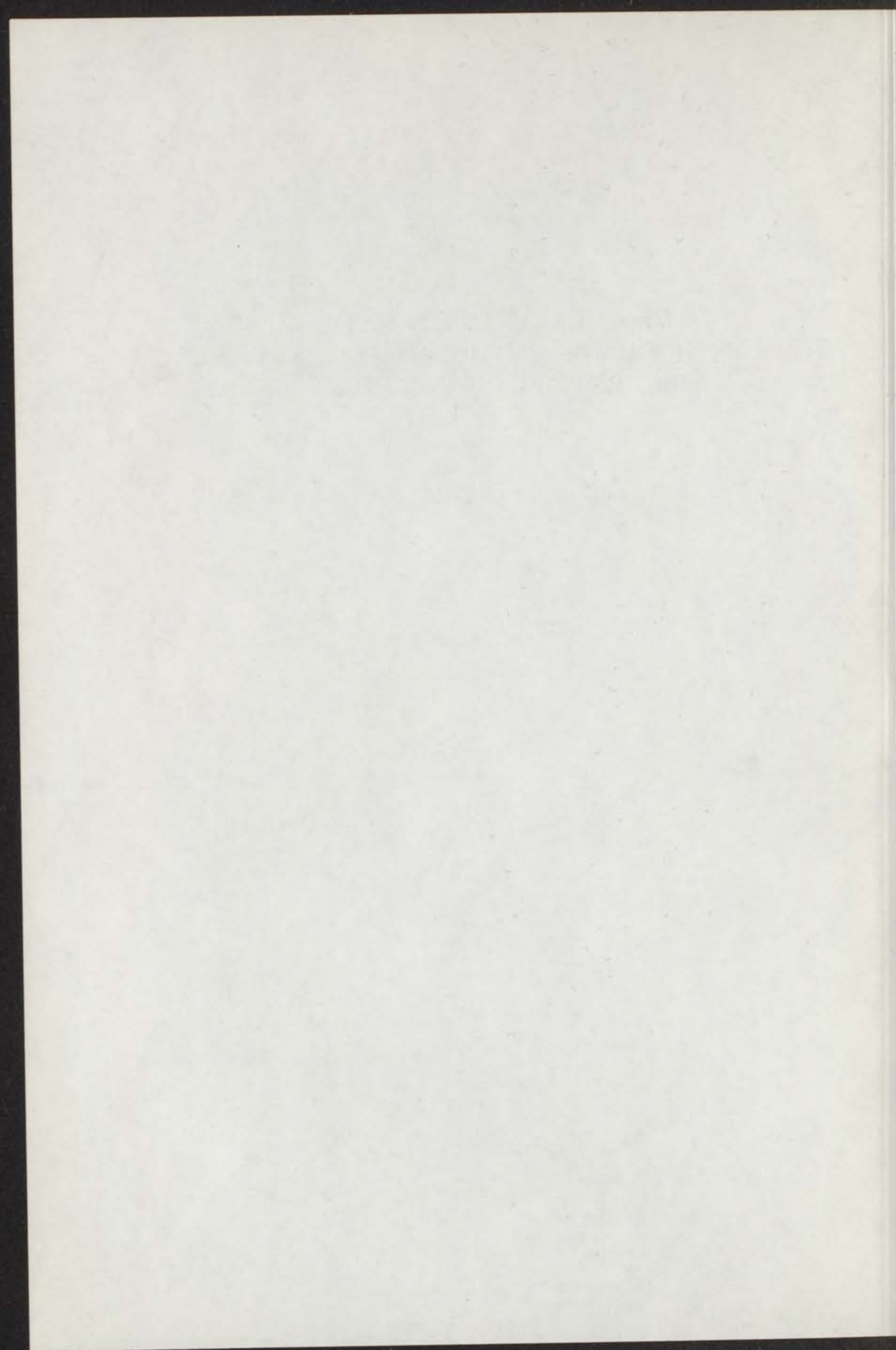
W.H. Darden, Jr., *Biochem. Biophys. Res. Commun.,* 45 (1971) 1205

8. Gezien de huidige verkeersintensiteit, dient voor bromfietsers een proeve van bekwaamheid vereist te zijn en zouden bestuurders van landbouwtrekkers een rijbewijs dienen te bezitten.

9. De grote mate van eenvormigheid in de bouw van de moderne eensgezinswoningen bevordert de leefbaarheid van Nederland niet.

J. Haveman   Leiden, 23 mei 1973

PHOTOREACTIONS OF HYDRAZINE COMPOUNDS WITH CHOROPLASTS AND SUBCHLOROPLAST PARTICLES FROM SPINACH



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HYDRAZOBENZENE OXIDATION BY 2,6-DICHLOROPHENOL-INDOPHENOL IN A PHOTOREACTION CATALYZED BY SYSTEM 1 OF PHOTOSYNTHESIS

HYDRAZINE COMPOUNDS AS DONORS FOR PHOTOSYSTEM 2

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A. E. COHEN, HOOGLERAAR IN DE FACULTEIT DER LETTEREN, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP WOENSDAG 23 MEI 1973 TE KLOKKE 16.15 UUR

DOOR

JACOB HAVEMAN

GEBOREN TE BARNEVELD IN 1944

1973

DRUKKERIJ J. H. PASMANS, 'S-GRAVENHAGE

Promotor: Prof.Dr. L.N.M. Duysens

En al ware het, dat ik de gave der profetie had,
en wist al de verborgenheden en al de wetenschap;
en al ware het dat ik al het geloof had,
zoodat ik bergen verzette, en de liefde niet had,
zoo ware ik niets.

1 *Korinthe* 13:2

Aan mijn ouders en mijn broer
Aan Els

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ABBREVIATIONS

Tris	tris(hydroxymethyl)aminomethane
Tricine	N-tris(hydroxymethyl)methylglycine
DCIP	2,6-dichlorophenolindophenol
DCMU	3-(3',4'-dichlorophenyl)-1,1-dimethylurea
CCCP	carbonylcyanide 3-chlorophenylhydrazone
PMS	phenazine methosulphate
NADP	nicotinamide-adenine dinucleotide phosphate
ATP	adenosine triphosphate
Q	"quencher", the primary electron acceptor of system 2
C550	compound supposed to be identical with the primary acceptor of system 2, showing a band-shift near 550 nm upon reduction
P700	pigment absorbing near 705 nm, the primary photooxidant of system 1
Z	donor to the primary photooxidant of system 2
System 2 particles	subchloroplast particles enriched in photosystem 2 prepared by means of digitonin

CHAPTER I
INTRODUCTION

In chloroplasts of higher plants two photosystems in series are involved in the electron transport from water to $NADP^+$ (for a review see Duysens, 1964). One of the photosystems (photosystem 1) produces a strong reductant which is able to reduce $NADP^+$ and a weak oxidant. The other photosystem (photosystem 2) produces a strong oxidant which is able to oxidize water to oxygen and a weak reductant. The weak oxidant and reductant react with each other, via a number of intermediates, in a reaction that is possibly coupled to photophosphorylation.

Both photosystems are localized in the thylakoid, a membrane structure in the form of a closed flattened sack (Menke, 1962) with a length of about 200 - 600 nm (Sane *et al.*, 1970). The thylakoids are stacked together to form the grana which are just observable by means of the light microscope. The grana, which contain both photosystems, are interconnected by means of intergrana lamellae (or stroma lamellae) which contain only photosystem 1 (Sane *et al.*, 1970; Goodchild and Park 1971). A chloroplast (length 3 - 10 μ) is a set of grana surrounded by a membrane envelope. The interlamellar space within the chloroplast (stroma) contains enzyme systems for the synthesis of carbohydrates and certain proteins and nucleic acids. The photooxidants of both photosystems are presumably situated at the inside of the thylakoid, the photoreductants at the outside. Upon illumination the inside of the thylakoid acidifies due to proton uptake (Jagendorf and Uribe, 1966) and a potential across the thylakoid membrane is developed (Witt *et al.*, 1968). This membrane potential is supposed to be the cause of the 515 nm absorption change. For a review concerning the implications of pH gradients and membrane potentials on photophosphorylation see Greville (1969) or Walker and Crofts (1970). In the same reviews there is a short survey of the uncoupling actions of ion transporting antibiotics like valinomycin and nigericin, of the classic uncouplers like CCCP and of substituted amines like methylamine.

The chloroplast membranes may be disrupted to give a separation of membrane fragments enriched in one of the photosystems by means of detergents (for a review see Boardman 1970) or by physical means *e.g.* by means of the "French press" technique as was first demonstrated by Michel and Michel-Wolwertz (1969), and more recently by Sane *et al.*, (1970).

The electron transport chain.

Fig. 1 shows a simplified scheme of the photosynthetic electron transport. According to this scheme electrons are transported from

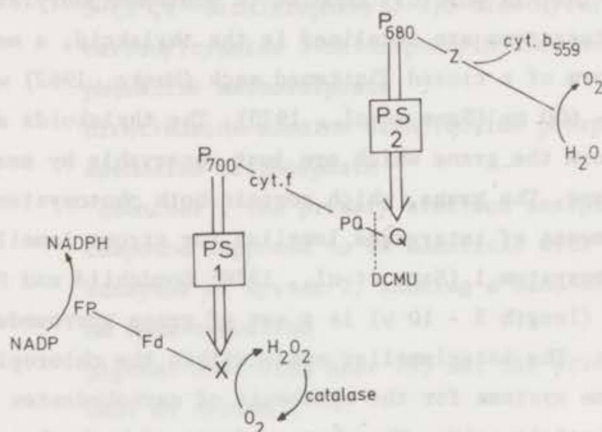


Fig. 1. Schematic representation of electron flow in photosynthesis. PS1 and PS2: the light harvesting pigment systems of photosystem 1 and 2 respectively; PQ: plastoquinone; Fd: ferredoxin; FP: the flavoprotein ferredoxin-NADP-reductase; Z: hypothetical component, donor to the primary photooxidant of system 2. For further explanation see the text.

water to the primary photooxidant of system 2 *via* a chain of unknown redox components (Kok *et al.*, 1970; Joliot *et al.*, 1969, 1971; Cheniae, 1970); from the primary acceptor (photoreductant) of system 2 to the primary photooxidant of system 1 *via* a chain which includes plasto-

quinone (Amesz, 1973) and cytochrome f (Duysens and Amesz, 1962); and from the primary acceptor of system 1 to $NADP^+$ *via* ferredoxin and the flavoprotein ferredoxin-NADP-reductase. Cyclic electron flow around photosystem 1, coupled to photophosphorylation, is possible: in that case ferredoxin reduces cytochrome f possibly via cytochrome b_6 (Arnon *et al.*, 1965; Schwartz, 1967). In the presence of PMS a cyclic electron flow occurs by reduction of PMS by the photoreductant of system 1 and reoxidation of this reduced PMS *via* cytochrome f. ATP produced by a reaction coupled to electron transport and NADPH are used for the reduction of CO_2 in the Calvin cycle (Calvin and Bassham, 1962).

P700 is the primary photooxidant of system 1. The oxidized *minus* reduced difference spectrum of P700 shows peaks near 430 and 700 nm indicating that it is a special type of chlorophyll. Its midpoint redox potential is +430 mV at pH 7 (Kok, 1961).

P680 is the presumed counterpart of P700 in photosystem 2, its redox potential must be higher than that of P700 because of its ability to oxidize water to O_2. Absorption changes due to P680 occur at 435 and at 680 nm (Döring *et al.*, 1967, 1969). They decay rapidly in the dark (200 μsec) presumably because of a back reaction with Q^-. Due to the high redox potential of the photooxidant of system 2 destructive reactions may occur when the normal electron transport from water is blocked *e.g.* by Tris-treatment. The pigment bleaching observed by K. Yamashita *et al.* (1969) and by Itoh *et al.* (1969) is due to such a destructive reaction.

"X" stands for the primary electron acceptor of system 1. Hiyama and Ke (1971) observed absorption changes in the blue region with different kinetics from P700 and called it P430 after the location of the maximum in its difference spectrum. They supposed P430 to be identical with X. Its midpoint potential proved to be -470 mV, low enough to reduce ferredoxin (Ke, 1973). There is evidence from ESR (electron spin resonance) measurements at liquid helium temperature that a bound form of ferredoxin is reduced (Malkin and Bearden, 1971) but how far this reduction is related to P430 is still unclear.

Q is the primary electron acceptor of photosystem 2 (Duysens and Sweers, 1963), its redox state is the main determinant of the fluores-

cence yield of chlorophyll a_2 (the chlorophyll of system 2). Its midpoint potential was estimated by Kok *et al.* (1966) at +180 mV for cell free particles of *Scenedesmus* mutant No. 8 (lacking P700) which value was unaffected by the pH (6 to 9) and by Cramer and Butler (1969) at -35 mV (pH 7.0) or at -95 mV (pH 8.0). Cramer and Butler observed in their fluorescence titrations a second quenching process with a much lower midpoint potential (-270 mV at pH 7.0). The significance of this process in photosynthetic electron transport is still unclear. In the presence of DCMU reduction of plastoquinone by reduced Q is inhibited (Duysens and Sweers, 1963; Duysens, 1972) and the fluorescence kinetics reflect the reduction of Q only. Knaff and Arnon (1969a, b) observed a light-induced bleaching at 550 nm attributed to a hypothetical compound called C550. They and especially Butler and coworkers obtained evidence that the state of C550 is influenced by the redox state of the primary electron acceptor of system 2. Butler and Okayama (1971) observed that at liquid nitrogen temperature the absorption changes corresponded to a bandshift by about 2 nm towards shorter wavelength of a compound (C550) absorbing at about 545 nm. Titration experiments gave a midpoint potential of -50 mV (Erixon and Butler 1971). The chemical nature of C550 is unknown, it might be β-carotene, bound to the reaction center of system 2 (Okayama and Butler, 1972).

Cytochrome b_{559} appears to be tightly bound to the chloroplast lamellae, it is localized in photosystem 2 (Boardman and Anderson, 1967). The role of cytochrome b_{559} in the electron transport chain remains unclear. Bendall and Sofrova (1971) supposed it to play a role in oxygen evolution. More recent evidence (Cramer and Böhme, 1972; Cox and Bendall, 1972) suggest that oxidation of cytochrome b_{559} occurs *via* a side-path of the oxygen evolution chain.

Luminescence (or delayed fluorescence) is caused by a back reaction of "early photoproducts" (Strehler, 1957). It originates from photosystem 2 (Goedheer, 1963; Bertsch *et al.*, 1967). According to the interpretation of Van Gorkom and Donze (1973) the 1 msec delayed fluorescence as measured by means of a phosphorescope is due to a backreaction between reduced Q (Q^-) and the primary photooxidant (P^+) in the presence of oxidized Z (Z^+). If Z is in the reduced state P^+ will react

faster with Z than with Q^- and the decay of the delayed fluorescence will be very fast. Van Gorkom and Donze explain Zankels 35 µsec component (Zankel, 1971) in this way. For a review concerning delayed fluorescence, see Kraan (1971).

Artificial electron donors and acceptors.

Hill (1939) made the discovery that illuminated chloroplasts are able to evolve oxygen if provided with ferrioxalate. Other investigators discovered that many other electron acceptors such as DCIP, ferricyanide, benzoquinone, methylviologen will sustain the oxygen evolution at a high rate. Mehler (1951, Mehler and Brown, 1952) showed that oxygen can also be reduced by chloroplasts. The reduction product is H_2O_2 if catalase is inhibited by sodium azide. The reduction of oxygen is a system 1 reaction and may be catalyzed by autoxidizable dyes such as methylviologen. Artificial electron acceptors (Hill oxidants) are widely used as tools for the study of photosynthesis. The same is true for artificial electron donors. In 1960 Vernon and Zaugg showed that photosystem 1 was able to reduce $NADP^+$ upon illumination when supplied with electrons from the donor couple ascorbate-DCIP. Other donors for system 1 are PMS (Jagendorf and Margulies, 1960), N,N,N',N' tetramethylphenylenediamine (Trebst, 1964) and diaminodurene (Trebst and Pistorius, 1965) all reduced by means of ascorbate.

Photosystem 2 is also able to oxidize artificial donors instead of water after destruction of the enzymic steps leading to oxygen evolution: *p*-phenylenediamene (T. Yamashita and Butler, 1968a, b, 1969), hydroxylamine (Bennoun and Joliot, 1969; Izawa *et al.*, 1969), semicarbazide (T. Yamashita and Butler, 1969; Vernon and Shaw, 1969a, b), ascorbate (Izawa *et al.*, 1969; Böhme and Trebst, 1969), Mn^{2+} (Kenten and Mann, 1955; Bachofen, 1966; Izawa, 1970; Ben Hayyim and Avron, 1970), hydrazine (Mantai and Hind, 1971; Heath, 1971), *sym.* diphenylcarbazide (Vernon and Shaw, 1969a, b), diketogulonate (Habermann *et al.*, 1968), tetraphenylboron (Homann, 1972), and H_2O_2 (Inoué and Nishimura, 1971). These artificial electron donors can be used to detect activity in sub-

chloroplast particles, where the oxygen evolution mechanism is destroyed (see *e.g.* Vernon and Shaw, 1969a).

This thesis reports experiments in which several hydrazine compounds were demonstrated to be effective electron donors in different light driven redox reactions. The effects of these compounds on fluorescence, delayed fluorescence, cytochrome b_{559} oxidation and on pigment bleaching have been studied. In order to determine whether system 1 or 2 participated action spectra were determined and the effects of DCMU were studied. Some titration experiments to determine the redox potential of the primary acceptor of system 2 in subchloroplast particles enriched in system 2 are also reported. Part of the results have already been published: Haveman and Donze (1971), Haveman *et al.* (1972).

CHAPTER II

MATERIALS AND METHODS

1. Preparation of chloroplasts and subchloroplast particles.

To prepare chloroplasts the washed leaves of spinach *(Spinacea oleracea)* obtained from the local market were ground at maximum speed in a homogenizer (Braun Starmix) for half a minute to a few minutes depending on the quantity. The grinding medium was 50 mM Tricine-KOH buffer, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$ and 0.4 M sucrose. The homogenate was filtered through a perlon gauze (mesh width approx. 25 μ) and the filtrate was centrifuged for a few minutes up to 7000 *g* or, if larger quantities had to be centrifuged, for 15 min at 3000 *g*. The chloroplast pellet was resuspended in the same medium as used for grinding and stored in an ice bath. Samples were used for experiments within a few hours after preparation. Just before the experiments the chloroplast suspension was diluted in the grinding medium without sucrose. Chlorophyll concentrations were estimated according to Arnon (1949).

In order to inhibit the oxygen evolving system, according to Yamashita and Butler (1968 a) the chloroplasts were exposed for 5 min to a 0.2 M Tris-HCl buffer, pH 9.0, centrifuged and taken up in the above mentioned Tricine buffer without sucrose.

To prepare digitonin subchloroplast particles we followed the method of Boardman and Anderson (1964) with slight modifications. The preparation of the chloroplasts was done in 50 mM phosphate buffer, pH 7.8, 10 mM KCl and 0.4 M sucrose. Then they were incubated for 30 min at 4° C in buffer without sucrose with digitonin: 1 g detergent to 60 mg chlorophyll as used by Vernon and Shaw (1969 a) to give the cleanest separation in system 1 and system 2 particles; the final concentration of digitonin was 1%. The resulting mixture was first centrifuged for 10 min at 3000 *g* to remove unbroken chloroplasts. Subsequent centrifugation at 25000 *g* for 30 min yielded a sediment consisting of chloroplast fragments enriched in photosystem 2 (system 2 particles) which

were normally taken up in a small quantity of a solution containing 50 mM phosphate buffer, pH 7.8, 10 mM KCl and 0.4 M sucrose. This suspension was stored in small plastic sacks ("Cryofoil", Cryoson N.V., Midden Beemster, The Netherlands) of 1 ml content in liquid nitrogen.

Chloroplast fragments enriched in photosystem 1 (system 1 particles) were obtained by recentrifugation of the 25000 *g* supernatant at 144000 *g* for 1 h, and were stored in liquid nitrogen in the same way. In all experiments the freshly thawed particles were used diluted in 50 mM phosphate buffer, pH 7.8, 10 mM KCl without sucrose. The system 2 particles had a chlorophyll *a/b* ratio of 2.0 to 2.3. The content of P700 in these particles was estimated to be about 1 to 800-1000 chlorophyll molecules. It was determined by measuring the absorbance decrease at 705 nm in saturating blue-green light in the presence of 1 mM ascorbate and 20 μM DClP. This value was compared with the absorbance of the suspension at 680 nm, corrected for scattering by subtracting the apparent absorbance at 750 nm. It was assumed that the molar differential extinction coefficient of P700 at 705 nm was the same as the molar extinction coefficient of chlorophyll *in vivo* at 680 nm. The system 1 particles had a chlorophyll *a/b* ratio of 4.5 to 4.9 and a corresponding P700/chlorophyll ratio of 1/200-250.

Other subchloroplast particles were prepared with Triton X-100 according to the method of Vernon, Shaw and Ke (1966). Only the fragments enriched in photosystem 2 were used. Chloroplasts prepared in 50 mM phosphate buffer, pH 7.8, 10 mM KCl and 0.4 sucrose were incubated with Triton X-100 for 1 h in phosphate buffer without sucrose (1 g detergent to 40 mg chlorophyll; final concentration of Triton X-100 1.5% by weight). The resulting mixture was first centrifuged for 20 min at 15000 *g* to remove large fragments. Centrifugation of the supernatant for 2 h at 144000 *g* yielded a sediment enriched in photosystem 2 (chlorophyll *a/b* ratio 2.3, P700/chlorophyll about 1/800). The sediment was taken up in buffer and stored in liquid nitrogen as was done with the digitonin subchloroplast fragments.

Sane, Goodchild and Park (1970) obtained chloroplast fragments enriched in one of the photosystems by means of the "French Press"technique. We obtained subchloroplast fragments in this way using the "Yeda Press"

(Yeda Research and Development Co. Ltd., Rehovot, Israel): chloroplasts suspended in 50 mM Tricine buffer, pH 7.8, 150 mM KCl and 2 mM $MgCl_2$ were passed through it two times at a pressure of 200 atm, provided by a high pressure nitrogen gas tank. Separation of the chloroplast fragments was achieved by means of centrifugation for 1 h at 51000 *g* in a 15-55% linear sucrose gradient in 50 mM Tricine buffer, pH 7.8, 10 mM KCl and 2 mM $MgCl_2$. Three bands were obtained (see Fig.12b) of which the two lower ones had a chlorophyll *a/b* ratio of 2.3. This indicates that both bands were enriched in system 2, but as will be discussed in the next Chapter the photochemical properties were different. The top band had a chlorophyll *a/b* ratio of 5.2 indicating that it contained mainly system 1.

2. Spectrophotometry and the estimation of quantum yields.

Changes in absorption induced by illumination were measured with an Aminco-Chance spectrophotometer (American Instrument Company, Silver Spring, Md., U.S.A.) in the split-beam mode. This spectrophotometer was equipped with side illumination for one of the two 1 x 1 x 4 cm^3 cuvettes. In this way DCIP reduction was measured at 600 nm, $NADP^+$ reduction at 340 nm, carotenoid bleaching at 490 nm (Itoh *et al.*, 1969; K. Yamashita *et al.*, 1969) and cytochrome b_{559} at 560 nm.

The device for the actinic side illumination was constructed in our laboratory. The intensity of actinic light was measured by deflecting part of it by means of a glass plate (45°) to a S1 vacuum phototube. The actinic light, provided by a quartz iodine lamp, passed a filter set, which except for the measurements of the quantum yields and action spectra, consisted of a Schott RG 645 glass filter and a Balzers Calflex C-1. These filters isolated a band between approximately 645 and 750 nm giving an intensity at the place of the cuvette of 25 $mW.cm^{-2}$ as measured by an YSI-Kettering model 65 radiometer (Yellow Springs Instrument Company, Ohio, U.S.A.). This intensity saturated the photochemical reactions studied. The photomultiplier was protected from the actinic light by means of a Corning CS 4-96 glass filter and/or an appropriate Balzers interference filter.

For action spectra the actinic light was filtered by means of a Balzers interference filter and a Balzers Calflex C filter. The maxima of the interference filters used occurred at the following wavelengths in nm (within brackets the half band width is given in nm) 632(12), 641(11), 659(12), 666(12), 670(12), 683(11), 690(14), 699(12), 711(14), 716(11), 724(14) and 736(16). The intensity of the incident light was measured by the S1 vacuum phototube which was calibrated by means of the YSI-Kettering radiometer. The detector of the radiometer (diameter of the sensitive area: 3mm) was placed in the center of the illuminated area of the cuvette (for technical reasons the cuvette was omitted; the loss of light originating from reflection by one cuvette wall was estimated at 5.7%). The phototube response proved to be linear at the various wavelenghts with light intensity as measured by the YSI radiometer. The fraction of light absorbed by the chloroplasts or subchloroplast particles was measured for each filter by means of a Zeiss PMQ II spectrophotometer equipped with opal glass (Shibata, Benson and Calvin, 1959). In order to correct for scattering the measured extinction at 750 nm was subtracted from the measured extinction at each wavelength; it was thus assumed that the extinction at 750 nm was totally due to light scattering and that the light scattering was independent of wavelength. (Amesz, Duysens and Brandt, 1961).

The fact that hydrazobenzene, like hydrazine (Heath, 1971), caused a considerable dark reduction of DCIP was an experimental complication. At concentrations of 50 μM hydrazobenzene and 60 μM DCIP in the absence or presence of system 2 particles or chloroplasts the absorption change was about 0.1 A per min. The rate of the dark reaction was strongly dependent on the pH (see Fig. 2). It was, at least at pH 7.8, proportional to the concentration of hydrazobenzene at a fixed concentration of DCIP. In order to be able to correct for the dark reaction rate, measurements of light-induced DCIP reduction in the presence of hydrazobenzene were done as follows: a small volume of a concentrated alcoholic solution of hydrazobenzene was added to the stirred mixture of chloroplast material and DCIP in the sample cuvette; meanwhile the absorption changes were recorded. The stirring was done by means of a rotating rod in the form of a screw at the top of the sample cuvette (see Fig. 3). A short time after

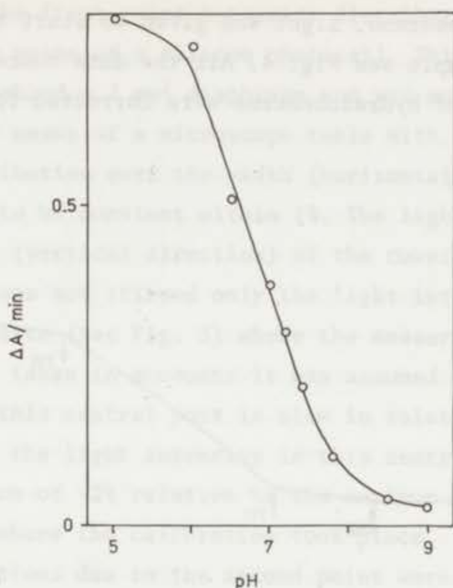


Fig. 2. The pH dependence of the dark reduction of DCIP by hydrazobenzene. Hydrazobenzene was added as a concentrated alcoholic solution via a microliter syringe to the stirred sample cuvette of the Aminco-Chance spectrophotometer containing 60 µM DCIP solution in 50 mM Tricine-KOH-buffer, 10 mM KCl and 2 mM $MgCl_2$; the pH was adjusted by adding KOH. The final concentration of hydrazobenzene was 50 µM (0.1 % alcohol).

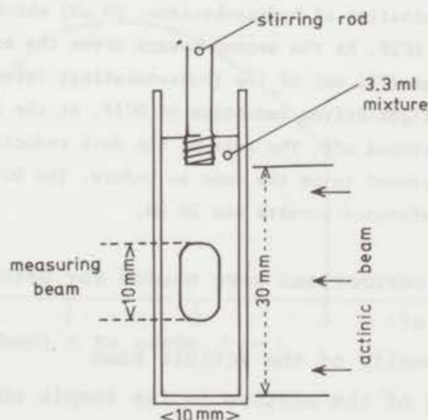


Fig. 3. Schematic cross-section of the sample cuvette in the Aminco-Chance spectrophotometer, perpendicular to the measuring beam.

injecting the hydrazobenzene, light was given to start the light-induced processes. For an example see Fig. 4. All the data concerning DCIP reduction in the presence of hydrazobenzene were corrected for the dark reduction.

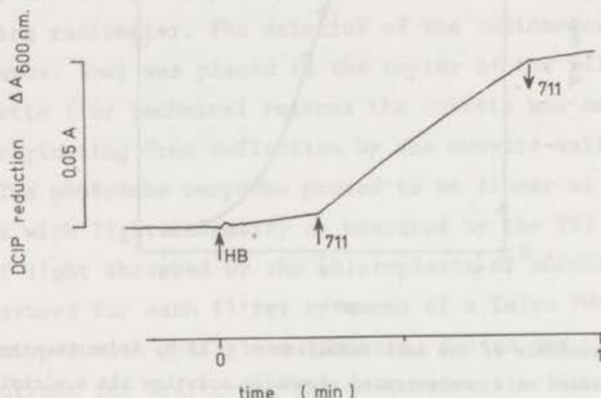


Fig. 4. DCIP reduction by chloroplasts (5 μg chlorophyll/ml) in the presence of hydrazobenzene. At the first upward arrow (zero time) hydrazobenzene (HB) is added via a microliter syringe to the stirred reaction mixture in the sample cuvette (final concentration of hydrazobenzene: 20 μM) which addition started the dark reduction of DCIP. At the second upward arrow the actinic light, here a monochromatic beam (711 nm) of low (subsaturating) intensity, was switched on, which started the light-driven reduction of DCIP. At the downward arrow the actinic light was switched off, the rate of the dark reduction after this period of actinic light proved to be the same as before. The DCIP concentration, in both sample and reference cuvette was 20 μM.

A number of corrections were needed for determining the quantum efficiency because of:

(1) Inhomogeneity of the actinic beam

(2) Stirring of the mixture in the sample cuvette in the case of addition of hydrazobenzene

(3) Light absorption by DCIP, especially at the shorter wavelengths (630 nm).

To check the first point intensity distribution of the actinic light was measured by means of a silicon photocell. This cell was covered with an opal glass behind a 2 mm^2 diaphragm and was moved perpendicular to the actinic beam by means of a microscope table with cross feeds. The light intensity distribution over the width (horizontal direction) of the cuvette appeared to be constant within 1%. The light intensity distribution over the height (vertical direction) of the cuvette is shown in Fig. 5. If the cuvette was not stirred only the light intensity in the central part of the cuvette (see Fig. 3) where the measuring beam passed the cuvette had to be taken in account: it was assumed that diffusion from other parts to this central part is slow in relation to the light-induced processes. When the light intensity in this central part was averaged it gave a correction of -2% relative to the maximum intensity in the center of the cuvette where the calibration took place.

The corrections due to the second point were necessary because of the fact that not only the chloroplasts in the area of the measuring beam participated in the process but also the chloroplasts of other parts of the cuvette (Fig. 3). The average intensity was smaller: -9% due to

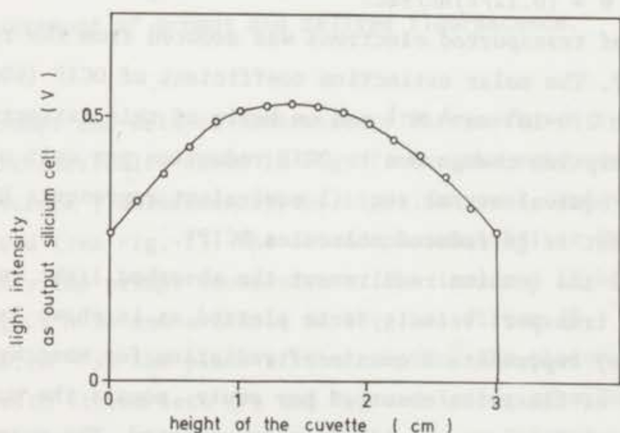


Fig. 5. Light intensity distribution of the actiniv beam over the height of the sample cuvette of the Aminco-Chance spectrophotometer. The light intensity was measured by means of a silicon photocell (see the text).

the fact that also chloroplasts of the unilluminated area were involved and -16% due to averaging of the light intensity over the whole illuminated area of the cuvette (see Fig. 5), the total correction was -25%.

Due to the fact that DCIP has a very broad absorption spectrum a considerable fraction of the total absorption in the wavelength region studied, especially at the shorter wavelengths (630 nm) was due to this compound. The absorption of the DCIP was measured with and without chloroplasts at the various wavelengths by means of the Zeiss PMQ II with opal glass or the Cary model 14 with scattered transmission accessory. In all experiments concerning the estimation of quantum yields we worked with diluted suspensions of chloroplasts (5 μg chlorophyll/ml) to have a low optical density (0.25 A, 44% absorption) so the absorption of light, in W/cm^3, in the center of the cuvette does not differ markedly from that over the whole depth of the cuvette.

The absorbed light intensity, after applying the above mentioned corrections, was measured in $Watt.cm^{-3}$ and was converted into $nE(instein).cm^{-3}.sec^{-1}$ for each wavelength by means of the equation $1\ E = Nh(c/\lambda)Watt.sec$, where N is Avogadro's number, h is Planck's constant, c is the velocity of light and λ the wavelength. We used as approximation: $1\ W = (0.12/\lambda)nE/sec$.

The number of transported electrons was deduced from the rate of bleaching of DCIP. The molar extinction coefficient of DCIP (600 nm) was assumed to be $2.0 \cdot 10^4\ cm^{-1}.M^{-1}$ and on basis of this extinction coefficient, the absorption change due to DCIP reduction per unit of time was converted to equivalents/ml.sec. (1 equivalent represents N transported electrons, that is ½N reduced molecules DCIP).

To calculate the quantum requirement the absorbed light intensity and the electron transport velocity were plotted as is shown in Fig. 6. Since 1 E(instein) represents N quanta of radiation for monochromatic light the number of Einsteins absorbed per equiv. equals the quantum requirement in absorbed quanta per electron transported. The quantum yield is the inverse of this. Experimentally Lumry and Spikes (1957) found for the Hill reaction of chloroplasts that the plot as in Fig. 6 of v/I (V is rate of reaction, I is light intensity) is a straight line. We too found an approximate linear relationship (see Fig. 6). The quantum re-

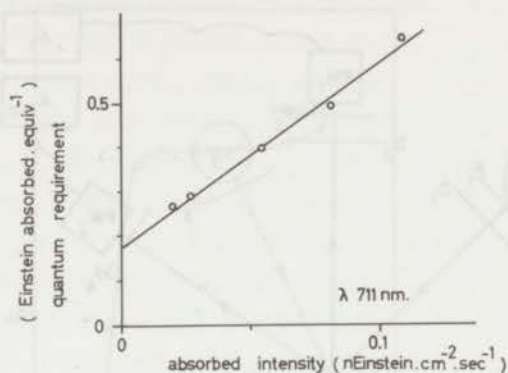


Fig. 6. Determination of the quantum yield of the DCIP reduction by chloroplasts at 711 nm with 20 μM hydrazobenzene, 20 μM DCIP and 5 μg chlorophyll/ml. The quantum requirement was found by linear extrapolation to zero intensity. The quantum yield is the inverse of this.

quirement apparently increases at higher light intensity. We calculated the quantum requirement by linear extrapolation to zero intensity.

3. Measurement of prompt and delayed fluorescence.

Prompt and delayed fluorescence were measured by means of the apparatus schematically shown in Fig. 7. For the measurement of delayed fluorescence (luminescence) the light source L_1 and photomultiplier PM_1 were used (see Fig. 7). The second photomultiplier PM_2 measured simultaneously the prompt fluorescence. The incident light was filtered by a Corning Cs 4-96 and a Schott BG 18 glass filter (F_1) giving an intensity of 5 $mW.cm^{-2}$ at the place of the cuvette. The photomultipliers were provided with filter sets (F_2 and F_4) consisting of a Schott BG 665 glass filter and interference filters (Balzers or Schott) with maximum transmittancy at 680 nm.

The same apparatus (Fig. 7) could be used to determine the rate of $NADP^+$ reduction by the increase in fluorescence around 450 nm (Amesz, 1964). The exciting light (L_1) then was provided by a mercury lamp and

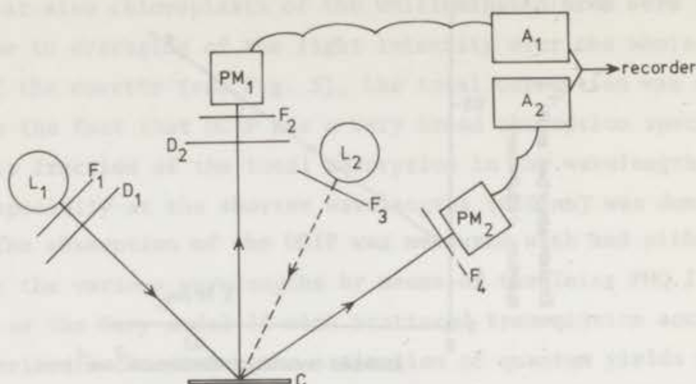


Fig. 7. Schematic representation of the apparatus used for fluorescence and luminescence measurements. L_1 and L_2: light sources (usually quartz iodine lamps). PM_1 and PM_2: photomultipliers for luminescence and fluorescence respectively. D_1 and D_2: synchronously rotating discs, each with 10 holes. Frequency of rotation: 50 Hz. When the discs were positioned in such a way relative to each other that, when D_1 was shutting off the light from L_1, D_2 transmitted the luminescence emitted by the sample, the apparatus acted like a Becquerel phosphoroscope. The dark time in that case was about 1 msec after each flash. See the text for further details.

was filtered through a Schott UG 11 glass filter (F_1); actinic light was provided by a second incident beam (L_2) and was filtered by a Schott RG 665 glass filter (saturating intensity). The photomultiplier (PM_1, D_2 removed) was protected from actiniv light by means of a Schott GG 400 and a Corning Cs 4-96 glass filter, and a Balzers broadband "K_3" interference filter (F_2).

4. Oxygen measurements.

Oxygen consumption or evolution was measured by means of a Clark type electrode (Yellow Springs Instrument Company). The reaction chamber was illuminated from one side by an Aldis projector fitted with a 500 W incandescent lamp. The light passed first a 0.1 M $CuSO_4$ solution (1 cm

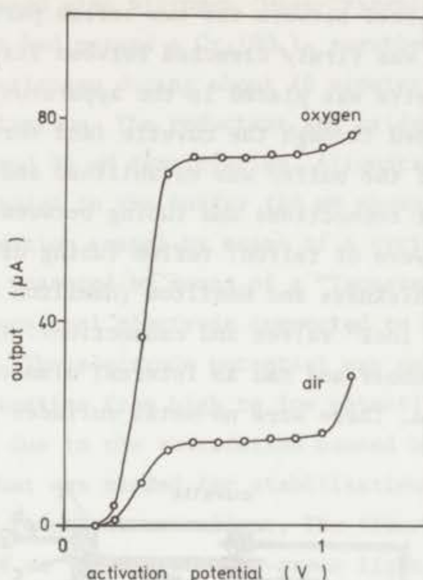


Fig. 8. Calibration curve of the oxygen electrode, respectively for water saturated with air and with oxygen. In water saturated with air the oxygen concentration is 0.28 mM (Winker, quoted by Delieu and Walker, 1972).

light path) and was filtered by means of a Schott RG 645 glass filter, providing a saturating intensity for the photochemical reactions studied. Fig. 8 shows a calibration curve of the electrode. The measurements to be described in the next Chapter were done at an operating voltage of 0.8 V. The current (ΔμA), measured over 470Ω, per mM oxygen appeared to be 72 μA/mM at 20° C for the experiments described.

5. Potentiometric titration of the prompt and delayed fluorescence.

Redox titrations of the prompt and delayed fluorescence of system 2 particles prepared by means of digitonin were performed with the flow system schematically shown in Fig. 9. The system 2 particles (about 1 ml containing 15 μg chlorophyll/ml) were precipitated on a General Electric "Nuclepore"filter (3.10^7 pores.cm^{-2}; diameter of the pores about 0.5 μ).

The filter was then placed between the two teflon parts of the flow cuvette and the cuvette was firmly clenched between its support and a quartz plate. The cuvette was placed in the apparatus described in Fig. 7. The buffer was pumped through the cuvette (and through the filter). The redox potential of the buffer was established and measured in the titration vessel. Most connections and tubing between the cuvette and the titration vessel were of telfon: teflon tubing of 1 mm internal diameter and 1 mm wall thickness and Hamilton (Hamilton Co., Whittier, Calif., U.S.A.) "luer lock" valves and connections. The tubing in the pump was of silicon rubber and had an internal diameter of 4 mm and a wall thickness of 3 mm. There were no metal surfaces in the system.

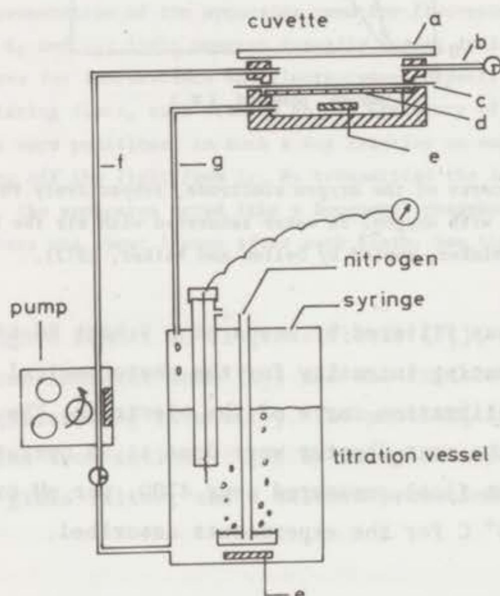


Fig. 9. Schematic representation of the apparatus used for titration of the redox potential of the prompt and delayed fluorescence. a: quartz plate, b: outlet of teflon tubing and a valve for eventually occurring bubbles. c: Nuclepore filter, d: supporting grid, e: magnetic stirrer, f and g: teflon tubing. (See the text for further details.)

The presence of oxygen caused much trouble during a titration, so before each titration the whole flow system was evacuated during a couple

of minutes and gassed with nitrogen (Hoek-Oxygenium, Schiedam, The Netherlands) which had passed a $Cr_2(SO_4)_3$ scrubber. The titration vessel was bubbled with nitrogen during about 10 minutes before the titration and during the titration. The reductant and oxidant were, respectively, 25 mM dithionite and 25 mM ferricyanide. Aliquots of the reductant or the oxidant were added to the buffer (50 mM phosphate buffer pH 6.8, 10 mM KCl in the titration vessel by means of a syringe. The redox potential in the vessel was measured by means of a "Tacussel" (Solea, Lyon, France) combined platinum-calomel electrode connected to a digital pH/mV meter (Philips PW 9408). The electrode potential was calibrated by means of quinhydrone. A titration from high to low potential (or *vice versa*) took about 20 minutes, due to the retardation caused by the flow system and due to the time that was needed for stabilisation of the redox potential after addition of reductant or oxidant. The fluorescence was excited by a weak (20 - 40 $\mu W.cm^{-2}$) beam of blue-green light, filtered by a Corning 4-96 and a Schott BG 18 glass filter (F_1 in Fig. 7).

6. Origin of chemicals.

Digitonin, 2,5-dichlorophenylhydrazine, N,N'-phtaloylhydrazine, semicarbazide, *sym.* diphenylcarbazide and N,N-diphenylhydrazine were obtained from Fluka AG, Buchs, Switzerland; hydrazinoethnol from Schuchardt GmbH, München, Germany, DCMU from K and K Laboratories Inc., U.S.A.; ferredoxin from Sigma Chemical Comp., St. Louis, U.S.A.; catalase and NADP from Boehringer and Söhne GmbH, Mannheim, Germany; valinomycin and nigericin were a gift from Dr. B.G. Pressman and Dr. C.W. Pettinga (Eli Lilly and Comp.); CCCP was a gift from Dr. P.G. Heytler (DuPont de Nemours) to Dr. L.N.M. Duysens; Triton X-100 was obtained from Baker Chemicals N.V., Deventer, The Netherlands; all other chemicals were obtained from E. Merck, Darmstadt, Germany, or BDH Chemicals Ltd., Poole, England. The chemicals were of the quality "pro analysi" or the purest quality available, N,N-diphenylhydrazine could only be obtained in the practical grade (minimum content 95%). All chemicals were used without further purification.

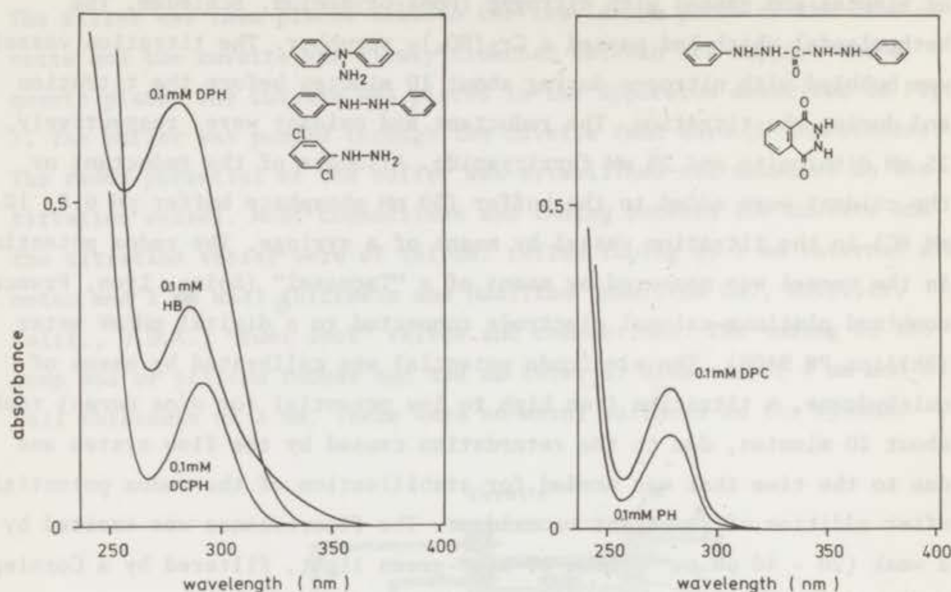


Fig. 10. Absorption spectra of some of the compounds used in the experiments described in this thesis as electron donors of photosystem 2. They all were diluted from an alcoholic stock solution in 50 mM Tricine-KOH buffer pH 7.8, 10 mM KCl and 2 mM $MgCl_2$ to a final concentration of 0.1 mM (0.2 % alcohol). DPH, diphenylhydrazine; HB, hydrazobenzene; DCPH, dichlorophenylhydrazine; DPC, diphenylcarbazide; PH, phtaloylhydrazine.

Hydrazobenzene (N,N'-diphenylhydrazine), N,N-diphenylhydrazine, dichlorophenylhydrazine, phtaloylhydrazine, diphenylcarbazide, valinomycin, nigericin, PMS, CCCP and DCMU were dissolved in ethanol and were used at a final concentration of less than 0.3% ethanol.

Absorption spectra of the compounds used as electron donor in this thesis are shown in Fig. 10, with the exception of hydrazinoethanol and semicarbazide which, even at a concentration of 10 mM, do not show an appreciable absorption above 250 nm.

CHAPTER III

RESULTS AND DISCUSSION

1. Hydrazine compounds as artificial electron donors of several electron transport reactions.

In Table 1 the effect is shown of the substituted hydrazines on DCIP reduction by system 2 particles prepared with digitonin. Without added donor the activity was rather low. Apparently the oxygen evolving mechanism was damaged by the digitonin treatment. As in Tris-treated chloroplasts (T. Yamashita and Butler, 1969; Vernon and Shaw, 1969b), artificial donors were able to enhance DCIP reduction, replacing water oxidation. From the donors mentioned in Table 1, hydrazobenzene attracts attention by its DCMU insensitivity. With freshly prepared chloroplasts in saturating red light the rates of DCIP reduction in μmoles.mg chlorophyll^{-1}.h^{-1} were 115 and 0 in the absence and presence of 5 μM DCMU respectively. In the presence of 100 μM hydrazobenzene these rates were 264 and 235, showing that addition of hydrazobenzene practically removed DCMU inhibition. On the other hand at a low concentration (2 μM) of hydrazobenzene the DCIP reduction, with 10 μM DCIP in system 2 particles was inhibited for 90% by 5 μM DCMU. The inhibitory action of DCMU appears to be a function of the hydrazobenzene concentration. This is further elaborated in the experiments shown in Fig. 11. In Fig. 11a the rates of DCIP photoreduction by system 2 particles are shown as a function of the hydrazobenzene concentration in a double reciprocal plot, in the presence and absence of DCMU. The crossing of the extrapolated lines on the ordinate could be interpreted to indicate that hydrazobenzene removes the inhibition by displacing DCMU, in analogy to competitive enzyme inhibition (see, however, the following paragraph). For phtaloylhydrazine (Fig. 11b) the percentage of inhibition by DCMU does not increase with decreasing donor concentration.

The observation that the DCMU inhibited non-cyclic photophosphorylation in chloroplasts with methylviologen as acceptor, in the presence

TABLE 1

ARTIFICIAL ELECTRON DONORS TO PHOTOSYSTEM 2 AS INHIBITORS OF THE CAROTENOID BLEACHING AND AS DONORS TO DCIP REDUCTION IN SYSTEM 2 PARTICLES

Carotenoid bleaching was measured at 490 nm in the presence of 5 μM CCCP and the initial slope of the kinetic curve was determined. DCIP was measured at 600 nm with 60 μM DCIP present. Chlorophyll concentration in both cases: 15 μg/ml.

Donor	*Concn (mM)*	*%inhibition carotenoid bleaching*	*DCIP reduction (μmoles·mg chlorophyll^{-1}·h^{-1})*	
			Without DCMU	*With 5 μM DCMU*
	-		18.4	0.0
Semicarbazide	0.8	62		
	5		25.4	0.0
	8	89		
	20		43.2	0.0
Hydrazinoethanol	0.5	52		
	1	81		
	5	96	40.0	0.0
	20		54.4	0.0
Phtaloylhydrazine	0.1	64	41.3	0.0
	0.2	86		
	0.5	99	64.8	0.0
sym-Diphenylcarbazide	0.005	37		
	0.01	47		
	0.05	84		
	0.1		36.7	0.0
	0.5		59.2	0.0
Dichlorophenylhydrazine	0.0005	41		
	0.005	87		
	0.05	96		
	0.1		75.2	2.9
	0.5		91.2	6.1
Hydrazobenzene	0.0002	62		
	0.0005	88		
	0.001	94		
	0.05		115.2	46.3

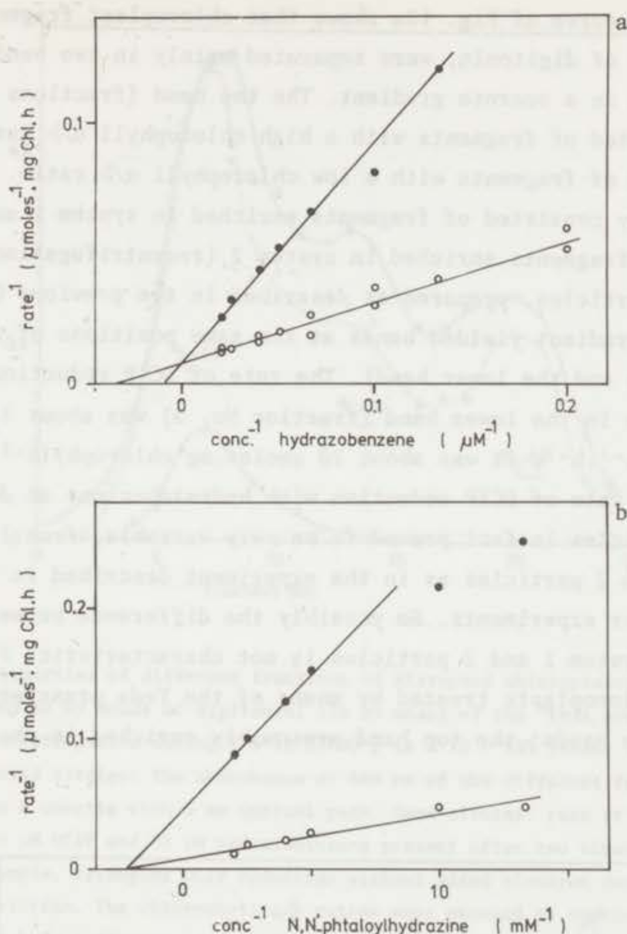
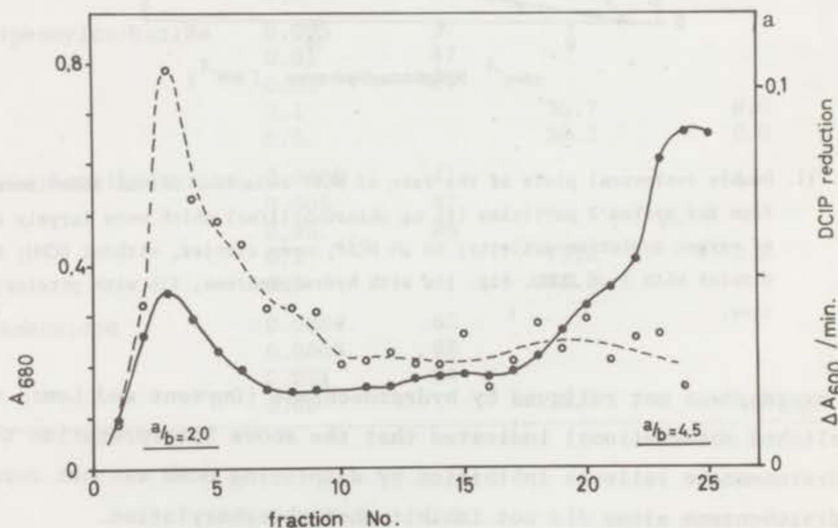


Fig. 11. Double reciprocal plots of the rate of DCIP reduction *versus* donor concentration for system 2 particles (15 μg chlorophyll/ml) which were largely devoid of oxygen evolution activity; 60 μM DCIP; open circles, without DCMU; solid circles with 1 μM DCMU. Fig. 11a with hydrazobenzene, 11b with phtaloylhydrazine.

of oxygen, was not relieved by hydrazobenzene (Duysens and Lems, unpublished observations) indicated that the above interpretation that hydrazobenzene relieves inhibition by displacing DCMU was not correct; hydrazobenzene alone did not inhibit photophosphorylation.

The solid curve of Fig. 12a shows that chloroplast fragments, prepared by means of digitonin, were separated mainly in two bands upon centrifugation in a sucrose gradient. The top band (fractions of high number) consisted of fragments with a high chlorophyll *a/b* ratio and the lower band of fragments with a low chlorophyll *a/b* ratio. The top band apparently consisted of fragments enriched in system 1 and the lower band of fragments enriched in system 2 (recentrifugation of system 1 and 2 particles, prepared as described in the previous Chapter, in a sucrose gradient yielded bands at the same positions of respectively the top and the lower band). The rate of DCIP reduction with hydrazobenzene in the lower band (fraction No. 3) was about 170 μmoles. mg chlorophyll^{-1}.h^{-1}; it was about 20 μmoles.mg chlorophyll^{-1}.h^{-1} in the top band. The rate of DCIP reduction with hydrazobenzene as donor by system 1 particles in fact proved to be very variable, from 12% of the rate of system 2 particles as in the experiment described in Fig. 12a, up to 70% in other experiments. So possibly the difference between the activities of system 1 and 2 particles is not characteristic. Fig. 12b shows that chloroplasts treated by means of the Yeda press were separated into three bands: the top band presumably enriched in photosystem 1,



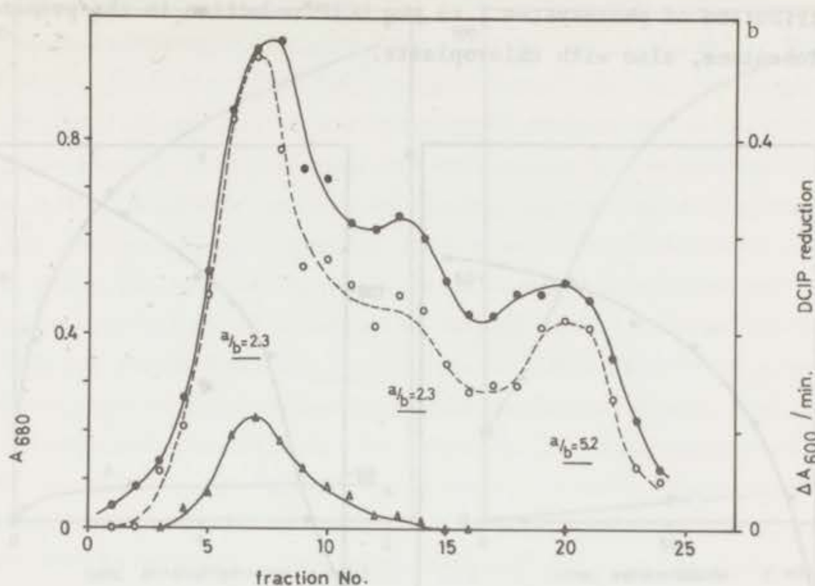
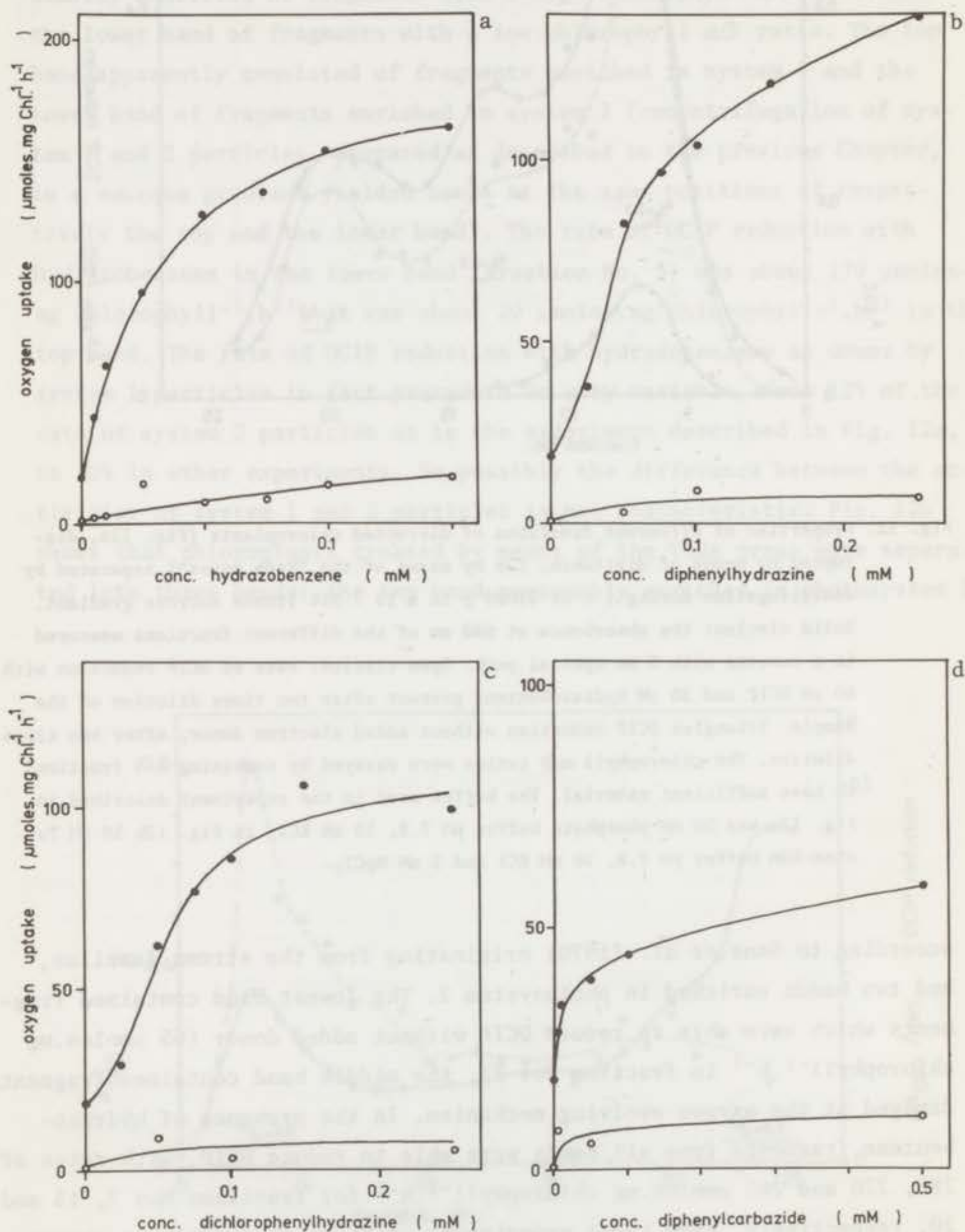


Fig. 12. Properties of different fractions of disrupted chloroplasts (Fig. 12a, disrupted by means of digitonin; 12b by means of the "Yeda press") separated by centrifugation during 1 h at 51000 *g* in a 15 - 55% linear sucrose gradient. Solid circles: the absorbance at 680 nm of the different fractions measured in a cuvette with 5 mm optical path. Open circles: rate of DCIP reduction with 60 μM DCIP and 50 μM hydrazobenzene present after two times dilution of the sample. Triangles DCIP reduction without added electron donor, after two times dilution. The chlorophyll *a/b* ratios were essayed by combining 2-3 fractions to have sufficient material. The buffer used in the experiment described in Fig. 12a was 50 mM phosphate buffer pH 7.8, 10 mM KCl; in Fig. 12b 50 mM Tricine-KOH buffer pH 7.8, 10 mM KCl and 2 mM $MgCl_2$.

according to Sane *et al.* (1970) originating from the stroma lamellae, and two bands enriched in photosystem 2. The lowest band contained fragments which were able to reduce DCIP without added donor (65 μmoles.mg chlorophyll^{-1}.h^{-1} in fraction No. 7), the middle band contained fragments damaged at the oxygen evolving mechanism. In the presence of hydrazobenzene fragments from all bands were able to reduce DCIP, with rates of 285, 220 and 265 μmoles.mg chlorophyll^{-1}.h^{-1} for fractions Nos 7, 13 and 20, respectively. From these experiments it seems reasonable to suppose

a contribution of photosystem 1 to the DCIP reduction in the presence of hydrazobenzene, also with chloroplasts.



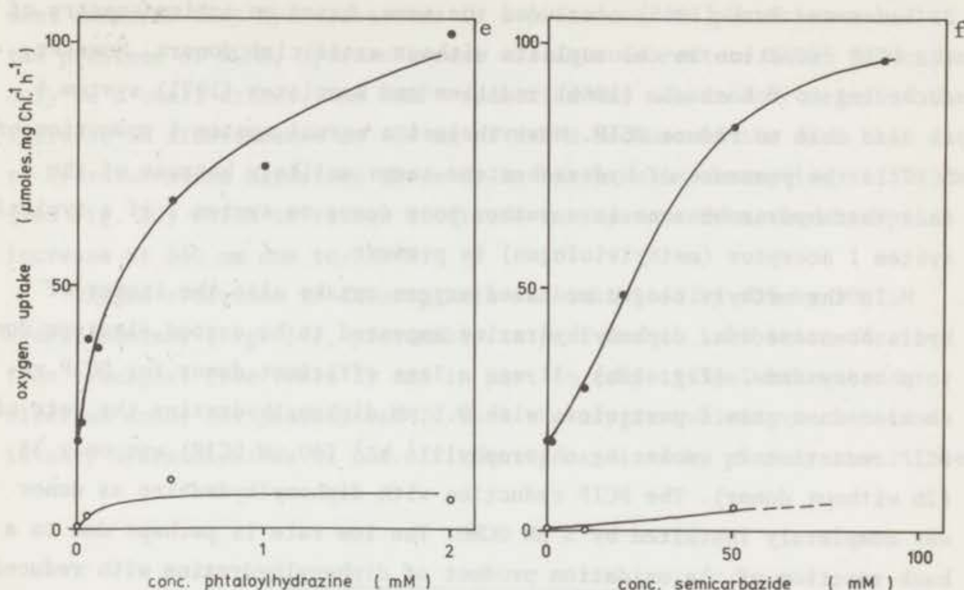


Fig. 13. The effect of hydrazine compounds on the methylviologen-mediated oxygen uptake in Tris-washed chloroplasts. The reaction mixture contained: Tris-washed chloroplasts, 10 µg chlorophyll/ml, 50 µM methylviologen, 0.5 mM methylamine, 0.5 mM NaN_3 and the concentrations of the different hydrazine compounds as indicated. Solid circles, without DCMU, open circles with 5 µM DCMU.

Fig. 13 shows that in Tris-treated chloroplasts, with methylviologen as acceptor and hydrazobenzene and other hydrazine compounds as donor, electron transport was largely inhibited by DCMU. This indicates that in this system these substances act mainly as system 2 donors. The small DCMU insensitive oxygen uptake may be caused by electron donation to the electron transport chain between photoreactions 1 and 2. In agreement with this the hydrazine compounds mentioned were able to enhance the rate of dark reduction of photooxidized P700 in system 1 particles in a way similar to ascorbate *plus* DCIP. The fact that DCIP reduction with system 2 particles, in which some P700 activity is still present (Table 1), and with Tris-treated chloroplasts (not shown), with the mentioned donors except hydrazobenzene and dichlorophenylhydrazine was totally inhibited by DCMU suggests that DCIP is an acceptor mainly for photosystem

2. Sauer and Park (1965) concluded the same, based on action spectra of the DCIP reduction in chloroplasts without artificial donors. However, according to Kok *et al.* (1966) and Lien and Bannister (1971) system 1 is also able to reduce DCIP. Nevertheless a normal system 1 reduction of DCIP in the presence of hydrazobenzene seems unlikely because of the fact that hydrazobenzene is a rather poor donor to system 1 if a typical system 1 acceptor (methylviologen) is present.

In the methylviologen mediated oxygen uptake also the isomer of hydrazobenzene *viz.* diphenylhydrazine appeared to be a good electron donor to photosystem 2 (Fig. 13b). It was a less efficient donor for DCIP reduction in system 2 particles: with 0.5 mM diphenylhydrazine the rate of DCIP reduction in µmoles.mg chlorophyll^{-1}.h^{-1} (60 µM DCIP) was only 38 (26 without donor). The DCIP reduction with diphenylhydrazine as donor was completely inhibited by 5 µM DCMU. The low rate is perhaps due to a back-reaction of the oxidation product of diphenylhydrazine with reduced DCIP.

TABLE 2

INFLUENCE OF ADDED HYDRAZOBENZENE ON THE NADP$^+$ REDUCTION IN CHLOROPLASTS

Measured by means of the fluorescence of NADPH at 450 nm, the control rate being approx. 80 µmoles·mg chlorophyll^{-1}·h^{-1} as measured by the absorption change near 340 nm, assuming a molar extinction coefficient for NADPH of $6.2 \cdot 10^3$ cm^{-1}·M^{-1}. The reaction mixture contained chloroplasts in Tricine buffer, 30 µg chlorophyll/ml and in addition 8 µg/ml ferredoxin, 0.2 mM NADP$^+$ and where indicated 0.1 mM hydrazobenzene and/or 1 µM DCMU.

Additions	*Rate as percentage of control*
-	100
Hydrazobenzene	74
DCMU	5
Hydrazobenzene + DCMU	13

The effect of added hydrazobenzene on NADP$^+$ reduction by freshly prepared chloroplasts is shown in Table 2. The result of this experi-

ment confirms that hydrazobenzene is a poor donor to photosystem 1. In the presence of DCMU, hydrazobenzene was able to restore $NADP^+$ reduction only to a small extent. The $NADP^+$ reduction was measured by means of the increase in fluorescence at 450 nm of NADPH because of the fact that due to hydrazobenzene oxidation there is an increase in absorption at 320 nm (see Fig. 20) which is rather difficult to separate from the absorption increase at 340 nm due to NADPH.

Oxygen evolution in chloroplasts was inhibited by 40% by 100 μM hydrazobenzene (Fig. 14), presumably in part due to inhibition of electron transport (see Table 2) and in part to competition with water as electron donor for photosystem 2. When the oxygen evolving mechanism is intact, hydrazobenzene is not efficiently oxidized by photosystem 2 (see

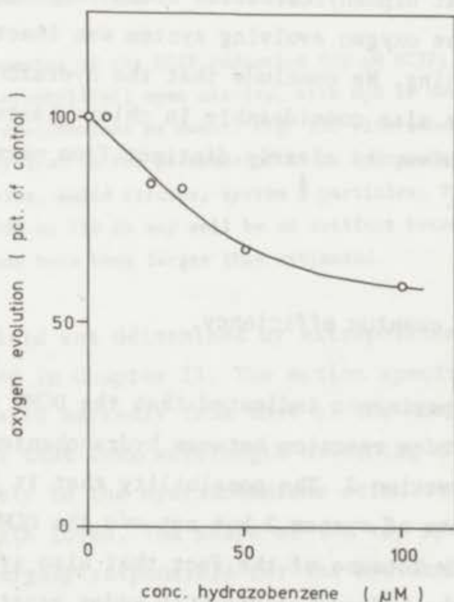


Fig. 14. The inhibition of oxygen evolution by hydrazobenzene. The reaction mixture contained chloroplasts, 30 μg chlorophyll/ml, 0.4 mM $NADP^+$ and 10 μg ferredoxin/ml.

TABLE 3

HYDRAZOBENZENE OXIDATION BY CHLOROPLASTS AND TRIS WASHED CHLOROPLASTS.

The hydrazobenzene oxidation was measured at 340 nm, assuming a molar extinction coefficient of $1.2 \cdot 10^4$ $cm^{-1}.M^{-1}$ for azobenzene at this wavelength (Fig. 20), in the presence of 0.5 mM methylamine, 50 μM methylviologen and 0.1 mM hydrazobenzene. Chlorophyll concentration: 15 μg/ml.

	Rate (μmoles.mg chlorophyll^{-1}.h^{-1})	
	Without DCMU	*With 5 μM DCMU*
Chloroplasts	15	7
Tris washed chloroplasts	113	9

also Table 3). This is consistent with experiments by Vernon and Shaw (1969b) who showed that diphenylcarbazide could only donate electrons to photosystem 2 if the oxygen evolving system was inactivated in some way, *e.g.* by Tris washing. We conclude that the hydrazobenzene DCIP redox reaction, which is also considerable in chloroplasts with an intact oxygen evolving mechanism, is clearly distinct from normal electron transport by system 2.

2. Action spectra and quantum efficiency.

The foregoing experiments indicated that the DCMU insensitive part of the light-driven redox reaction between hydrazobenzene and DCIP is not caused by photoreaction 2. The possibility that it is sensitized exclusively by pigments of system 2 but not *via* the DCMU-sensitive photoreaction 2 is unlikely because of the fact that also system 1 particles are able to sustain the hydrazobenzene-DCIP redox reaction. So, in spite of the fact that DCIP, with other donors, appears to be an acceptor for photosystem 2, photosystem 1 seems to be involved under certain conditions in the hydrazobenzene DCIP redox reaction. In order to clarify this point, action spectra of the quantum yield of DCIP reduction in chloroplasts were determined with water and with hydrazobenzene as donor.

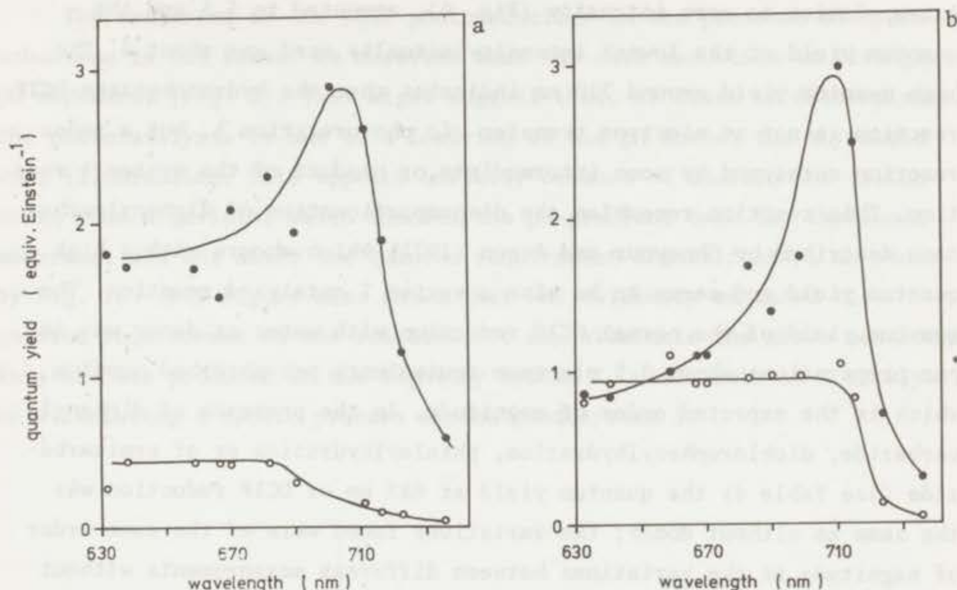


Fig. 15. Action spectra of the DCIP reduction (20 μM DCIP). Fig. 15a with chloroplasts, 5 μg chlorophyll/ml; open circles, with H_2O as donor, solid circles with 20 μM hydrazobenzene as donor. Fig. 15b with subchloroplast particles, 5 μg chlorophyll/ml in the presence of 20 μM hydrazobenzene; open circles, system 2 particles, solid circles, system 1 particles. The decrease in quantum yield beyond 700 or 710 nm may well be an artifact because the scattering in this region may have been larger than estimated.

The quantum yield was determined by extrapolation to zero light intensity as described in Chapter II. The action spectrum with hydrazobenzene as donor deviates markedly from that of the normal DCIP reduction (Fig. 15a) and shows that long wavelength absorbing chlorophyll contributes more effectively to the hydrazobenzene stimulated reaction than the short wavelength forms. The shape of the top spectrum suggests that system 1 is largely responsible for the hydrazobenzene-DCIP photoreaction at low intensity. The quantum yield was not lowered by the addition of DCMU: The initial parts of the rate *versus* intensities curves with and without DCMU coincided (Fig. 17). Around 710 nm the quantum yield (electrons per quantum) for the hydrazobenzene-DCIP reaction exceeded 2 for most preparations: *e.g.* the quantum yield at 711 nm calculated by

extrapolation to zero intensity (Fig. 6), amounted to 5.5 and the quantum yield at the lowest intensity actually used was about 4. The high quantum yield around 710 nm indicates that the hydrazobenzene-DCIP reaction is not an electron transfer *via* photoreaction 1, but a redox reaction catalyzed by some intermediate or product of the system 1 reaction. This reaction resembles the disproportionation of diphenylcarbazone described by Shneyour and Avron (1971) which occurs with a high quantum yield and seems to be also a system 1 catalyzed reaction. The quantum yield of the normal DCIP reduction with water as donor was in our preparations about 0.5 electron equivalents per absorbed quantum which is the expected order of magnitude. In the presence of diphenylcarbazide, dichlorophenylhydrazine, phtaloylhydrazine or of semicarbazide (see Table 4) the quantum yield at 683 nm of DCIP reduction was the same as without donor; the variations found were of the same order of magnitude as the variations between different measurements without added donor. In the presence of donor (with the exception of hydrazobenzene) the ratio of the yields at 683 and at 711 nm was about half of that without donor, indicating that under these conditions (low light intensity) some system 1 activity may contribute to the DCIP reduction if a donor is present.

TABLE 4

THE QUANTUM YIELD OF THE DCIP REDUCTION IN CHLOROPLASTS AND THE RATIO OF THE YIELDS IN RED AND FAR RED LIGHT IN THE PRESENCE OF SEVERAL HYDRAZINE COMPOUNDS.

The quantum yields were measured at 683 nm and at 711 nm with chloroplasts (see Chapter II), 15 μg chlorophyll/ml, in the presence of 20 μM DCIP.

Addition	*Yield at 683 nm*	*Ratio of the yields 683/711*
-	0.45	2.8
20 μM Hydrazobenzene	2.4	0.5
50 μM *sym.* Diphenylcarbazide	0.48	1.6
50 μM Dichlorophenylhydrazine	0.32	1.5
1 mM Phtaloylhydrazine	0.44	1.4
8 mM Semicarbazide	0.34	1.5

The mechanism of the DCIP photoreduction in the presence of hydrazobenzene is not known. We observed that the dark reduction is strongly pH dependent (Fig. 2). This might suggest that, at least in chloroplasts, the photocatalysis is due to a lowering of the pH inside the thylakoid upon illumination. This appears unlikely because of addition of valinomycin *plus* nigericin, which removes the pH-gradient over the thylakoid membrane, did not alter the quantum requirement significantly, as shown by Fig. 16. This Figure also shows that PMS evidently enhanced the quantum requirement of the reaction. It may eliminate the above mentioned intermediate produced in the light by system 1, which acts as a catalyst, by stimulating a cyclic process around photosystem 1.

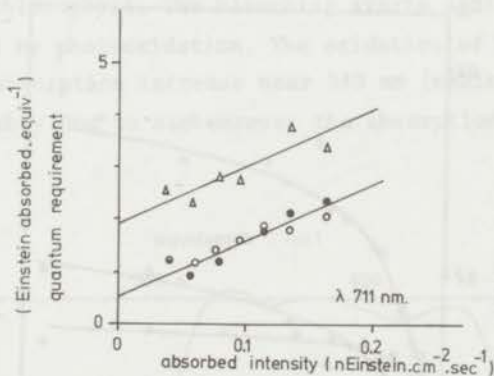


Fig. 16. The influence of added PMS and valinomycin *plus* nigericin on the quantum yield at 711 nm of the DCIP reduction by chloroplasts in the presence of hydrazobenzene; 5 μg chlorophyll/ml, 20 μM DCIP and 20 μM hydrazobenzene. Open circles, without further additions; solid circles, with 0.5 μM valinomycin and 1 μM nigericin; triangles, with 50 μM PMS.

Fig. 15b shows that the quantum yield for the hydrazobenzene-DCIP reaction was much higher in system 1 than in system 2 particles. This finding is consistent with the conclusion that a system 1 reaction is involved. Surprising is that at shorter wavelengths the quantum yields of the system 1 particles were appreciably lower than at 710 nm. This may indicate that in system 1 particles the pigments of photosystem 1

absorbing at longer wavelengths are predominantly active. The peak near 710 nm is not pronounced in the spectrum with system 2 particles. In these particles the pigments of system 2 participate in the normal DCIP reduction *via* the reaction centers of system 2 and an appreciable fraction of the activity will be due to contamination with system 1 (according to Vredenberg and Slooten, 1967, "system 2 particles" contain about 30% system 1). Between the various samples of digitonin subchloroplast particles the quantum yields of the hydrazobenzene-DCIP reaction showed large fluctuations: from values below 0.5 to more than 2 for system 1 particles. However, the relative heights of the spectra for system 1 and system 2 particles were roughly similar to those of Fig. 15b.

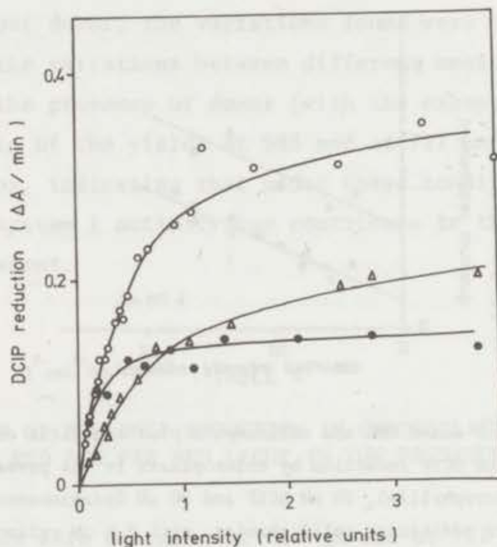


Fig. 17. Light intensity dependence of the DCIP reduction in chloroplasts. The reaction mixtures contained: 20 μM DCIP, 5 μg chlorophyll/ml. Triangles, without further additions; open circles, with 20 μM hydrazobenzene; solid circles, with 20 μM hydrazobenzene and 5 μM DCMU. The actinic light was filtered by means of a Balzers "K6" interference filter and a Calflex C-1 giving a band from approx. 620-675 nm.

3. Pigment bleaching in system 2 particles.

In system 2 particles, as in Tris-treated chloroplasts (K. Yamashita *et al.*, 1969; Itoh *et al.*, 1969) pigments are bleached by photooxidation. Additions of 5 μM CCCP enhanced the rate of photobleaching about twofold. The pigment bleaching was followed at 490 nm in the carotenoid band, where the bleaching, which occurred also in other spectral regions (Fig. 18), was most pronounced. Table 1 shows that addition of hydrazine compounds prevented the pigment bleaching presumably be reducing the oxidized donor of system 2. Hydrazobenzene prevented pigment bleaching at very low concentrations (Fig. 19a). There is an effect even at a concentration of 0.2 μM, at which 1 molecule hydrazobenzene is present per 75 molecules of chlorophyll. The bleaching starts again when hydrazobenzene is exhausted by photooxidation. The oxidation of hydrazobenzene is indicated by an absorption increase near 340 nm (maximum at 320 nm), which was presumably due to azobenzene: the absorption spectrum of azo-

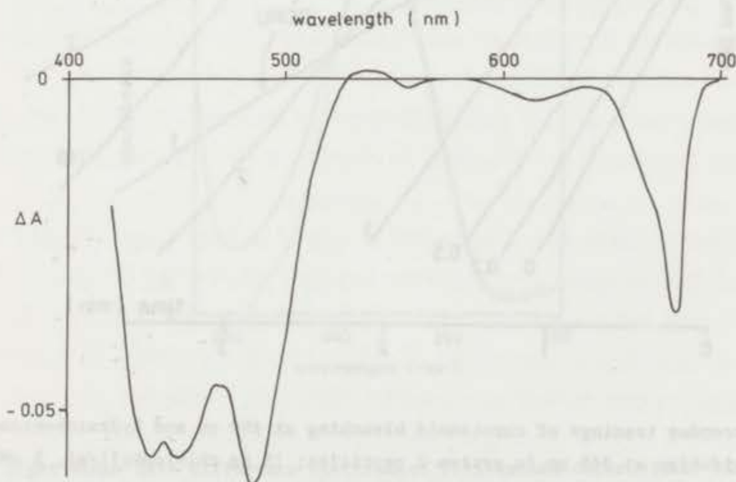


Fig. 18. The light *minus* dark difference spectrum of digitonin system 2 particles (15 μg chlorophyll/ml) in the presence of 5 μM CCCP. The spectrum was recorded after 3 min of illumination by saturating red light.

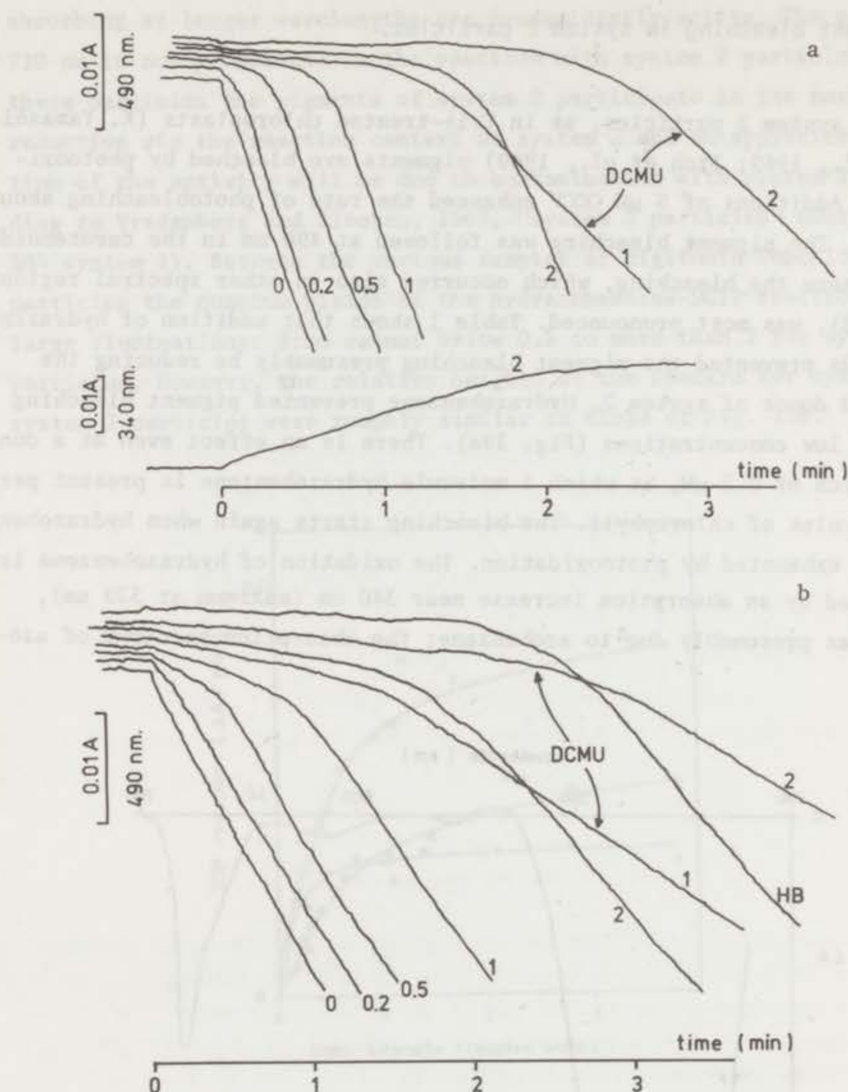


Fig. 19. Recorder tracings of carotenoid bleaching at 490 nm and hydrazobenzene photooxidation at 340 nm in system 2 particles; 15 μg chlorophyll/ml, 5 μM CCCP was added to stimulate carotenoid bleaching. Fig. 19a: 0, without further additions; 0.2, 0.5, 1 and 2 with 0.2, 0.5, 1 and 2 μM hydrazobenzene respectively; 0.5 μM DCMU added where indicated. Fig. 19b: 0, without further additions; 0.2, 0.5, 1 and 2 with 0.2, 0.5, 1 and 2 μM dipenylhydrazine respectively; 0.5 μM DCMU and 2 μM hydrazobenzene added where indicated. The inhibition by 0.5 μM DCMU in the absence of donor was the same as observed after exhaustion of the donor.

benzene was the same as that of the product formed in the light from hydrazobenzene (Fig. 20). DCMU inhibited carotenoid bleaching, apparently by inhibiting electron transport. As Fig. 19a shows, it also retarded the hydrazobenzene consumption. Fig. 19b shows (with another sample of system 2 particles) that also the isomer of hydrazobenzene, diphenylhydrazine is very effective in preventing carotenoid bleaching. In these experiments no electron acceptor was added, O_2 possibly acted as such. The bottleneck at the electron acceptor side of system 2 which limits the rate of electron transport to about 4 μmoles.mg chlorophyll^{-1}.h^{-1} (based on a molar extinction coefficient for azobenzene of $1.2 \cdot 10^5$ at 340 nm, Fig. 20) explains the fact that low concentrations of hydrazobenzene are not limiting and are thus effective in these particles.

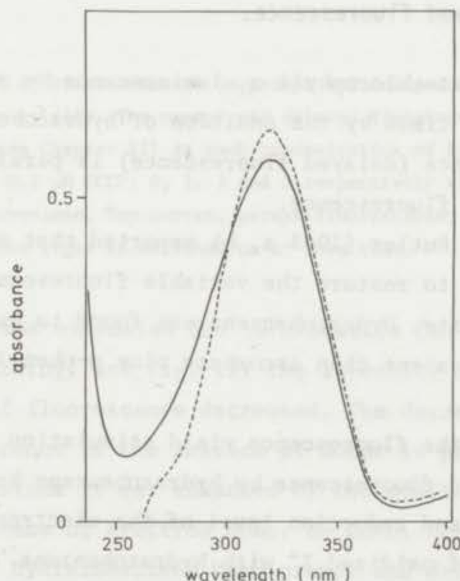


Fig. 20. Light *minus* dark difference spectrum of Tris-washed chloroplasts (15 μg chlorophyll/ml) after 3 min illumination with saturating red light in the presence of 50 μM hydrazobenzene, 0.5 mM methylamine and 50 μM methylviologen (dashed curve). The other curve represents the absorption spectrum of 30 μM azobenzene diluted from an alcoholic solution in buffer (0.2% final alcohol concentration): 50 mM Tricine-KOH buffer pH 7.8, 10 mM KCl and 2 mM $MgCl_2$.

Donors requiring lower concentrations for good rates of DCIP reduction require also lower concentrations for inhibiting carotenoid bleaching (see Table 1). The three compounds requiring the highest concentration can be easily dissolved in buffer, the other require an organic solvent: ethanol. It seems likely that the more effective donors which are more hydrophobic, dissolve more readily in the membranes. So partitioning of these donors between medium and chloroplast membranes is in favour of the latter, which is the cause that low external concentrations are sufficient. This partitioning is worked out quantitatively by Izawa and Good (1965) for herbicides (DCMU).

4. Effect of addition of hydrazobenzene and other hydrazines on prompt and on 1 msec delayed fluorescence.

Fig. 21 shows that chlorophyll a_2 luminescence by system 2 particles is diminished 2-3 times by the addition of hydrazobenzene. This decrease in luminescence (delayed fluorescence) is paralleled by a stimulation of the prompt fluorescence.

T. Yamashita and Butler (1968 a, b) reported that artificial electron donors were able to restore the variable fluorescence yield of Tris-washed chloroplasts. Hydrazobenzene was found to have the same effect but to a larger extent than ascorbate *plus* *p*-phenylenediamine (see Fig. 22).

Presumably both the fluorescence yield stimulation and the diminishing of the delayed fluorescence by hydrazobenzene have a common cause, *viz.* an increased reduction level of the electron donor Z of system 2 by a reaction of oxidized Z^+ with hydrazobenzene. The stimulation of the prompt fluorescence yield was expected since the rate of photoreduction of Q which causes the increase in fluorescence (Duysens and Sweers, 1963) is enhanced by the presence of electron donor. The delayed fluorescence which is due to a backreaction between Q^- and the primary photooxidant in the presenze of Z^+ diminished because Z^+ is reduced more rapidly by hydrazobenzene and reduced Z reacts faster with the primary photooxidant of system 2 than Q^- does (see also Chapter I). When the

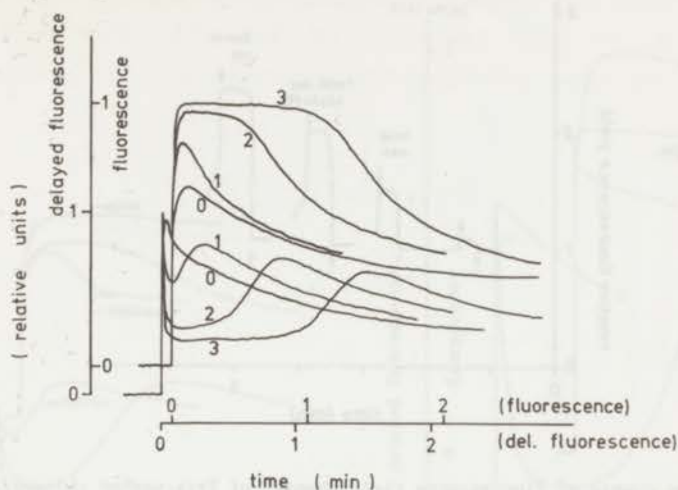


Fig. 21. The effect of hydrazobenzene on prompt and delayed chlorophyll fluorescence in system 2 particles. The prompt and delayed fluorescence are measured simultaneously (see Chapter II) at each concentration of hydrazobenzene. 75 μg chlorophyll/ml; 0.2 μM CCCP; 0, 1, 2 and 3 respectively without and with 1, 2 and 3 μM hydrazobenzene. Top curves, prompt fluorescence; lower curves delayed fluorescence. The light is switched on at zero time.

hydrazobenzene was exhausted due to oxidation (as in the experiments with carotenoid bleaching, see Fig. 19) the intensity of delayed fluorescence increased and of fluorescence decreased. The decrease in both prompt and delayed fluorescence in the absence of donor is presumably caused by pigment bleaching since it was enhanced by the addition of CCCP, and inhibited in the presence of electron donor or DCMU. During illumination in the presence of hydrazobenzene both prompt and delayed fluorescence returned to their original level in a shorter time in the presence of CCCP than in its absence: hydrazobenzene oxidation was faster (about 10 times in the presence of 0.2 μM CCCP), probably due to acceleration of the reduction of Z^+ by hydrazobenzene. CCCP might enhance the reactivity of Z^+ (*cf.* Renger, 1971). Analogous effects were observed with carotenoid bleaching: hydrazobenzene caused a longer delay in the bleaching if no CCCP was present. In the experiment shown in Fig. 21 the initial delayed

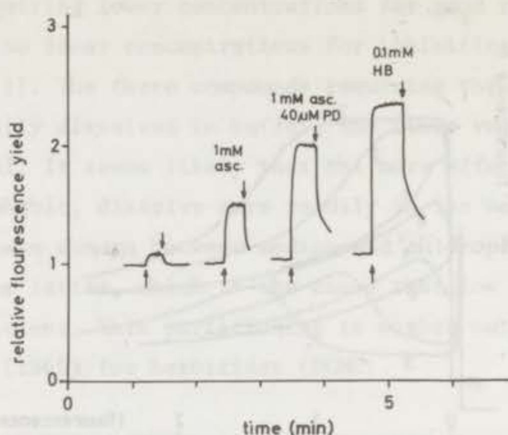


Fig. 22. Time course of fluorescence yield changes of Tris-washed chloroplasts (75 μg chlorophyll/ml) upon illumination with actinic light in the presence of the additions as indicated; asc, ascorbate; PD, *p*-phenylenediamine; HB, hydrazobenzene. The fluorescence was excited by a weak modulated blue-green beam, originating from L_1 (See Fig. 7). The actinic beam was provided by L_2 and filtered by means of a Corning Cs 4-96 and a Schott BG18 glass filter; intensity at the place of the cuvette about 5 $mW.cm^{-2}$. Upward and downward pointing arrows indicate switching on and off the actinic light, respectively. The amplifier (A_1, Fig. 7) responded only to the modulated fluorescence excited by L_1 but not to the steady fluorescence excited by L_2.

fluorescence emission was diminished 2 times by the addition of 0.2 μM CCCP. Perhaps also this may be explained by an enhancement of the reactivity of Z^+ by which the actual concentration of Z^+ is diminished due to a reaction with some donor, maybe a carotenoid.

The effect of catalase shown in Fig. 23 indicates that the effect of hydrazobenzene on prompt and delayed fluorescence with Triton system 2 particles is in part due not to a direct effect of the donor but to H_2O_2. The H_2O_2 may be formed by reduction of oxygen (as in the experiments with carotenoid bleaching, no electron acceptor was added), perhaps *via* Q^-, perhaps *via* system 1 in a Mehler reaction (Mehler, 1951). H_2O_2 can act as electron donor to system 2 (Inoué and Nishimura, 1971). Addition of H_2O_2 to Triton system 2 particles stimulated the prompt fluorescence and diminished the delayed fluorescence emission (Van Gorkom and Haveman, in preparation).

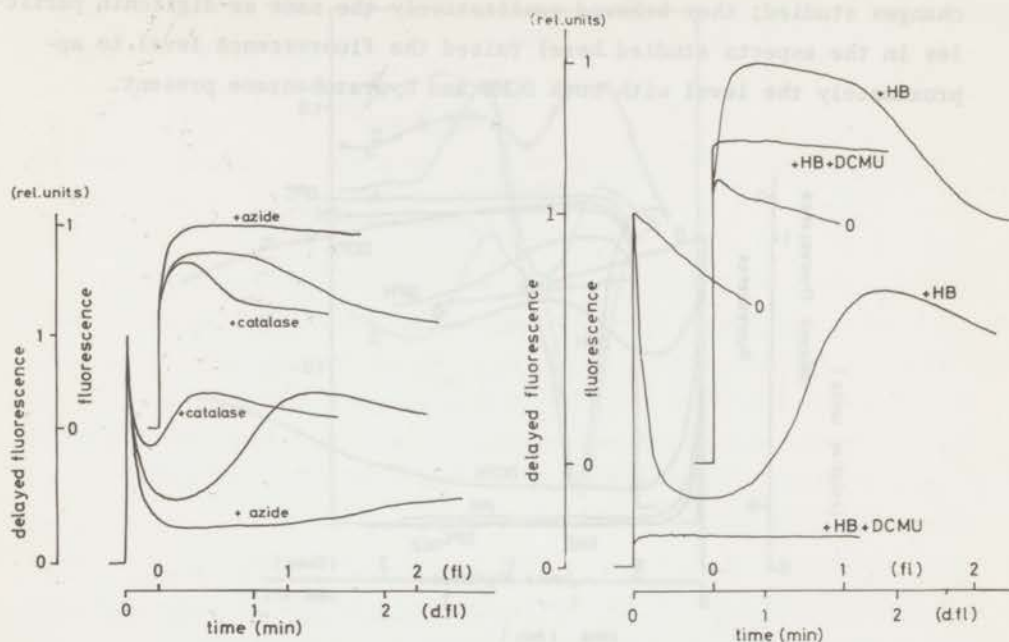


Fig. 23. The influence of catalase and sodium azide on the effects of hydrazobenzene on prompt and delayed fluorescence in particles enriched in system 2 prepared by means of Triton-X-100; 75 µg chlorophyll/ml, 2 µM hydrazobenzene; 1 mM NaN_3 or 20 µg/ml catalase added where indicated. Top curves, prompt fluorescence; lower curves, delayed fluorescence. Further conditions, see Chapter II.3.

Fig. 24. The effect of DCMU on the fluorescence rise induced by hydrazobenzene. The cuvette contained: particles enriched in system 2 (Triton), 75 µg chlorophyll/ml, 2 µM hydrazobenzene (HB) and 5 µM DCMU where indicated. Further conditions, see Chapter II.3.

In the presence of DCMU there is no stimulation of the fluorescence yield by hydrazobenzene (Fig. 24). This paradoxal finding was further investigated by Van Gorkom and Haveman (in preparation). The conclusion was that in these particles in addition to Q another quencher has to be reduced which is reduced by Q^-. Addition of DCMU to the Triton system 2 particles (which we used because they did not have light-induced changes in fluorescence yield without donor, which might interfere with the

changes studied; they behaved qualitatively the same as digitonin particles in the aspects studied here) raised the fluorescence level to approximately the level with both DCMU and hydrazobenzene present.

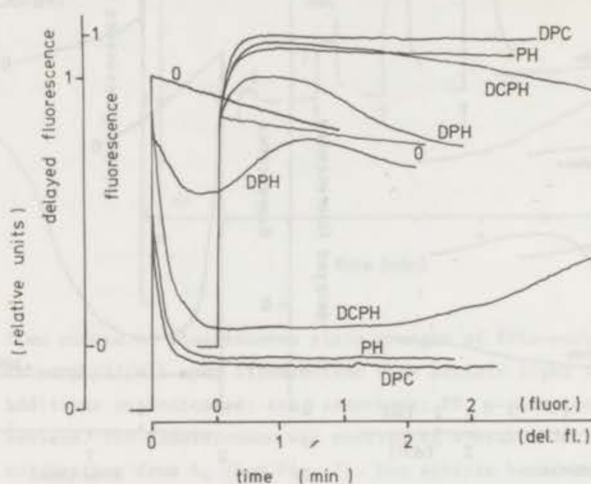


Fig. 25. The effects of various donors on prompt and delayed fluorescence of particles enriched in system 2 (Triton), 75 µg chlorophyll/ml; DPH, with 2 µM diphenylhydrazine; DCPH, with 2 µM dichlorophenylhydrazine; PH, with 200 µM phtaloylhydrazine; DPC, with 200 µM diphenylcarbazide. Further conditions, see Chapter II.3.

In Fig. 25 are shown the effects of some other hydrazine compounds on prompt and delayed fluorescence with Triton system 2 particles. They all acted like hydrazobenzene. For phtaloylhydrazine and diphenylcarbazide higher concentrations than for hydrazobenzene were necessary.

5. Cytochrome b_{559} oxidation in system 2 particles.

(This work was done in cooperation with H.J. van Gorkom)

In Fig. 26 the light-induced changes in the α- and β-band region of cytochromes of the system 2 particles are shown. They were determined by scanning spectra before and during illumination and subtracting them

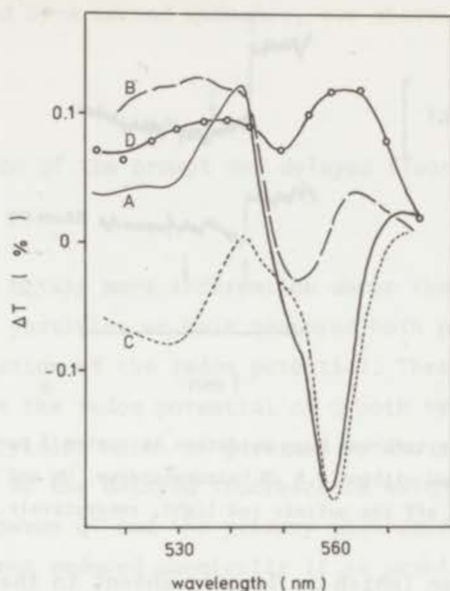


Fig. 26. Light *minus* dark difference spectra of system 2 particles. 50 μg chlorophyll/ml, in the presence of 0.5 μM nigericin, 1 cm light path. Curve A without further additions, Curve B with 10 μM hydrazobenzene, Curve C is Curve A *minus* Curve B, Curve D with 1 μM CCCP. The half band width was about 2.5 nm. The noise level was about 0.01% of the transmitted light.

(Curves A and B) or by measuring the absorption change upon illumination at a fixed wavelength using a new sample for each point (curve D). Nigericin was added to minimize contributions of the 515 nm change which affect the spectrum up to 545 nm. A photooxidation of cytochrome b_{559} is evident.

The photooxidation was completely inhibited by 5 μM DCMU (not shown) indicating that this photooxidation is due to photosystem 2 activity. The photooxidation was largely prevented by 10 μM hydrazobenzene (curve B) (for 50% by 0.8 μM hydrazobenzene). Addition of hydrazobenzene did not change the baseline in the dark and did not accelerate the (very) slow reduction of cytochrome b_{559} in the dark after illumination (see Fig. 27). These results indicate that cytochrome b_{559} photooxidation can be observed if the photooxidant of system 2 is not reduced by the

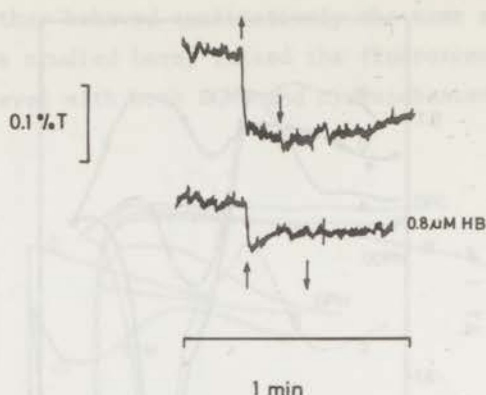


Fig. 27. Kinetics of the cytochrome b_{559} oxidation in system 2 particles, 15 μg chlorophyll/ml, with and without 0.8 μM hydrazobenzene. Up and downward arrows: switching on and off the actinic red light, respectively.

water splitting system (which is largely absent in these system 2 particles) or by hydrazobenzene. Knaff and Arnon (1969 c) using Tris-treated chloroplasts and Erixon and Butler (1971) using untreated chloroplasts at liquid N_2 temperature presumably could observe cytochrome b_{559} for the same reason.

Hydrazobenzene efficiently competes with cytochrome b_{559} as a donor to photosystem 2. Phtaloylhydrazine at a concentration of 0.5 mM prevented pigment bleaching but did not appreciably prevent cytochrome b_{559} oxidation, which suggests different points of entry for hydrazobenzene and phtaloylhydrazine, or a less efficient reduction by the latter compound.

Curve D suggests that in the presence of 1 μM CCCP some photoreduction occurred, which was inhibited by 5 μM DCMU. In the presence of CCCP presumably cytochrome b_{559} shifts from its high potential form to its low potential form (see Cramer and Böhme 1972).

The shoulder at 550 nm and the increase in absorption near 540 nm in the difference spectrum (curve A, Fig. 26) may be due to C550. If this is true, then C550 is not influenced by addition of hydrazobenzene (curve B), which is in contrast with the effects of hydrazobenzene on fluorescence. This finding may agree with the conclusion that the fluorescence

may be determined by a second quencher, see above. (Van Gorkom and Haveman, in preparation).

6. Redox titration of the prompt and delayed fluorescence from system 2 particles.

In order to obtain more information about the photochemical properties of system 2 particles we have measured both prompt and delayed fluorescence as a function of the redox potential. These titrations should give information about the redox potential of Q both by direct titration of the fluorescence yield, which is governed by the redox state of Q, and by the titration of the delayed fluorescence which originates from a back-reaction between Q^- and the primary photooxidant. After the primary acceptor Q has been reduced chemically it is unable to accept electrons upon excitation of the reaction center and the primary photooxidant will stay in its reduced form, therefore a backreaction yielding delayed fluorescence should not occur under these conditions.

Fig. 28a shows a titration of the prompt fluorescence. The reductive titration with dithionite was started at a high redox potential after addition of a small quantity of potassium ferricyanide. After the reductive titration the oxidative titration with ferricyanide immediately followed. The concentration of ferricyanide after both titrations never exceeded 0.5 mM. The midpoint potentials were -60 mV and +85 mV respectively for the reductive and oxidative titration. The delayed fluorescence was measured simultaneously with the prompt fluorescence, the result of the titrations is shown in Fig. 28b. In this case, the midpoint potentials were -90 mV and +50 mV,respectively, for the reductive and oxidative titration. The origin of the discrepancy between the oxidative and reductive titrations is not clear, perhaps it is due to retardations in the flow system of the titration set-up or to an imperfect "reversibility" of the redox reactions. The fluorescence returns in the oxidative back-titration to nearly exactly its original level.

In Fig. 29 the results of both reductive titrations are replotted according to the Nernst equation (Clark, 1960). The slopes of the lines

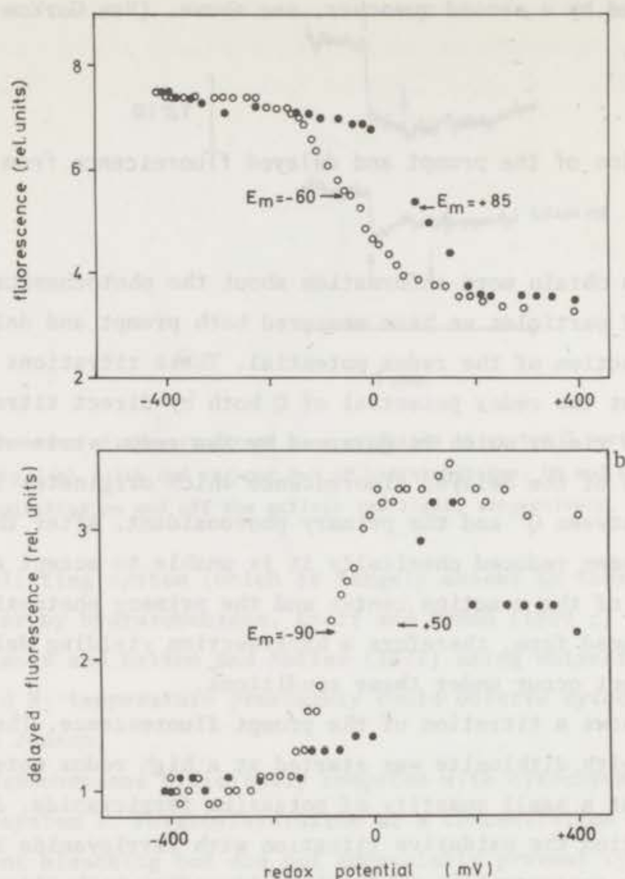


Fig. 28a. Relative fluorescence intensity of system 2 particles at pH 7.8 as a function of the redox potential of the medium. Open circles: reductive titration by sequential additions of aliquots of dithionite, solid circles: subsequent oxidative titration by sequential additions of small aliquots of ferricyanide. See the text for further explanation.

Fig. 28b. Relative intensity of delayed fluorescence of system 2 particles as a function of the redox potential of the medium measured simultaneously with the prompt fluorescence. Open circles reductive titration, solid circles oxidative titration.

were 0.7 for prompt fluorescence and 0.8 for delayed fluorescence, indicating that the reduction of Q is a 1 electron process. However, the

deviation from 1, especially with the prompt fluorescence data, may suggest that a second fluorescence quenching process is interfering with the quenching by Q. The midpoint potential of this second process

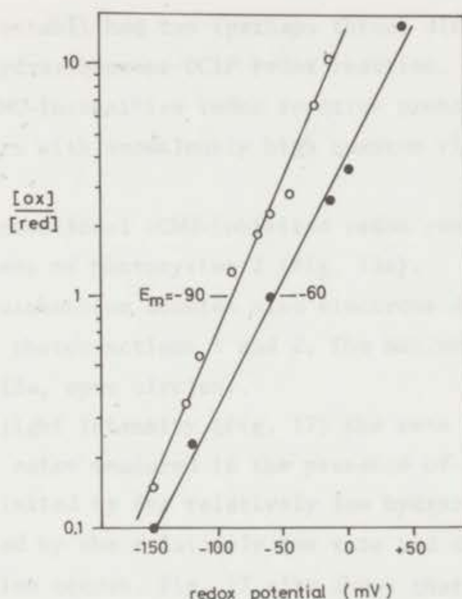


Fig. 29. Replotting of data from Fig. 28 according to the Nernst equation. Open circles: reductive titration of the delayed fluorescence, solid circles: reductive titration of the prompt fluorescence.

may be slightly higher than that of Q, so it is difficult to recognize. If this is true the titration of the delayed fluorescence may give a more precise estimate of the midpoint potential of Q because it is only dependent on the redox potential of Q and not of the supposed other quencher.

If we take the values obtained by titration of the delayed fluorescence as the limits between which the redox potential of Q must lie: -90 mV and +50 mV at pH 7.8 than there is a rather good agreement with the data of Cramer and Butler (1969). They found midpoint potentials of -95 and -90 mV at pH 8.0 for the oxidative and reductive titration respec-

tively. In contrast to their results, however, we never found a quenching process at a very low redox potential (about -300 mV at pH 8.0). Apart from this difference it may be stated that our results strongly indicate that the primary photochemical reaction is basically the same as that of intact chloroplasts.

CHAPTER IV.

CONCLUSIONS

We have established two (perhaps three) different photoreactions driving the hydrazobenzene-DCIP redox reaction.

(1) A DCMU-insensitive redox reaction probably catalyzed by system 1, which occurs with anomalously high quantum yields of 2-5 near 710 nm (Fig. 15).

(2) A conventional DCMU-inhibited redox reaction driven by the reaction centers of photosystem 2 (Fig. 13a).

(3) Hydrazobenzene donates also electrons directly to the redox chain between photoreactions 1 and 2. The maximum rate of this reaction is low (Fig. 13a, open circles).

At high light intensity (Fig. 17) the rate of reaction (1) (represented by the rates measured in the presence of DCMU and 20 μM hydrazobenzene) is limited by the relatively low hydrazobenzene concentration, as is indicated by the relatively low rate and the low intensity at which saturation occurs. Fig. 17 also shows that without DCMU at high intensity the reaction rate is about equal to the sum of the rate of the reaction with DCMU and that of the "normal" Hill reaction (without hydrazobenzene). However, at low light intensity, below saturation, addition of DCMU to the chloroplasts with hydrazobenzene did not decrease the reaction rate, at least not by the amount of the normal Hill reaction. The same is true at high light intensity if the concentration of hydrazobenzene is high (100 μM). The explanation of this phenomenon may be that the system 1 reaction is stimulated by the addition of DCMU, so that the loss in activity due to inhibition of the normal Hill reaction is approximately compensated by an increase in the rate of the system 1 catalyzing reaction. This only occurs if the hydrazobenzene concentration is not limiting the system 1 reaction. In the presence of DCMU the components of the redox chain between Q and system 1 tend to get oxidized and one of these components or a substance oxidized by it, may thus be responsible for catalyzing the hydrazobenzene-DCIP reaction. The obser-

vation that the high quantum yield of the system 1 catalyzed reaction is diminished by the addition of PMS is in agreement with this conclusion. PMS is able to eliminate the oxidized component(s), responsible for the catalysis, in a cyclic reaction around system 1. The observation with system 2 particles that at higher concentrations of hydrazobenzene the percentage of inhibition by DCMU is less than at lower concentrations (Fig. 11a) may also be explained by system 1 activity. At higher concentrations of hydrazobenzene the system 1 reaction is apparently more pronounced. The observation that with system 2 particles the quantum yield of the DCIP reduction with 20 μM hydrazobenzene present is as high at 711 nm as at 683 nm also indicates that a system 1 reaction is involved. (Fig. 15b).

The hypothesis that hydrazobenzene is a very efficient donor to photosystem 2, if the oxygen evolving mechanism is destroyed in some way (Tris-wash, digitonin treatment *etc.*), is supported, not only by the relatively high rate of the DCMU-sensitive hydrazobenzene DCIP reaction, but also by other effects (see below and *e.g.* Table 3).

Also the isomer of hydrazobenzene, N,N-diphenylhydrazine and 2,5 dichlorophenylhydrazine and N,N'-phtaloylhydrazine appeared to be donors to photosystem 2. Although less efficient than hydrazobenzene, these donors were more or as efficient as the already known donors semicarbazide and *sym*-diphenylcarbazide. The DCIP reduction with these donors was inhibited by DCMU but at low light intensity (Table 4), with untreated chloroplasts, where presumably the hydrazines were not able to act as donors to photosystem 2, some contribution of system 1 may be observable. With methylviologen mediated oxygen uptake all the hydrazines appeared to donate mainly to photosystem 2, as is indicated by the DCMU sensitivity; a small part of the activity, however, is due to direct electron donation to the system 1 reaction (Fig. 13).

Pigmentbleaching, in system 2 particles, caused by destructive activity of accumulated oxidized components produced by photosystem 2, is prevented by hydrazobenzene and the other compounds by donation of electrons to system 2.

Bound cytochrome b_{559} is oxidized in system 2 particles. Hydrazobenzene prevents this oxidation. Phtaloylhydrazine, even at concentra-

tions which nearly totally inhibit pigment bleaching is unable to do so.

Also the effects of hydrazobenzene and the other hydrazine compounds on prompt and delayed fluorescence in subchloroplast particles prepared by means of digitonin or of Triton-X-100 can be explained by the electron donation to system 2. The effects, at least under the conditions described here, are complicated by the production of H_2O_2, which on its turn acts as a donor to system 2.

Redox titrations of both prompt and delayed fluorescence of system 2 particles indicated that the midpoint potential of the primary electron acceptor (photoreductant) of system 2 lies between -90 mV and +50 mV (pH 7.8). Deviation of the fluorescence titration curve from a one electron transport process suggested that the fluorescence yield was also influenced by a second quencher with a slightly higher midpoint potential (Fig. 28, 29). Also the effect of DCMU on the light induced fluorescence yield changes of subchloroplast particles in the presence of hydrazobenzene, indicated participation of a second quencher. How far both observations refer to the same quencher is still unclear.

Hydrazobenzene may, because of its unusual high efficiency as donor, also for future research prove to be a valuable tool for the study of photosystem 2.

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SUMMARY

1. The photooxidation of hydrazobenzene with dichlorophenolindophenol (DCIP), methylviologen and $NADP^+$ as electron acceptors were studied in spinach chloroplasts, Tris-treated chloroplasts and subchloroplast particles, in the presence and absence of dichlorophenyldimethylurea (DCMU). The hydrazobenzene-DCIP photoreaction was much less inhibited by DCMU than the reactions with the other acceptors.

2. For chloroplasts the action spectrum of the quantum yield for the hydrazobenzene-DCIP redox reaction showed a maximum at 710 nm, indicating system 1 participation. Two or more electrons were transported per quantum absorbed at 710 nm.

3. It is concluded from these and other experiments that hydrazobenzene can be oxidized in at least two photoreactions. In the first place it acts as an efficient electron donor for system 2. This reaction is inhibited by 5 μM DCMU. A second hydrazobenzene-DCIP reaction, which is not inhibited by DCMU, is presumably catalyzed *via* an oxidized component of the redox chain between Q and system 1.

4. Addition of hydrazobenzene prevented efficiently cytochrome b_{559} oxidation, pigment bleaching and emission of luminescence and stimulated the light induced fluorescence increase of chlorophyll *a* in system 2 particles. These effects can be explained by the efficient electron donation to photosystem 2.

5. N,N-Diphenylhydrazine, N,N'-phtaloylhydrazine and dichlorophenylhydrazine were found to be relatively efficient donors to photosystem 2. They acted like hydrazobenzene, though less efficiently, with pigment bleaching, chlorophyll fluorescence and luminescence and different electron transport reactions. The redox reaction with DCIP was inhibited by DCMU.

6. The relative yield of fluorescence and luminescence of chlorophyll *a* of "system 2 particles" was measured as a function of the redox potential. The results of these redox titrations indicate that the midpoint potential of the primary electron acceptor Q, of system 2 lies between -90 mV and +50 mV at pH 7.8. The results suggest that a second fluorescence quenching process is involved.

SAMENVATTING

1. De fotooxydatie van hydrazobenzeen met dichlorofenolindofenol (DCIP), methylviologeen en $NADP^+$ als electronen acceptoren werd bestudeerd in spinazie chloroplasten, Tris gewassen chloroplasten en subchloroplast deeltjes, met en zonder het herbicide DCMU. De hydrazobenzeen-DCIP redox reactie was veel minder gevoelig voor DCMU dan de reacties met andere acceptoren.

2. Met chloroplasten vertoonde het actiespectrum van het quantum rendement van de hydrazobenzeen-DCIP reactie een piek bij 710 nm, hetgeen wijst op deelname van fotosysteem 1 aan deze reactie. Per geabsorbeerd quant bij 710 nm werden er twee of meer electronen getransporteerd.

3. Uit deze en andere experimenten volgt dat de fotooxydatie van hydrazobenzeen op tenminste twee manieren kan gebeuren: ten eerste in een DCMU gevoelige systeem 2 reactie en ten tweede in een DCMU ongevoelige reactie met DCIP die vermoedelijk wordt gecatalyseerd door een geoxydeerd product uit de keten tussen beide fotosystemen.

4. Toevoegen van hydrazobenzeen aan subchloroplast deeltjes verrijkt met fotosysteem 2 ("systeem 2 deeltjes") voorkwam efficient de fotooxydatie van cytochroom b_{559}, de pigmentbleking en de luminescentie emissie en het stimuleerde de fluorescentie emissie. Deze effecten kunnen worden verklaard door de effectieve electronendonatie van hydrazobenzeen aan fotosysteem 2.

5. N,N-Diphenylhydrazine, N,N'-phtaloylhydrazine en 2,5-dichlorophenylhydrazine bleken ook goede donoren voor fotosysteem 2. Ze vertoonden de zelfde werking als hydrazobenzeen, maar minder effectief, bij pigmentbleking, fluorescentie, luminescentie en verschillende electronen transport reacties. DCIP reductie in aanwezigheid van deze donoren werd geremd door DCMU.

6. Zowel de luminescentie als fluorescentie emissie van systeem 2 deeltjes werd gemeten als functie van de redox potentiaal van het medium. De resultaten van deze redox titratie wezen erop dat de "midpoint" potentiaal van de primaire electronen acceptor Q van system 2 lag tussen -90 mV en + 50 mV bij pH 7.8. De titratie van de fluorescentie deed vermoeden dat er sprake was van een tweede quencher met waarschijnlijk een iets hogere "midpoint" potentiaal dan Q.

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LEVENSLOOP

In 1961, na het behalen van het eindexamen H.B.S.B aan de H.B.S. "Beeklaan" te 's-Gravenhage, begon ik mijn studie in de faculteit der Wiskunde en Natuurwetenschappen aan de Rijks Universiteit te Leiden. In oktober 1965 legde ik het candidaatsexamen, letter K', af en in mei 1968 het doctoraal-examen Biologie met als hoofdvakken plantenfysiologie en biochemie, en als bijvak biofysica, respectievelijk onder de leiding van de hoogleraren Dr A. Quispel, Dr H. Veldstra en Dr L.N.M. Duysens. In juni 1968 werd ik benoemd tot wetenschappelijk medewerker bij het Laboratorium voor Biofysica in dienst van de Rijks Universiteit te Leiden en begon aan het onderzoek dat geleid heeft tot dit proefschrift. Van november 1970 tot juni 1972 was ik in dienst van de organisatie Z.W.O. Sinds juni 1972 ben ik in het kader van mijn militaire dienstplicht als ornitholoog verbonden aan de Sectie Luchtmachtbedrijfsveiligheid van de Luchtmachtstaf.