Isolation and Identification of an Oxygen Reducing Factor (ORF) from Isolated Spinach Choloroplast Lamellae

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From the water extracts of heat-treated spinach chloroplast lamellae, five compounds were isolated by column chromatography and identified by paper chromatography, UV-, IR-, ^{1}H-NMR- and ^{13}C-NMR spectrometry: besides the major component, sucrose, 1 g lyophilized material contained 197 mg oxalic acid, 4.6 mg uridine and uracil and 22 µg lumiflavin. Only the isolated lumiflavin stimulated photosynthetic oxygen reduction and H_2O_2 production by illuminated chloroplast lamellae. Evidence is presented that the stimulation of photosynthetic oxygen activation by water extracts of heat-treated chloroplast lamellae is most likely due to flavin derivative(s) (probably FAD) released from thylakoid-bound ferredoxin-NADP reductase by heat- and other treatments. Lumiflavin is artifactually derived from the solubilized flavin coenzymes during the isolation procedure.

Introduction

Numerous reports and reviews [1–4] appeared in the past on the preparation of compounds from plant material possibly involved as redox cofactors at the reducing side of photosystem I (PS I). Some of these factors were postulated to function as primary electron acceptors of PS I. The activities of the above factors were measured as stimulation of photosynthetic electron transport (PET) and/or photophosphorylation, using isolated chloroplast lamellae. The structures of these compounds remained unresolved, however, although proposals for possible structures have been made in some cases [1]. In 1966, Vernon and Ke [5] supposed that reduced chlorophyll (Chl) with a strongly negativ redox potential might be the reductant at the reducing side of PS I; Fuller and Nugent [6] suggested a pteridine derivative as primary electron acceptor while Wang [7] presented evidence for a flavinlike substance. In 1969, Yocum and San Pietro [8] isolated a compound with an absorption maximum at 270 nm; due to its activity in PET, it was called "ferredoxin reducing substance" (FRS). Similarly Regitz, Berzborn and Trebst [9] reported on a substance, $S_{L\text{-eth.}}$, solubilized from ether-treated chloroplast lamellae. This compound was also suggested to function between the primary electron acceptor of PS I and ferredoxin, possibly containing *p*-coumaryl-mesotartaric acid and 3-methyl-6,7-methylenedioxy quercetagetine [10, 11] as prosthetic groups [12]. $S_{L\text{-eth.}}$ thus showed properties also described as "P-compound" and "310-factor" [13]. The structures of the compounds described by Krogman and Stiller [14], Gee *et al.* [15] and Black *et al.* [16] remained unresolved, although a pteridine was suggested [17] as the active group of "Phosphodoxin", extracted and partially purified by Black *et al.* [16].

The question concerning the primary acceptor of PS I was solved by Malkin and Bearden [18], Hiyama and Ke [19] and Leigh and Dutton [20]: a thylakoid-bound non-heme-iron (NHI) was identified as the "true" electron acceptor of PS I.

There are, however, reports on activities of compounds isolated from bluegreen algae and chloroplasts, different to the ones suggested as primary electron acceptors of PS I:

a) the activity of a "cytochrome reducing substance" (CRS) reported by Fujita *et al.* [21] has been shown to be due to an FE^{III+}/EDTA-complex [22] and thus most probably artifactual;

b) an oxygen reducing factor from sugar beet leaves, stimulating the hydrogen peroxide photoproduction by isolated chloroplast lamellae has been identified as 3-hydroxytyramine [23];

c) water-soluble factors from Chenopodium bonus henricus leaves catalyzing oxygen photoreduction by isolated chloroplast lamellae were not identified, but there was a similarity with reactions catalyzed by certain quinones [24];

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d) Honeycut and Krogman [25, 26] described an oxygen reducing substance "ORS", extracted from the cyanobacteria *Anabaena.* This factor with an appearant molecular weight of ca. 3000 to 4000 showed an absorption maximum at 265 nm. The chemical nature of the compound(s) was not identified;

e) isolated chloroplast lamellae from spinach release a low molecular weight and heat stable "oxygen reducing factor" (ORF) upon heat treatment. This factor is not identical with the described compound isolated from sugar beet leaves, 3-hydroxytyramine [23] but bears some resemblance to o-diphenols as far as the products of photosynthetic oxygen reduction, H_2O_2 (and not $O_2^{\cdot -}$!) and other properties (inhibition of H_2O_2 production by ascorbate) of the catalysis are concerned [27, 28].

As outlined above, none of the chloroplast-bound autooxidizable cofactors of PET with absorption maxima in the UV near 260 to 270 nm has so far been identified. We here report on the isolation and identification of a flavin as the active substance in the UV-absorbing, crude ORF preparations.

Results and Discussion

Chemical nature of ORF

Preparation of ORF crude powder: 100 kg of deveined spinach leaves were homogenized in batches of 10 kg with 6 *l* grinding solution containing 120 ml 1 M Tris-HCl buffer pH 8.0, 120 ml NaCl (1 M) -solution and 5760 ml 0.4 M Sucrose solution. The homogenate was filtered through a nylon net and the filtrate was centrifuged for 30 min at 13 200 × *g*. The green pellet was resuspended with 2 l 0.1 M Tris-HCl buffer pH 8.0 and centrifuged for 30 min at ca. 20 000 × *g*. The resulting pellet was suspended in 1.3 l H_2O. Batches of 5 ml were heat-treated (5 min at 80 °C) and recentrifuged for 30 min at 20 000 × *g*. The pale-green supernatant (SN) was recentrifuged twice at 20 000 × *g*. The clear, yellow SN was evaporated at 30 °C under reduced pressure and the resulting concentrate was lyophilized. From 100 kg spinach leaves we obtained 112 g "ORF crude powder".

Activity of "ORF crude powder": As already reported [27, 28] ORF-preparations stimulate oxygen

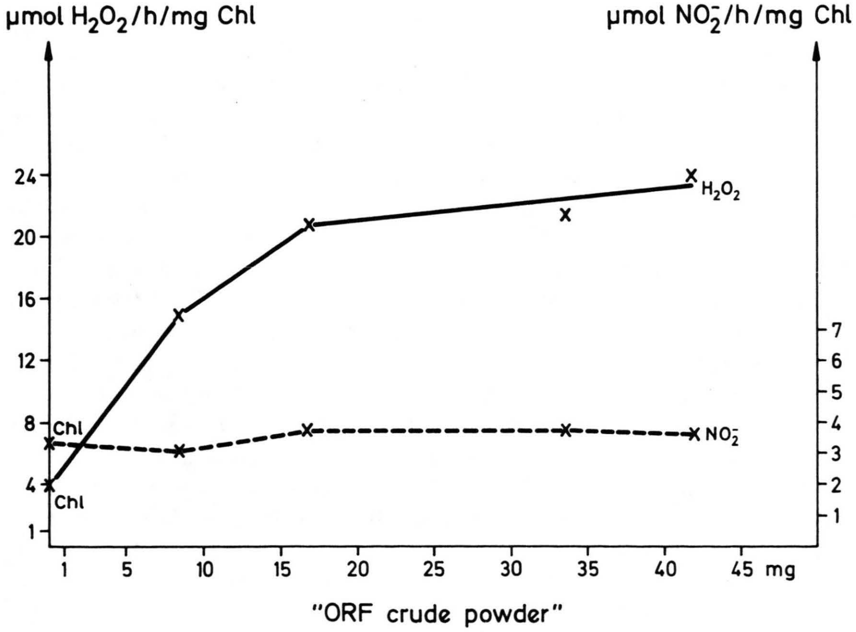


Fig. 1. Activities of crude ORF preparations from isolated spinach chloroplast lamellar. a) H_2O_2-formation; b) $O_2^{\cdot -}$-formation. Reaction mixture: 2 ml contained: 100 µmol phosphate buffer pH 7.8 5 µmol NH_4Cl; 5 µmol $MgCl_2$; 0.5 mM KCN; in the $O_2^{\cdot -}$ test: 1 µmol NH_2OH; chloroplast lamellae with 100 µg Chl. Addition: increasing amounts of "ORF crude powder" (see text). Reaction conditions: the reaction vessels were illuminated (white light, 30 klux from the bottom) for 10 min at 18 °C. After the reaction, the mixture was centrifuged for 15 at 5000 × *g* and the SN was tested for H_2O_2 and NO_2^- (see experimental).

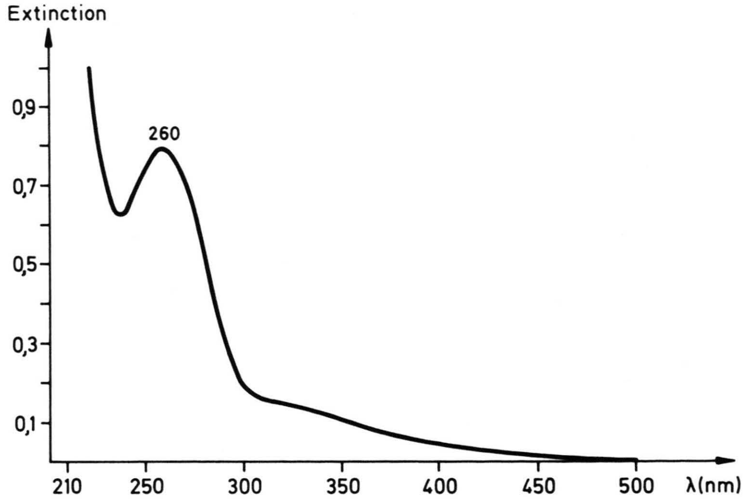


Fig. 2. UV spectrum of "ORF crude powder" (1.32 mg in 2 ml H_2O).

uptake and concomitant H_2O_2 production by illuminated chloroplast lamellae. In contrast to other autoxidizable electron acceptors of PS I as paraquat or anthraquinone, superoxide radical ion ($O_2^{\cdot -}$) is not an observable intermediate with ORF or 3-hydroxytyramine as catalysts of oxygen reduction.

As shown in Fig. 1, and apparent saturation of H_2O_2 production is observed with ca. 20 mg "ORF crude powder" (2 ml choroplast suspension with 100 µg Chl) while $O_2^{\cdot -}$ formation (measured as hydroxylamine oxidation, *cf.* ref. [29] and [30]) is not stimulated after ORF additions up to 40 mg. ORF crude powder, dissolved in H_2O shows an absorption maximum at 260 nm and a shoulder at ca. 315 nm (Fig. 2).

Isolation and identification of sucrose, oxalic acid, uridine, uracil and lumiflavin from ORF crude powder: Sucrose and oxalic acid have been determined as major components of ORF crude powder. 8.7 g of ORF crude powder dissolved in 5 ml H_2O were eluted from a DE_{52} (Whatman)-column (3 × 30 cm) with 350 ml H_2O (flow rate: ca. 100 ml/h). The effluent had no ORF activity; the concentrated effluent showed and NMR (^1H)-spectrum identical with the one of authentic sucrose (data not shown).

Sucrose was used for the preparation of the chloroplast lamellae and thus artificially introduced into the ORF Preparation: for the preparation of 112 g of ORF crude powder from 100 kg spinach, 10.5 kg sucrose was used! After elution with 350 ml H_2O, the above DE_{52} column was eluted with 800 ml 0.1 M NaCl solution. The yellow effluent was evaporated to dryness and HCl conc. was added. Cristalline NaCl was filtered off, again evaporated to dryness and taken up in 10 ml H_2O. This procedure was repeated 3 times. The final residue was taken up in 10 ml H_2O and eluted with 150 ml H_2O from Dowex 50 WX 4 (200 – 400 mesh) column (2 × 8 cm). After evaporation, a yellow partially cristalline residue was obtained which was dissolved in 3 ml H_2O. After fractionation from Sephadex G 10 (1 × 25 cm), 12 fractions with 4 ml each were collected. Fractions 5 and 6 yielded 153 mg pale yellow cristals which after recristallization had a melting point at 109 °C. The aqueous solution was acidic and showed no ^1H-NMR signal in D_2O except an intensified solvent signal; upon addition of Ca^{2+}-ions, Ca-oxalate was identified. The IR spectra of the isolated cristals and of authentic oxalic acid were identical. Neither the isolated cristals nor authentic oxalic acid showed any ORF activity.

Uridine, uracil and lumiflavin were identified as follows: 10.2 g of ORF crude powder were dissolved in 5 ml H_2O and chromatographed on DE_{52} (3 × 25 cm) and eluted with 2.4 l H_2O (flow rate: 100 ml/h). 8 fractions with 300 ml each were collected and concentrated to dryness at 30 ° under reduced pressure. As shown in Fig. 3, only fractions 2 to 4 exhibited significant ORF activity; fraction 1 contained most of the sucrose. Fractions 2 to 4 were combined and rechromatographed on a DE_{52} column (1 × 32 cm) and eluted with H_2O. 20 fractions with 15 ml each were collected (flow rate: 20 ml/h). Frac-

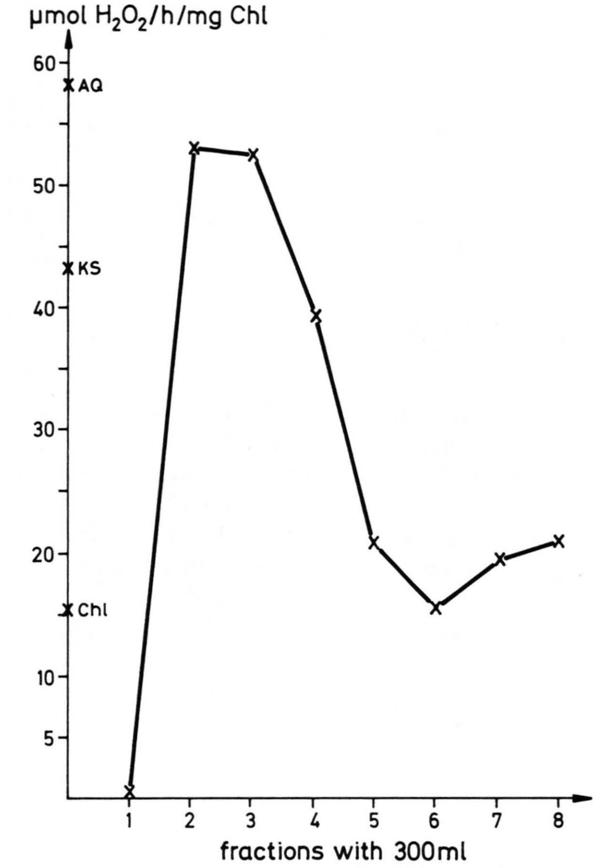


Fig. 3. ORF activities of fractions eluted from DE_{52} with 240 ml H_2O 5% of each fraction (after evaporation) were tested for the stimulation of H_2O_2 formation by illuminated chloroplast lamellae as described in Fig. 1 and under "experimental".

Table I. Spectral characteristics and ORF activities of five fractions eluted from DE_{52}-column. The experimental conditions were as described in Fig. 1 and in the text. 5% of the total fraction were evaporated to dryness and tested for ORF activity.

Column fractions combined	$\lambda_{max.}$	New signature	Colour in H_2O	ORF activity [μmol H_2O_2/h × mg Chl^{-1}]
control (H_2O)	–	–	–	3.2
1–4	268	I	pale yellow	7.7
5–8	260	II	yellow green	27.5
9–11	–	III	colourless	6.9
12–14	254	IV	turbid	6.9
15–20	250	V	colourless	5.3

Table II. Spectral characteristics and ORF activities of four fractions diluted from Sephadex G 10-column. The experimental conditions were as described in Fig. 1 and Table I. 5% of fractions A to D were used for the activity tests.

Column fractions combined	$\lambda_{may.}$	New signature	Colour in H_2O	ORF activity [μmol/h × mg Chl^{-1}]
control (H_2O)	–	–	–	6.7
1–3	–	A	colourless	6.7
4–8	260	B	colourless	8.9
9–12	264	C	yellow green	32.1
13–15	–	D	colourless	8.9

tions with identical UV spectra were combined. The spectral characteristics and the activities of the 5 combined fractions (I–V) are summarized in Table I.

As shown in Table I, only fraction II with an absorption maximum at 260 nm strongly stimulated H_2O_2 production by illuminated chloroplast lamellae. Fraction II was concentrated by evaporation and 1.5 ml aqueous solution were chromatographed on a Sephadex G 10 column (1 × 25 cm) and eluted with H_2O (flow rate: 0.5 ml/min). 15 fractions with 4 ml each were collected, and again fractions with identical absorption maxima were combined, yielding 4 new fractions (A–D) with the following properties (Table II): As shown in Table II, the above gel filtration yields a separation of material (fraction B) absorbing at 260 nm (ca. 720 optical density units) which exhibits no ORF activity and material (fraction C) absorbing at 264 nm (ca. 0.65 optical density units), representing all ORF activity. Identification of the chemical nature of fractions B and C was performed as follows:

a) fraction B was evaporated to dryness and weighed: the yield was 4.2 mg from 10.2 g ORF crude powder. Data of a 200 MHz–^{1}H-NMR spectrum in D_2O are shown in Table III: two signals, b and e show lower intensities as compared to the 8 other signals, suggesting a mixture of two compounds. The main component obviously has 8 different, not D_2O exchangeable protons while the minor component has only 2 not D_2O exchangeable protons. Coupling and chemical shifts of the signals together with data from the literature [31] indicate a pyrimidine base and a pyrimidine nucleoside actually uracil and uridine, respectively. This was confirmed by comparison with the UV-, ^{1}H-NMR and

Table III. ^{1}H-NMR data of fraction B (Table II) in D_2O. See text, "experimental" and Fig. 5a.

Data	Signal (see Fig. 5a)									
	a	b	c	d	e	f	g	h	i	j
δ [ppm]$^\alpha$	7.82	7.46	5.86	5.84	5.73	4.30	4.18	4.08	3.87	3.75
Relative intensity	1	0.4	1	1	0.4	1	1	1	1	1
Multiplicity	d	d	d	d	d	dd	tr	m	dd	dd
J [Hz]	8.2	7.6	4.2	8.2	7.6	4.2 5.5	5.5	5.5 3.0 4.2	−12.5 3.0	−12.5 4.2

α H_2O-signal (4.75 ppm) as intern standard; d = doublet; dd = double doublet; tr = triplet; m = multiplet.

^{13}C-NMR spectra of authentic uridine and uracil, as shown in Figs. 4 and 5; authentic uridine and uracil have no ORF activity.

b) The ORF-active fraction C has an absorption spectrum similar to flavin derivatives. By comparison with different flavins, the lumiflavin spectrum is almost identical with that of the undiluted fraction C (Fig. 6). The identity of fraction C with lumiflavin was confirmed by paper chromatography in 5 different solvents as compared to R_f-values from the literature [32, 33].

Lumiflavin, other flavin derivatives as riboflavin, flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) exhibit ORF activities at concentrations between 10^{-5} and 10^{-6} M (Fig. 7).

From these data it seems to be clear that the oxygen reducing activity of ORF crude powder is due to a flavin derivative. The UV maximum of ORF crude extract at ca. 260 nm is merely due to uridine and uracil. As major components of ORF crude powder, as far as its "weight" is concerned, sucrose and oxalic acid have been identified.

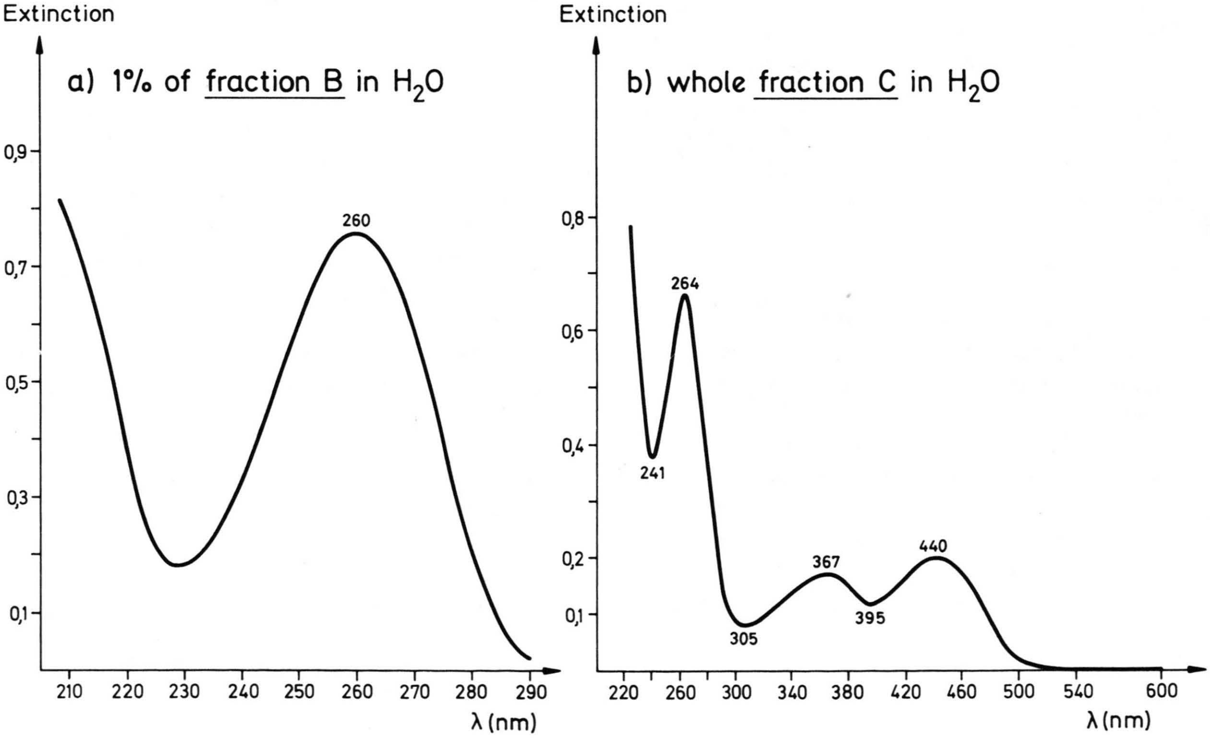


Fig. 4. Absorption spectra of fractions "B" and "C".

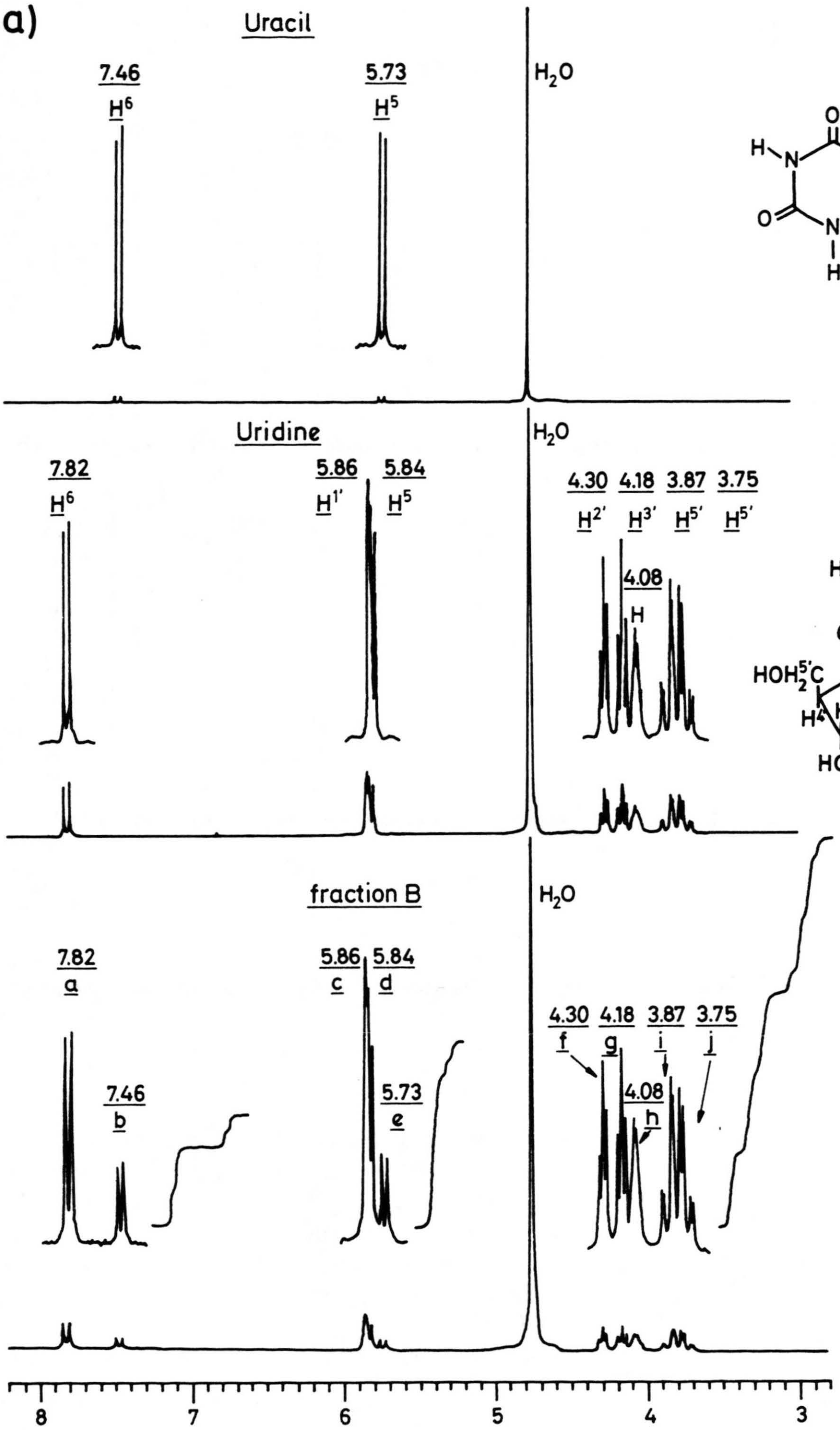
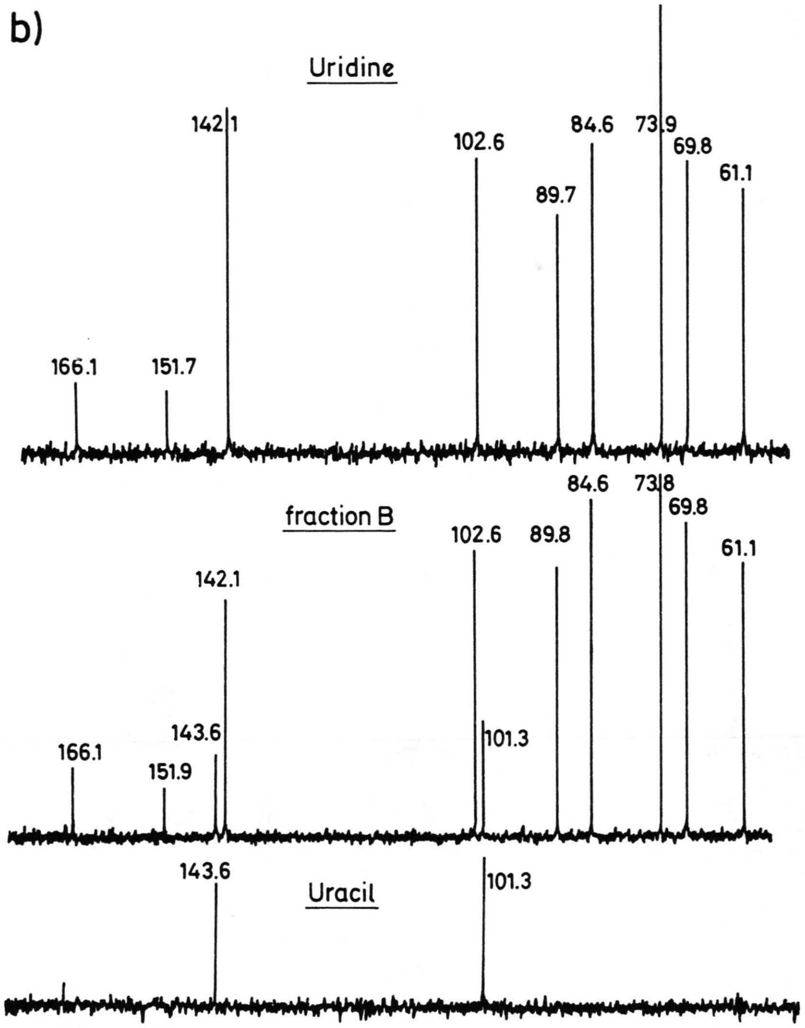


Fig. 5. NMR spectra of fraction "B" as compared to uridine and uracil. 5 a) 200 MHz-^{1}H-NMR spectra of fraction "B", 5 b) Decoupled ^{13}C-NMR spectra (50.3 MHz) of fraction "B" in D_2O with acetone ($\delta = 30.5$ ppm) as intern standard. Because of partial saturation, the carbonyl carbons of uracil are neither visible in the spectrum of fraction B nor of authentic uracil.



Localization and identity of the flavin derivatives in chloroplast lamellae

In order to elucidate whether lumiflavin is identical with the ORF-active compound extracted from the chloroplast lamellae, we tried to extract lumiflavin from the ORF crude concentrate according to the procedure described by Müller and Moor [34]: 10.2 g ORF crude powder were dissolved in 100 ml H_2O and acidified with 3 ml conc. acetic acid. The turbid solution was extracted 5 times with 100 ml $CHCl_3$. After separation of the phases, the $CHCl_3$ emulsion was centrifuged for 30 min at 24000 × *g* and separated into 3 fractions:

1) The yellow-green chloroform phase was evaporated to dryness and the residue dissolved in 100 ml H_2O;
2) the water phase was combined with the water phases of the five extractions, evaporated to dryness and the residue dissolved in 100 ml H_2O;
3) the white pellet was redissolved in 100 ml H_2O.

The ORF activities of the 3 fractions was compared (Table IV). Surprisingly, the chloroform extract showed no ORF activity, but the waterphase was strongly active. This experiment suggests that not the lipophilic lumiflavin is the original ORF-

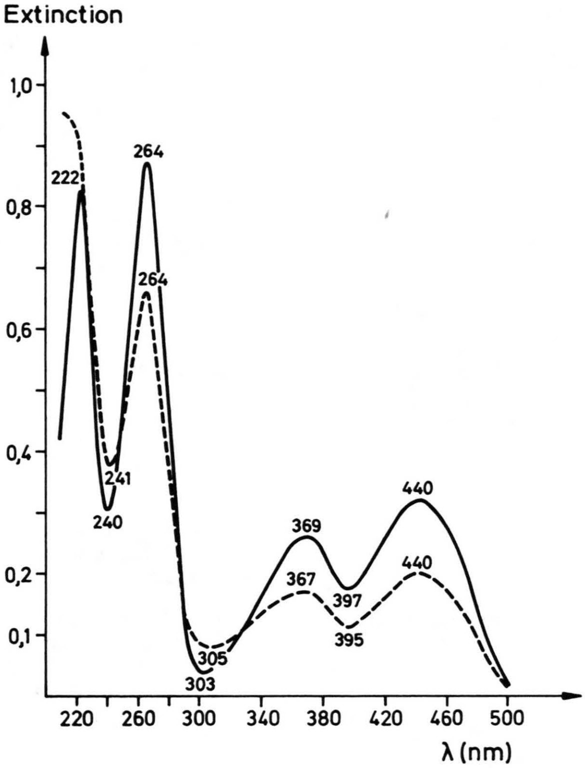
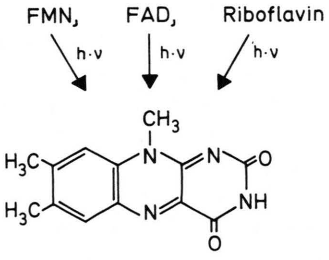


Fig. 6. Ablorption spectra of lumiflavin (—) (6.86×10^{-5} M) as compared to fraction "C" (- - -) (in H_2O).

active component extracted from the chloroplast lamellae but rather a hydrophilic component which is converted into lumiflavin during the purification procedure. It is known that several watersoluble flavins (as riboflavin, FMN and FAD) undergo light-stimulated conversions, where lumiflavin is the product [35], as depicted in the following scheme:



Lumiflavin
(6, 7, 9 - Trimethylisoalloxazin)

Since in an independent experiment even after darkening of the columns only lumiflavin was identified as ORF-active compound, we treated authentic

Table IV. Partitioning of ORF – activities after chloroform – extraction of aqueous solutions of ORF crude powder. For experimental conditions see "experimental" and Figure. For further details see text.

Additions	Activity [µmol H_2O_2/ h × mg Chl]
Controls:	
H_2O	6.5
caffeic acid (5×10^{-6} M)	16.8
anthraquinone-sulfonic acid (0.1 mM)	24.6
0.5 ml ORF-concentrate	16.4
0.5 ml chloroform extract	1.3
0.5 ml water phase	25.1
0.5 ml dissolved residue	11.9

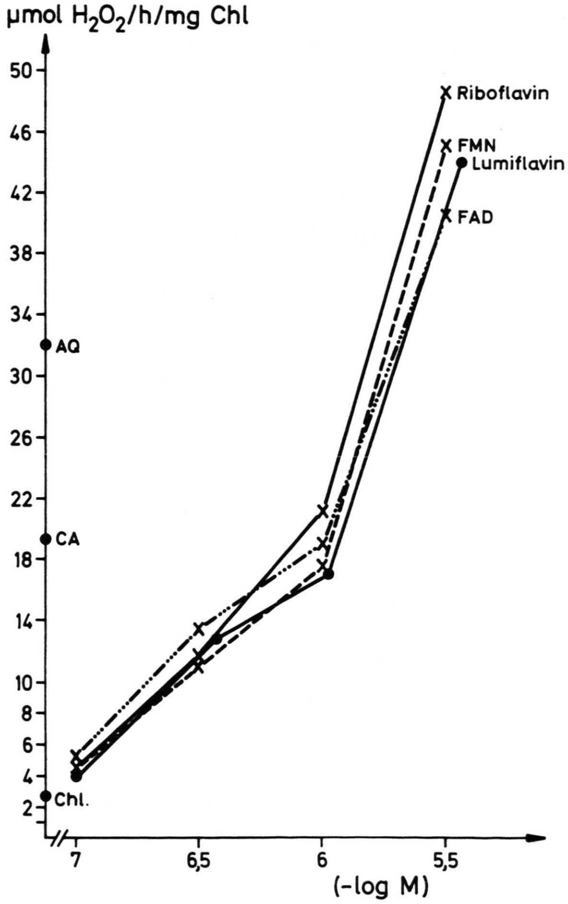


Fig. 7. Comparison of the ORF activities of riboflavin, FMN, FAD and lumiflavin.

riboflavin in an identical manner as described for the isolation of lumiflavin from ORF crude powder. The result of this procedure (elution from DE_{52}, paper chromatography) showed that both absorption spectra as well as R_f-values (data not shown) were identical to lumiflavin; from 150 mg riboflavin started with, we isolated 64.55 mg lumiflavin (43% yield). Since on the other hand FAD, FMN, riboflavin and lumiflavin exhibit almost identical oxygen reducing activities together with illuminated chloroplast lamellae, the above results suggest that the original ORF active component in the hot-water extracts from spinach chloroplast lamellae is not lumiflavin but rather another water-soluble flavin derivative.

Localization of the ORF-active flavin in the thylakoids

Ferredoxin-NADP reductase (E.C. 1.18.1.2.), a flavoprotein acting between ferredoxin and the terminal electron acceptor of PET, NADP, is tightly bound to the chloroplast lamellae and not solubilized (like ferredoxin) upon treatment of isolated whole chloroplast with hypotonic solvents We isolated this enzyme according to the procedure described by Shin *et al.* [36]: after aceton-fractionation, ammoniumsulfate precipitation and dialysis, the active enzyme is eluted from DE_{52} column (pretreated with 0.1 M Tris-HCl pH 7.3, containing 0.11 M Cl^-) with a linear gradient from 0 to 0.2 M NaCl. From 112 fractions with 12 ml each, the active fractions (for a quick test see refs. [37, 38]) No. 39 to 49 were again precipitated with ammoniumsulfate and dialysed.

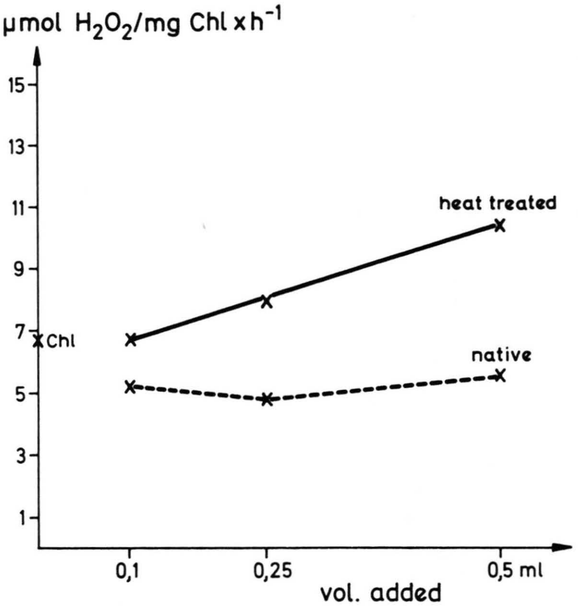


Fig. 8. Stimulation by heat-treated ferredoxin-NADP reductase of H_2O_2 production by illuminated chloroplast lamellae. 2 ml ferredoxin-NADP reductase with ca 6.5 mg protein were heat-treated (5 min at 80 °C) and centrifuged (15 min at 5000 × *g*). 1 ml SN corresponded to 3.7 mg protein with a diaphorase activity (*cf.* ref. [39]) of units × mg protein^{-1}.

Table V. ORF activities of several flavin derivatives as compared to ferredoxin-NADP reductase before and after heat-treatment. Oxygen consumption was measured polarographically.

Additions	Rates [μmol O_2 uptake/mg Chl × h^{-1}]	
	before addition	after addition
anthraquinone sulfonic acid (0.1 mM)	18.4	111.2
riboflavin (5×10^{-6} M)	15.7	112.6
FMN (5×10^{-6} M)	18.8	118.7
FAD (5×10^{-6} M)	14.9	73.9
lumiflavin (5×10^{-6} M)	21.0	110.4
ferredoxin-NADP reductase (0.67 mg protein)	24.4	13.9
ferredoxin-NADP reductase: SN after heat-treatment	16.4	46.8
ferredoxin-NADP reductase: pellet after heat-treatment	16.6	22.1

As shown in Table V, only heat treated ferredoxin-NADP reductase which lost all enzymic activities stimulated oxygen uptake by illuminated chloroplast lamellae as well as H_2O_2 formation (Fig. 8). Furthermore, ferredoxin-NADP reductase with 66 mg protein was heat-treated for 5 min at 80 °C and centrifuged for 5 min at 5000 × *g*. The yellow supernatant was evaporated and chromatographed on Sephadex G 10 in the absolute dark. Comparing the active compounds of the supernatant with authentic samples of FAD, FMN, riboflavin and lumiflavin by paper chromatography (three different solvents) identified the active compound as FAD (data not shown). Thus, most probably FAD is the original flavin derivative liberated from heat-treated isolated chloroplast lamellae. This compound, like many other artificial autoxidizable electron acceptors is highly active in stimulating (pseudocyclic) photophosphorylation and photosynthetic oxygen reduction, yielding H_2O_2 (Fig. 7) (*cf.* refs. [39, 40]). Since FAD is released in very small quantities among other strongly UV-absorbing substances (like uridine and uracil) it seems very likely that most of the compounds described as "endogenous" cofactors of photophosphorylation and/or oxygen reduction are identical with a very minor flavin contamination not visible by UV-spectroscopy but exhibiting their activities already at μM concentrations.

Experimental

Chloroplast lamellae were prepared from spinach according to Nelson *et al.* [41]. Photosynthetic oxygen uptake was measured polarographically with a Hansatech oxygen electrode (Bachofer, Reutlingen). Production of $O_2^{\cdot -}$ was determined as $NO_2^{\cdot -}$ formation from hydroxylamine [29, 30]. H_2O_2 was determined with the aid of NADH peroxidase (Boehringer, Mannheim, No. 10 3 64) as described by Dolin [42].

Incubations for photosynthetic oxygen activation were done as follows:

1) The reaction mixture contained: 100 μmol phosphate buffer pH 7.8; 5 μmol NH_4Cl; 5 μmol $MgCl_2$; 0.25 μmol KCN; 1 μmol NH_2OH (only in the experiments for the determination of $O_2^{\cdot -}$); chloroplast lamellae with 100 μg Chl; solutions with components to be tested for ORF-activity ad 2 ml.

2) The reaction mixtures were incubated at 22 °C for 10 min under illumination from the bottom (white light, 30 klux). Chl was determined according to Arnon [43].

Paper chromatography: Flavin derivatives were separated and identified by chromatography on Whatman No. 1 paper in the dark according to Whitby [32] and Huennekens *et al.* [33] with the following solvents:

a) 5% Na_2HPO_4 in H_2O;
b) *n*-butanol (4), acetic acid (1), H_2O (5); organic phase;
c) 160 g phenol, 30 ml *n*-butanol, 100 ml H_2O; org. phase. According to Kilgour *et al.* [44], 160 g phenol were slightly heated in 30 ml *n*-butanol and, after cooling, 100 ml H_2O were added;
d) Collidine-H_2O saturated (org. phase);
e) H_2O-saturated with isoamylalcohol.

As described by Kilgour *et al.* [44], the dried chromatograms were washed 3 times in chloroform after developing in solvents c or d; only then the flavins become vesible by fluorescence under 366 nm light (UV-Duo-Strahler, Desaga, Heidelberg).

^{1}H-NMR and ^{13}C-NMR spectra were taken with a 200 MHz NMR spectrometer, WP 200 (Bruker); IR spectra were taken with a Perkin Elmer Mod. 21. All preparations and reactions are described in the text or, if necessary, in the tables and figures.

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