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A FACTOR FROM SPINACH LEAVES INTERACTING WITH CHLOROPHYLLS

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SUMMARY

A factor has been isolated from spinach leaves that interacts with chlorophyll. This interaction is measurable as an increased light sensitivity and fluorescence capacity of the pigment in an aqueous medium. The factor is probably a protein.

Interaction was also observed with bacteriochlorophyll and magnesium tetrabenzporphyrin, but not with pheophytin.

Preliminary experiments indicate that the factor exhibits some activity in photoreduction and dark re-oxidation of 2,6-dichlorophenolindophenol by chloroplast fragments.

INTRODUCTION

During experiments with protein preparations from leaves of *Chenopodium album* it was found that chlorophyll bleaches readily upon illumination when added to fractions containing the soluble chlorophyll–protein complex CP 670 (*cf.* ref. 1). As colloidal chlorophyll in an aqueous medium is rather stable, this seemed remarkable. Hence, the phenomenon was investigated further. The compound responsible for the increased rate of bleaching of added chlorophyll was also found in spinach leaves. The experiments indicate that it is likely to be a protein. Certain properties of this compound, and of the complexes formed upon addition of different chlorophyll-like substances, are described in the present paper.

MATERIAL AND METHODS

Fresh spinach was obtained from the Botanical Gardens. Aqueous leaf extracts were prepared by precipitating the proteins first with $(NH_4)_2SO_4$ (30–60 % saturation), and thereafter with polyethyleneglycol. The precipitates were redissolved in 0.1 M phosphate buffer (pH 7.0). The method was the same as described for the preparation of CP 670 from Chenopodium leaves[1]. The final (polyethyleneglycol) precipitate was dissolved in 10 ml buffer for each 125 g of leaves. Preparations thus obtained were used in most experiments and will be called "crude". Further purification was done by means of filtration over a Sephadex G-100 column in 0.1 M phos-

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

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phate buffer (pH 7.0) at about 4°. The length and diameter of the column were 46 cm and 2 cm, respectively. The filtrate was collected in 5-ml fractions; collection of the fractions commenced as soon as the sample was applied to the Sephadex.

Protein concentrations were estimated by the biuret reaction, with albumin as a standard.

Fluorescence was determined as described by GOEDHEER[2]. The values were obtained by extrapolating the decreasing fluorescence yield, measured at 673 nm, to zero time.

Chlorophyll preparations from *Chlorella vulgaris*, *Anacystis nidulans* and *Rhodospirillum rubrum* were obtained by extracting the cells once or twice with methanol for 2 h in a refrigerator in the dark. The debris was precipitated by centrifugation for 20 min at 7000 × *g* at 0°.

The reaction mixture for bleaching experiments consisted of 0.1–0.4 ml crude spinach preparation, supplemented with 0.1 M phosphate buffer (pH 7.0) up to 1.2 ml, and 0.01–0.05 ml methanolic solution of extracted chlorophyll or of other pigments to be tested. Mixing was done in weak green light (*cf.* ref. 1).

For bleaching, a 100-W incandescent lamp at a distance of 10 cm was used. A 3-cm water filter was placed between the light source and the 1-cm sample cuvette.

Absorption spectra were measured in a Zeiss or a Beckman recording spectrophotometer.

The spinach chloroplast fragments used for 2,6-dichlorophenolindophenol (DCIP) photoreduction and reoxidation measurements were prepared according to SAN PIETRO AND LANG[3]. Photoreduction and oxidation were determined by measuring absorption changes at 570 nm in a mixture of 0.01 ml chloroplast fragments, 1 ml 0.1 M phosphate buffer (pH 7.0) (or purified active spinach preparation in the same buffer), 1.9 ml phosphate buffer containing 0.01 M KCl and 0.1 ml 12 mM DCIP in phosphate buffer. Irradiation, at room temperature, was done with the same arrangements as used for the bleaching experiments.

RESULTS AND CONCLUSIONS

Preparations from *Ch. album* leaves, obtained by precipitation of leaf proteins with $(NH_4)_2SO_4$ and polyethyleneglycol consecutively[1], cause an increased rate of aerobic photobleaching of added Chlorella chlorophylls as compared with that for the same amount of chlorophyll added to buffer. As the presence of the light-sensitive chlorophyll–protein complex CP 670 in these preparations complicated the absorption measurements, another source of the active factor was looked for. The high rate of photobleaching of added chlorophyll was also observed in the presence of protein precipitates from spinach, prepared in the same way as those from Chenopodium. As these preparations do not show an absorption maximum in the region of the red chlorophyll absorption band, they were used for all further experiments.

Fig. 1 shows the effect of illumination on bleaching of chlorophyll *a* in (A) buffer, (B) a dilute solution of a spinach preparation and (C) methanol. While scarcely any bleaching of colloidal chlorophyll or of chlorophyll in methanol occurs under conditions used, chlorophyll in aqueous solution rapidly bleaches in the presence of the spinach preparation.

The effect, henceforth called "bleaching effect", could not be evoked by albumin,

indicating that it is not a general protein effect. Moreover, as will be described below, most of the proteins present in the spinach preparations are inactive.

Polyethyleneglycol, that may be present at low concentration in crude preparations, is also inactive.

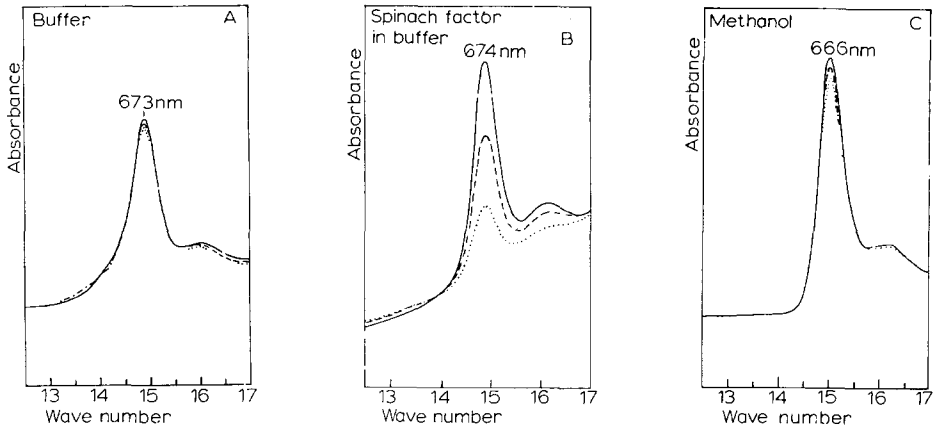

Fig. 1. Bleaching of the red chlorophyll *a* absorption band. A, in buffer; B, a dilute solution of a spinach preparation; C, methanol. Chlorophyll extracted from *A. nidulans*. ———, not irradiated; – – – –, irradiated 2 min; ·····, irradiated 5 min.

The fluorescence of the red chlorophyll *a* band in the presence of a spinach preparation is clearly increased as compared with that measured in a colloidal solution in buffer. The very weak fluorescence measured in the latter solutions is ascribed to the presence of impurities in the methanolic extract of the algal cells used as a chlorophyll source.

It seems reasonable to assume from the observations on bleaching and fluorescence that the (photochemical) properties of chlorophylls are altered by some interaction with a substance present in the spinach preparations.

Table I presents results on the influence of certain compounds on the bleaching effect. No influence of β-mercaptoethanol (50 mM) or *p*-chloromercuribenzoate (0.1 mM) was detected. This indicates that, as distinct from the chlorophyll–protein

TABLE I

INFLUENCE OF VARIOUS COMPOUNDS ON THE BLEACHING OF CHLOROPHYLL *a* IN THE PRESENCE OF THE SPINACH FACTOR

Reaction mixture: see text. Illumination time 5 min.

Compound added	*Bleaching of red absorption band (arbitrary units)*
None	100
p-Chloromercuribenzoate (0.1 mM)	97
β-Mercaptoethanol (50 mM)	91
Ascorbic acid (5 mM)	70
Urea (8 M)	7

complex from Chenopodium[1], SH-groups are not involved in the bleaching effect or in the "binding" of chlorophyll to the spinach factor.

The bleaching effect is inhibited by urea (about 8 M). Urea may influence the conformation of the factor interacting with chlorophylls, or it may interfere with the binding between chlorophylls and this factor.

The bleaching of chlorophyll in the presence of the spinach factor is somewhat inhibited by ascorbic acid (5 mM in 0.1 M phosphate buffer (pH 7.0)). This may indicate that the bleaching is a photooxidation. The original chlorophyll spectrum could not be restored by adding ascorbic acid to the bleached chlorophyll solution.

Heating of an active spinach preparation for 5 min at 75° results in a total loss of activity: apparently the active factor is thermolabile.

The active component from spinach is precipitated with $(NH_4)_2SO_4$ and polyethyleneglycol. This indicates that it may be a protein. In order to identify this active factor with more certainty, the spinach preparation was filtered through Sephadex G-100. Maximum bleaching activity (per unit volume) was found in the fractions appearing after the bulk of the proteins (Fig. 2). After the protein fractions, compounds with an absorption maximum at 260 nm, probably nucleic acids, are collected. These compounds are not active in producing the bleaching effect. The absorption spectrum of the most active fraction is shown in Fig. 3. It shows a maximum at about 275 nm and a shoulder at about 290 nm. These results suggest that

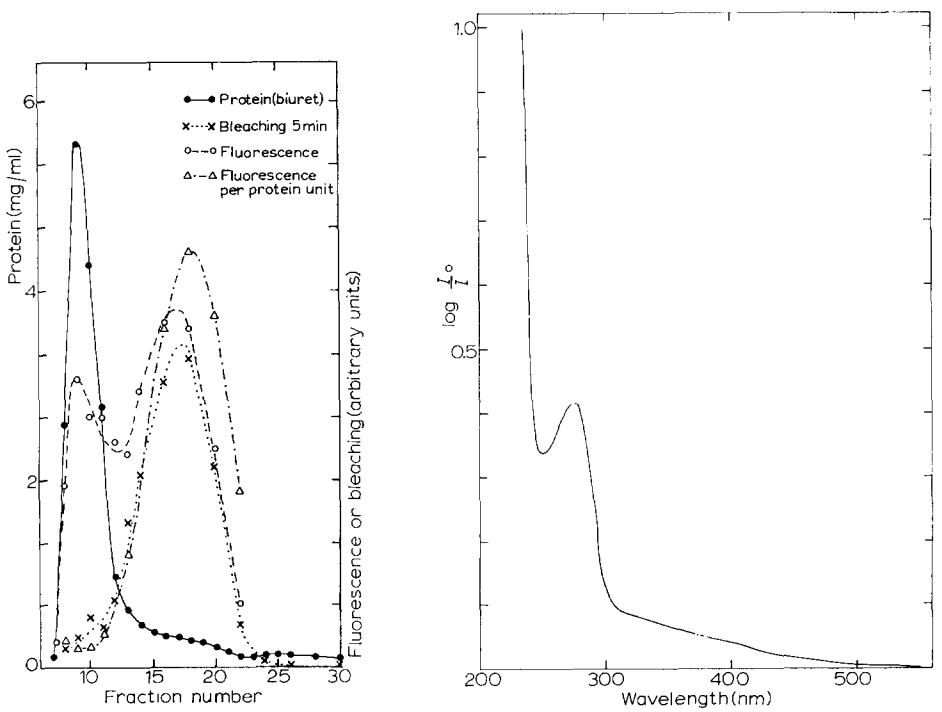

Fig. 2. Effect of Sephadex-filtered fractions from a spinach preparation on bleaching and fluorescence of chlorophylls *a* and *b*. Chlorophyll extracted from *Chl. vulgaris*.

Fig. 3. Absorption spectrum of a purified preparation of the spinach factor.

the active compound is a protein of relatively low molecular weight. The shoulder at 290 nm indicates the presence of tryptophan[4].

No clear absorption maximum was detected in the visible part of the absorption spectrum, either before or after treatment with $K_3Fe(CN)_6$ (10 mM or 1 mM). The active factor is probably not identical, therefore, with plastocyanin[5,6].

The bleaching of chlorophyll, measured in the presence of the spinach factor purified by Sephadex filtration, did not differ from that measured with the crude preparations. Apparently the inactive proteins have little influence on activity.

The fluorescence yield of chlorophyll, added to the same volume of different fractions obtained from the Sephadex column, is clearly increased in those fractions which contain the bulk of the proteins, and in the active fractions. Per unit of protein, however, fluorescence is highest in the fractions with maximum bleaching activity (Fig. 2). Assuming the active factor to be a protein, this indicates that the compound is far more capable of evoking chlorophyll fluorescence in an aqueous medium than the inactive proteins.

Generally, bleaching was measured with chlorophyll extracted from *Chl. vulgaris* or *A. nidulans*. The effect was clearest with extracts from the latter organism, probably because in this case chlorophyll *b*, which is somewhat more resistant to irreversible photobleaching than chlorophyll *a* (*cf.* ref. 7), is absent. In order to obtain some insight into the mechanism of action of the active factor, its influence on the bleaching of certain other chlorophyll-like compounds was studied.

Bacteriochlorophyll. Bacteriochlorophyll is more sensitive to air and illumination than is chlorophyll[8]. If a methanolic extract from *Rh. rubrum* is added to buffer containing the spinach factor, photobleaching of bacteriochlorophyll occurs at a much higher rate than in the absence of this factor (Fig. 4). Moreover, there are conspicuous changes in the absorption spectrum: the 850-nm absorption band (*cf.* refs. 9, 10), which is very prominent in colloidal bacteriochlorophyll, is much lower in the presence of the spinach preparation. This band is absent in methanolic solution. The

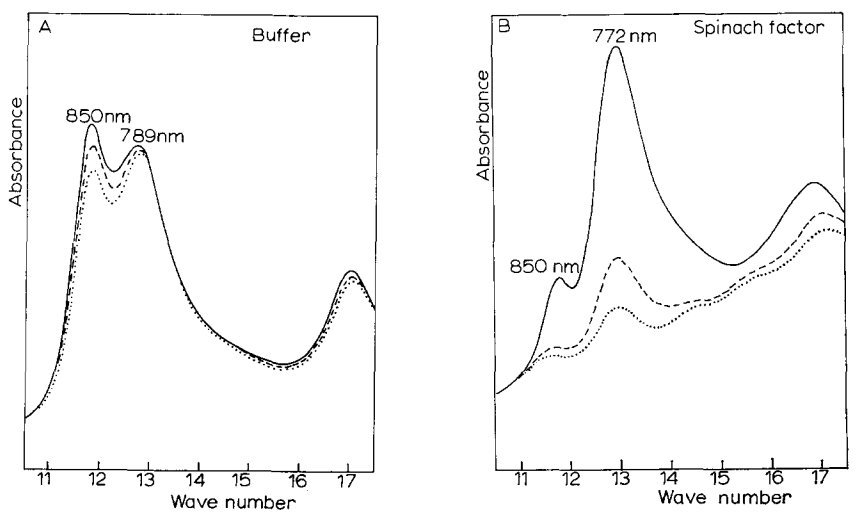

Fig. 4. Bleaching of the far-red bacteriochlorophyll absorption band. A, in buffer; B, a dilute solution of a spinach preparation. Bacteriochlorophyll extracted from *Rh. rubrum*. ———, not irradiated; – – – –, irradiated 0.5 min; ·····, irradiated 1 min.

main absorption maximum is much nearer to the maximum in methanol (771 nm) than the corresponding maximum in colloidal solution.

Pheophytin. Pheophytin was tested in order to study the influence of the presence of Mg^{2+} on the bleaching effect. Acidification of a methanolic extract from Chlorella or Anacystis with HCl (0.01 ml of 0.1 M or 0.2 M HCl per 0.3 ml extract) causes a change in the absorption spectrum: the absorption band in the red part of the spectrum decreases and shifts by a few nm to a shorter wavelength. This indicates the formation of pheophytin[11]. Addition of this acidified extract to buffer containing the active spinach factor does not result in a significant bleaching effect (Fig. 5). Care was taken not to lower the pH in the reaction mixture more than 0.05 pH unit.

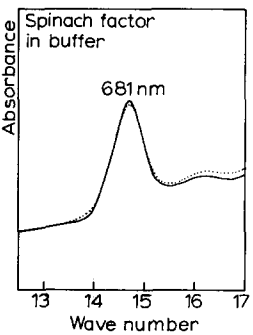

Fig. 5. Bleaching of the red pheophytin *a* absorption band. Pheophytin prepared from Anacystis chlorophyll extract. ———, not irradiated; · · · · · ·, irradiated 5 min.

Magnesium tetrabenzporphyrin. Magnesium tetrabenzporphyrin resembles chlorophyll only in having a porphin skeleton with Mg at its centre. There are no side chains. Some of its properties have been described by GOEDHEER AND SIERO[12]. A solution of magnesium tetrabenzporphyrin in methanol shows a slight but distinct bleaching effect if added to an active spinach preparation (Fig. 6).

Some preliminary experiments were done on the possible physiological activity of the spinach factor. Photoreduction and re-oxidation of DCIP by spinach chloroplast fragments were measured both in the presence and absence of the active compound. As a crude preparation from spinach causes an appreciable reduction of DCIP in the dark, all experiments were done with Sephadex-filtered preparations. In a number of experiments an activation of DCIP photoreduction in the presence

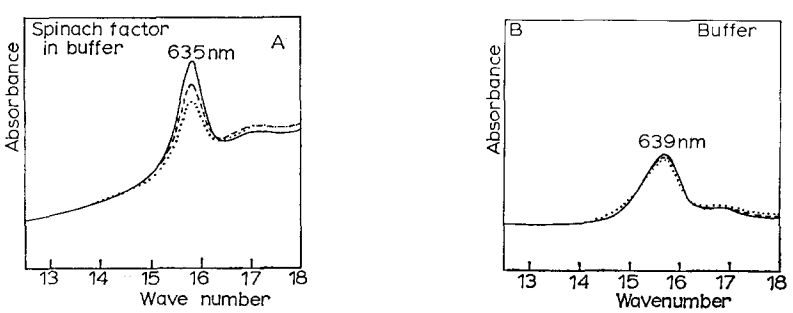

Fig. 6. Bleaching of the red magnesium tetrabenzporphyrin absorption band. A, a dilute solution of a spinach preparation; B, in buffer. ———, not irradiated; – – – –, irradiated 2 min; · · · · · ·, irradiated 5 min.

TABLE II

INFLUENCE OF THE SPINACH FACTOR ON DCIP PHOTOREDUCTION AND RE-OXIDATION BY CHLOROPLAST FRAGMENTS

Reaction mixture: see text. Illumination time 4 (Expts. 1–3) or 2 (Expts. 4–7) min. Re-oxidation measured during 4 min.

Expt. No.	*DCIP photoreduction* (%)		*DCIPH re-oxidation* (%)	
	Without spinach factor	*With spinach factor*	*Without spinach factor*	*With spinach factor*
1	56.9	57.9	13.0	18.3
2	47.8	58.5	5.5	18.3
3	37.6	43.0	3.9	10.3
4	45.9	57.4	8.0	23.4
5	48.1	54.5	10.6	21.5
6	26.3	30.0	4.2	8.9
7	30.0	29.1	5.4	8.5

of the spinach factor was found, while in all cases an increased rate of re-oxidation of DCIP in the dark was measured (Table II).

DISCUSSION

The experiments described demonstrate that a compound that interacts with chlorophyll can be isolated from spinach leaves. The interaction is deduced from increased light sensitivity and fluorescence yield of chlorophylls in an aqueous medium containing the active factor. Comparable results can be obtained by adding an arbitrary detergent, *e.g.* Tween or Triton X-100, to an aqueous chlorophyll solution[13,14]. The factor acts, however, more selectively than a detergent. No interaction could be measured with pheophytin, while Triton X-100 (1 % in buffer) was found to cause a strong fluorescence increase with pheophytin, comparable to that with chlorophyll.

The absence of a measurable interaction of the factor with pheophytin, together with a clear interaction with (bacterio)chlorophyll and, to a lesser degree, with magnesium tetrabenzporphyrin, suggests that the factor interacts *via* the Mg atom in the chlorophyll molecule.

The factor influences the shape and position of the absorption band in the red part of the spectrum of the chlorophyll-like compounds tested. In general, there is a tendency for the spectrum to be changed from that of colloidal chlorophyll to that of actually dissolved chlorophyll. However, the changes in the absorption band are rather complex; further work on this subject is in progress.

Although there is no proof, there are several indications that the active factor is a protein (low molecular). It is (1) precipitated with $(NH_4)_2SO_4$ and polyethyleneglycol, (2) thermolabile, (3) inactivated by 8 M urea, while (4) no activity was ever found in Sephadex fractions containing no protein.

The factor from spinach leaves, which is always isolated without any trace of chlorophyll, is soluble under physiological conditions. Therefore, it cannot be identical with the structural chloroplast protein, isolated by CRIDDLE AND PARK[15]. The spectrum

of the purified compound does not make it possible to identify the protein with any of the proteins known to be active in photosynthesis: heme proteins, flavoproteins[16,17], plastocyanin[5,6], ferredoxin[4,16], rubimedin[18]. Preliminary experiments indicate some physiological activity. This will be a subject of further investigations.

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