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PARTICLE FRACTIONATION FROM SPINACH LEAF HOMOGENATES AND DIFFUSION OF SPINACH PROTEIN FACTOR

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SUMMARY

1. Spinach leaves were homogenized in a phosphate or Tris buffer medium containing 0.35 M sucrose and were filtered through filter paper. The particle suspension thus obtained was fractionated by differential centrifugation.

2. The particle fractions sedimenting from 47000 to 105500 × *g* excreted Spinach Protein Factor, a soluble colourless protein that enhances the light sensitivity of colloidal chlorophylls.

3. Upon fractionation of the combined fractions mentioned under 2 on a sucrose gradient, three types of particle fractions showed up: (a) Colourless small particles (0.01–0.02 μ), presumably ribosomes. (b) A green fraction, mainly consisting of thylakoids (about 0.15 μ), characterized by a very high chlorophyll *a*/chlorophyll *b* ratio, a high 740/685–695 nm fluorescence (—196°) ratio, a high $NADP^+$ photoreduction capacity and a very low or no Hill reaction capacity. (c) A green fraction, mainly consisting of thylakoids (about 0.3 μ), characterized by a lower chlorophyll *a*/chlorophyll *b* ratio than that of the particle fraction mentioned under b, a lower 740/685–695 nm fluorescence (—196°) ratio, a lower $NADP^+$ photoreduction capacity and a high Hill reaction capacity. Spinach Protein Factor diffused from this particle fraction.

4. Evidence is presented that the particles, mentioned under 3b and 3c, are not chloroplast fragments formed during the homogenization procedure of the spinach leaves but were already present in the leaf.

5. A high Spinach Protein Factor activity from particle diffusates coincided with a high 2,6-dichlorophenolindophenol-oxidase activity.

6. The results are discussed with reference to Systems I and II of photosynthesis.

INTRODUCTION

Spinach leaves were found[1] to contain a water-soluble, colourless protein, which combines with colloidal chlorophylls; the complexed chlorophylls exhibit an increased light sensitivity[1,2]. The physiological function of the protein, which was called Spinach Protein Factor, is unknown. Previous experiments[3] suggested that the protein may accelerate certain oxidation reactions. Oxidative enzymes are present, among others,

Abbreviation: DCIP(H), 2,6-dichlorophenolindophenol.

in peroxisomes[4–8,33]. These peroxisomes are disrupted very easily, releasing their enzyme content into the surrounding medium. In the experiments presented below the possibility of Spinach Protein Factor being a peroxisome enzyme was investigated. This did not appear to be true, but Spinach Protein Factor nevertheless diffused from particles. The identity of these particles was examined.

MATERIAL AND METHODS

Leaves from nonsprayed spinach were obtained from the Botanical Gardens.

Preparation of particle fractions by differential centrifugation

40–50 g spinach leaves were deribbed, washed, cooled and homogenized in 100 ml cold buffer (0.02 M Tris buffer (pH 8.0) or 0.01 M phosphate buffer (pH 7.0)) containing 0.35 M sucrose in a Braun multipress provided with filter paper. The suspension was fractionated, in cooled centrifuges, as indicated in Table I. Fractions were numbered from I (heaviest particles) to V (lightest particles).

Diffusion of proteins

The pellets, obtained by differential centrifugation, were suspended in 0.01 M phosphate buffer (pH 7.0) without sucrose, to give an "osmotic shock". After storing overnight at about 4° the suspensions were centrifuged for 1 h at 120000 × *g* (bottom of the tube) in a Spinco centrifuge. The supernatant was assayed for proteins and enzymes.

Sucrose density gradient fractionation

A discontinuous sucrose gradient of six layers was prepared at 4° by pipetting 5 ml of respectively 1.4, 1.2, 1.0, 0.77, 0.56 and 0.35 M sucrose in 0.01 M phosphate buffer (pH 7.0) into a 34-ml centrifuge tube. Pellet Fractions IV and V, obtained by differential centrifugation, were suspended in 1.0 ml 0.01 M phosphate buffer (pH 7.0) containing 0.35 M sucrose, homogenized in a Potter tube and layered on top of the gradient. The sample was centrifuged for 1 h at 32000 × *g* (bottom of the tube) in a swinging bucket rotor SW-25 in a Spinco centrifuge. Fractions were removed by piercing the bottom of the tube with a needle.

To establish diffusion of proteins the fractions were kept overnight in a refrigerator; particles were then spun down at 144000 × *g* and the supernatant was assayed for proteins, 2,6-dichlorophenolindophenol (DCIP(H))-oxidase and Spinach Protein Factor activity. In these experiments no osmotic shock was applied.

Spinach Protein Factor activity

This is the ability of Spinach Protein Factor to enhance the light sensitivity of colloidal chlorophylls[2]. It was estimated by measuring its effect on the rate of decrease of the bacteriochlorophyll absorption maximum around 775 nm upon a 2-min illumination with a 100-W incandescent lamp, distance 20 cm. To remove excess heat a 3-cm water-filled cuvette was placed in front of the sample. Illumination occurred 10 min after mixing the Spinach Protein Factor preparation with bacteriochlorophyll (dark conversion of colloidal bacteriochlorophyll, *cf.* ref. 2).

The reaction mixture contained 1 ml or less of the sample to be assayed, sup-

plemented, if necessary, with 0.01 M phosphate buffer (pH 7.0) until 1.0 ml, 0.2 ml 1 M phosphate buffer (pH 7.0) (for obtaining a sufficient ionic strength[2]) and about 0.02 ml methanolic bacteriochlorophyll solution (absorbance in the reaction mixture about 0.4 at 775 nm). The absorbance decrease upon illumination is only a rough measure of Spinach Protein Factor activity of the preparation, proportionality is only approximate (*cf.* ref. 2).

Protein

Protein was determined after LOWRY *et al.*[9].

DCIPH-oxidase activity

The activity (*cf.* ref. 3) is measured as the enhanced rate, as compared to the control, of absorption increase at 650 nm from 0 to 1.0 in a reaction mixture containing DCIP and a slight excess of ascorbic acid. Activity is expressed in percents of the control. The reaction mixture was as follows: Sample to be essayed 0.5 ml, DCIP 2.4 μmoles, ascorbic acid (added at zero time) 2.55 μmoles. Reaction medium 0.05 M phosphate buffer (pH 7.0), final volume 3 ml. If the preparation to be assayed contained sucrose, the rate of absorption increase was compared with that of a control containing a comparable amount of sucrose.

Catalase activity

The activity was measured according to BEERS and SIZER[10].

Chlorophyll

Chlorophyll was estimated by the method described by BRUINSMA[11].

Hill activity

Hill activity was measured as the photoreduction rate of DCIP per unit chlorophyll. The reaction took place in a 0.01 M phosphate buffer solution (pH 7.0) containing 0.08 μmole DCIP and 30 μmoles KCl. Total volume 3 ml. Illumination (2 min) was provided by a 100-W incandescent lamp at a distance of 10 cm. A 3-cm water-filled cuvette was placed in front of the samples. Absorbance decrease at 570 nm was measured in a Spectronic 20 colorimeter.

$NADP^+$ photoreduction

This was measured, per unit chlorophyll, in a 0.05 M Tris medium (pH 7.8) containing 1 μmole $NADP^+$, 20 μmoles sodium ascorbate, 0.2 μmole DCIP, together with crude preparations of ferredoxin and ferredoxin–$NADP^+$ reductase. Total volume 3 ml. Illumination occurred as described under *Hill activity*. The illumination time was 2.5 min. Absorbance increase at 340 nm was measured in a Spectronic 20 colorimeter. No attempt was made to reach maximum rates.

Ferredoxin was prepared from spinach leaves according to TAGAWA AND ARNON[12]. The preparation was an extract of an acetone precipitate of spinach leaves in 0.05 M Tris buffer (pH 7.8) chromatographed three times on DEAE-cellulose. Ferredoxin–$NADP^+$ reductase was a redissolved $(NH_4)_2SO_4$ (40–65 % satn.) precipitate of the first filtrate of the acetone precipitate extract after filtering over DEAE-cellulose[13].

Absorption

Absorption spectra at room and liquid N_2 (—196°) temperatures were measured in a Cary-14R spectrophotometer.

Fluorescence

Spectra were measured with the apparatus described by GOEDHEER[14]. The preparation was mixed with glycerol, final concentration 60%, and measured in a perspex 1-mm cuvette placed against a window in a 3-walled Dewar vessel, filled with liquid N_2 (*cf.* ref. 15).

Electron microscopy

Particle preparations for electron microscopy were suspended in 0.01 M phosphate buffer (pH 6.5) containing 0.15 M KCl. Fixation occurred, in a medium containing 0.15 M KCl and 1 mM $MgSO_4$, with 1% OsO_4 for 1 h at room temperature. The pellet was dehydrated in acetone and embedded in a low-viscosity epoxy resin medium[16]. Sections were stained with uranyl acetate and lead citrate. Electron micrographs were prepared at the "Centre for Submicroscopical Research of Biological Objects", director Dr. P. F. Elbers, of the State University, at Utrecht.

RESULTS AND CONCLUSIONS

(1) Spinach Protein Factor diffusion from particles separated by differential centrifugation

Differential centrifugation of spinach leaf homogenates (Table I) yielded five green-coloured particle fractions. The diffusate from Pellet II exhibits a catalase activity peak (Table I). This indicates[8,17] that also at our experimental conditions (*cf.* ref. 4) spinach peroxisomes sediment at about 3500 × *g*. Glycolate oxidase[4,18] was also found to be maximum in Fraction II. Spinach Protein Factor activity appears to be highest in diffusates from Pellet IV and V (Fig. 1). Besides, an appreciable amount of Spinach Protein Factor was found in the supernatant after centrifugation at 105500 × *g* for 60 min. As the supernatant contained many cytoplasmic proteins, the Spinach Protein Factor content on a protein basis was low as compared with that of diffusates obtained from sedimented particles (Fig. 1). Although the quantities of Spinach Protein Factor found in different spinach preparations varied strongly, al-

TABLE I

CATALASE ACTIVITY OF DIFFUSATES IN BUFFER FROM SPINACH PARTICLE FRACTIONS, SEPARATED BY DIFFERENTIAL CENTRIFUGATION IN A BUFFER–SUCROSE MEDIUM

Fraction No.	*Centrifugal force (bottom of the tube)*	*Centrifugation time (min)*	*Vol. (ml)*	*Protein (μg/0.1 ml)*	*Catalase* $\left(\frac{\Delta A_{240\,nm}\ (10\ min)}{\mu g\ protein} \times 1000\right)$
I	500 × *g*	20	5	175	16
II	3 500 × *g*	20	5	118	38.5
III	12 000 × *g*	20	5	78	22.5
IV	47 000 × *g*	30	5	47	15
V	105 500 × *g*	60	5	52	9
Supernatant			±100	400	4

ways the same distribution pattern was found. From these results the following conclusions are drawn: (a) Spinach Protein Factor is not released by peroxisomes. (b) Spinach Protein Factor diffuses from small particles (the particles of Pellets IV and V).

As to the Spinach Protein Factor, found in the supernatant, it cannot be concluded from these experiments whether this is released by (damaged) particles or was already present in the leaf outside these particles.

(2) *Are the small particles, yielding Spinach Protein Factor, broken chloroplast fragments?*

Little, if any, Spinach Protein Factor diffuses from Pellets I and II. These are assumed to contain whole chloroplasts and relatively large chloroplast fragments. It might be possible that Spinach Protein Factor diffuses only from small chloroplast fragments, formed from whole chloroplasts during the homogenization procedure. If that were true, Pellets I and II might be expected to yield Spinach Protein Factor after breaking up. This hypothesis was tested by the following experiment: Pellets I and II were suspended in 10 ml 0.01 M phosphate buffer (pH 7.0). One half was sonicated 3 times for 30 sec at 800 kcycles, 200 W. During sonication the preparation was

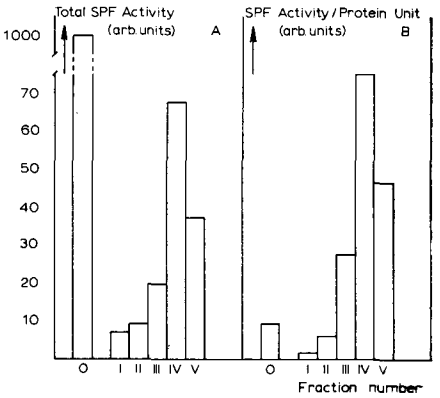


Fig. 1. Spinach Protein Factor (SPF) activity in diffusates of spinach particle fractions, separated by differential centrifugation in a buffer–sucrose medium. A. Total Spinach Protein Factor activity (activity per ml volume). B. Spinach Protein Factor activity per protein unit.

TABLE II

INFLUENCE OF SONICATION ON DIFFUSION OF SPINACH PROTEIN FACTOR FROM WHOLE CHLOROPLASTS AND LARGE CHLOROPLAST FRAGMENTS (FRACTIONS I AND II)

Comparison with Spinach Protein Factor diffusion from small particles (Fractions IV and V).

Fraction No.	*Centrifugal force*	*Spinach Protein Factor activity (arbitrary units)*	*Chlorophyll (mg)*	*Spinach Protein Factor activity per unit chlorophyll (arbitrary units)*
I + II untreated	3 500 × *g*	8	13.2	0.7
I + II sonicated	3 500 × *g*	30	13.2	2.3
IV + V	105 500 × *g*	324	0.25	1300

cooled with ice water. After standing overnight at about 4° the particles of treated and untreated suspensions were spun down (1 h, 144000 × *g*). Spinach Protein Factor activity of the supernatants was tested. Although a definite increase was measured upon sonication, Spinach Protein Factor activity was very small, per unit chlorophyll, as compared with that of diffusates from Particles IV and V (Table II).

It was ascertained that Spinach Protein Factor is not destroyed under the applied experimental conditions.

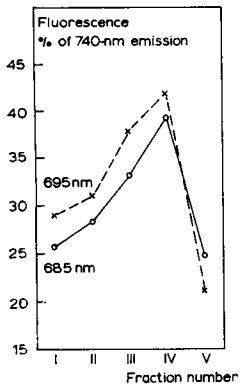
It seems very unlikely that many more chloroplasts were broken during the comparatively mild treatment of spinach leaves in the Braun multipress than in the sonication procedure. Therefore, it is concluded that Particles IV and V, yielding Spinach Protein Factor, are not formed by breaking up of whole chloroplasts.

(*3*) *Properties of large- and small-particle fractions*

(*a*) *Absorbance.* The absorption spectrum of Fractions I and II shows, in the red part of the spectrum, a maximum at about 677 nm and a distinct shoulder at 650 nm, due to chlorophyll *b*. In the fractions with smaller particles the absorption maximum is at about 678 nm and the chlorophyll *b* band gradually disappears; in Fraction V no chlorophyll *b* shoulder is visible in the spectrum at room temperature.

(*b*) *Fluorescence.* The fluorescence spectrum at —196° shows three maxima in the red part of the spectrum, at 685, 695 and 740 nm (*cf.* ref. 14), called F685, F695 and F740, respectively. The intensity of F685 and F695 gradually increases, from Fractions I to IV particles, as compared with F740, while in Fraction V a sudden decrease is observed (Fig. 2). F695 is higher than F685 in Fractions I–IV, in Fraction V F685 is higest.

(*c*) *Electron microscopy.* All fractions show thylakoids, the largest ones occurring in Fraction I and the smallest ones, and only relatively few, in Fraction V. In Fraction III some small particles (0.01–0.02 μ) show up. These are abundant in Fraction IV and especially in Fraction V.



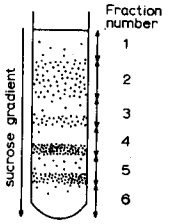


Fig. 2. Ratio of 740/685 and 740/695 nm fluorescence at —196°, measured in spinach particle fractions separated by differential centrifugation in a buffer–sucrose medium.

Fig. 3. Band formation and fraction separation upon sucrose density gradient centrifugation of spinach particles sedimenting between 12000 and 105000 × *g*.

(4) Fractionation on a sucrose density gradient of particles exhibiting high Spinach Protein Factor diffusion

The experiments described above show that Spinach Protein Factor can diffuse from relatively small particles. Properties of the small-particle fractions, as described above, allow no conclusion to be drawn as to the identity of the particles yielding Spinach Protein Factor. As Spinach Protein Factor combines with colloidal chlorophylls, it seemed particularly interesting to establish whether Spinach Protein Factor is released by green or colourless particles. Therefore, Pellets IV and V were fractionated further on a sucrose gradient. After centrifugation three fairly narrow green bands and one broad yellow-green band were visible, as well as two nearly colourless zones. These were separated as shown in Fig. 3. The fractions with increasing sucrose concentration were numbered 1–6. The chlorophyll content of Fractions 1–6, as related to total chlorophyll in spinach leaves, is shown in Table III.

TABLE III

CHLOROPHYLL CONTENT OF SPINACH PARTICLE FRACTIONS SEPARATED BY DIFFERENTIAL CENTRIFUGATION (FRACTIONS I–V) AND, THEREAFTER, SUCROSE DENSITY GRADIENT FRACTIONATION (FRACTIONS 1–6)

Fraction No.		*Chlorophyll (mg/50 g spinach leaves)*	*Chlorophyll (% of total chlorophyll)*
I–III		21.5	96.5
IV + V	1	0.02	—
	2	0.22	1
	3	0.13	0.5
	4	0.19	1
	5	0.19	1
	6	0.006	—

(5) Diffusion from particles separated on a sucrose gradient.

(a) Spinach Protein Factor. A clear Spinach Protein Factor activity peak is found in diffusates from Fractions 4 and 5 (Fig. 4). The particles were not treated with an osmotic shock. Apparently, this is not necessary for allowing Spinach Protein Factor to diffuse. This suggests that the diffusion is the result of a constant formation of Spinach Protein Factor within the particle.

(b) Proteins. Protein determination in the diffusates of Fractions 1–6 showed a clear maximum in the nearly colourless Fraction 1 (Fig. 4). This indicates that this fraction contains colourless protein-yielding particles. Assuming two kinds of particles to be present in the original Fractions IV + V, namely colourless small particles and larger coloured particles (thylakoids, *cf.* Section 3), it is concluded that the colourless particles are accumulated in Fraction 1. This would mean that Spinach Protein Factor, diffusing mainly from particles of Fractions 4 and 5, originates from green particles.

(c) DCIPH-oxidase. DCIPH-oxidase activity of Fractions 1–6 diffusates showed two maxima, namely in Fractions 1 and 4. The latter peak coincides with the Spinach Protein Factor activity maximum (Fig. 5). Both activities are measured per unit

volume of the diffusates. Fraction 1 contains a relatively large amount of protein. On a protein basis, oxidase activity is lower in Fractions 1 and 2 than in Fractions 3–5.

(6) *Properties of particles separated by sucrose density centrifugation*

(*a*) *Absorption.* The room temperature absorbance spectrum of Fraction 2 shows, in the red part of the spectrum, a maximum at 677 nm, whereas no chlorophyll *b* band at 650 nm is observed. Fraction 5 has its maximum absorbance at 676 nm and

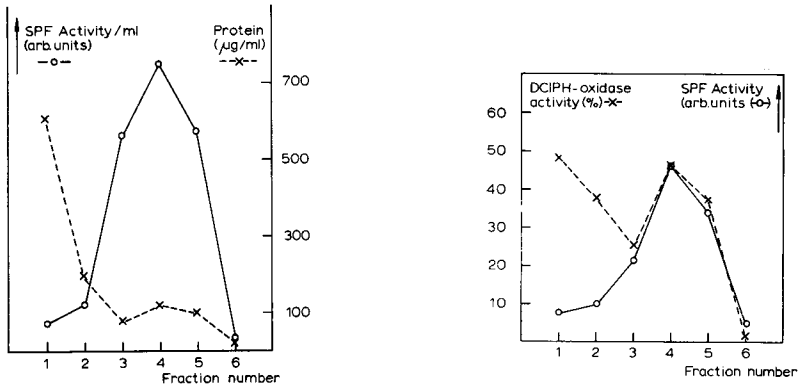


Fig. 4. Spinach Protein Factor (SPF) activity and protein content from diffusates of spinach small-particle fractions, which sedimented between 12 000 and 105 500 × *g* and were then separated on a sucrose density gradient.

Fig. 5. Spinach Protein Factor (SPF) and DCIPH-oxidase activity from diffusates of spinach small-particle fractions, which sedimented between 12 000 and 105 000 × *g* and were then separated by centrifugation on a sucrose gradient.

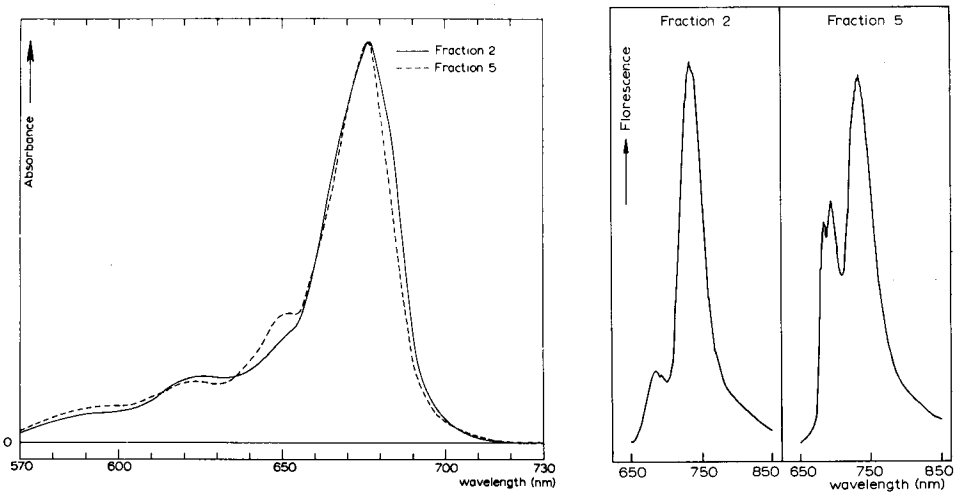


Fig. 6. Absorption spectra (−196°) of two spinach small-particle fractions, separated by sucrose density gradient centrifugation. Fraction 2: Low-density fraction of spinach particles, sedimented between 12 000 and 105 500 × *g*. Fraction 5: High-density fraction of spinach particles, sedimented between 12 000 and 105 500 × *g* (*cf.* Fig. 3).

Fig. 7. Fluorescence spectra (− 196°) of two spinach small-particle fractions separated by sucrose density gradient centrifugation. Fractions 2 and 5: *cf.* Figs. 3 and 6.

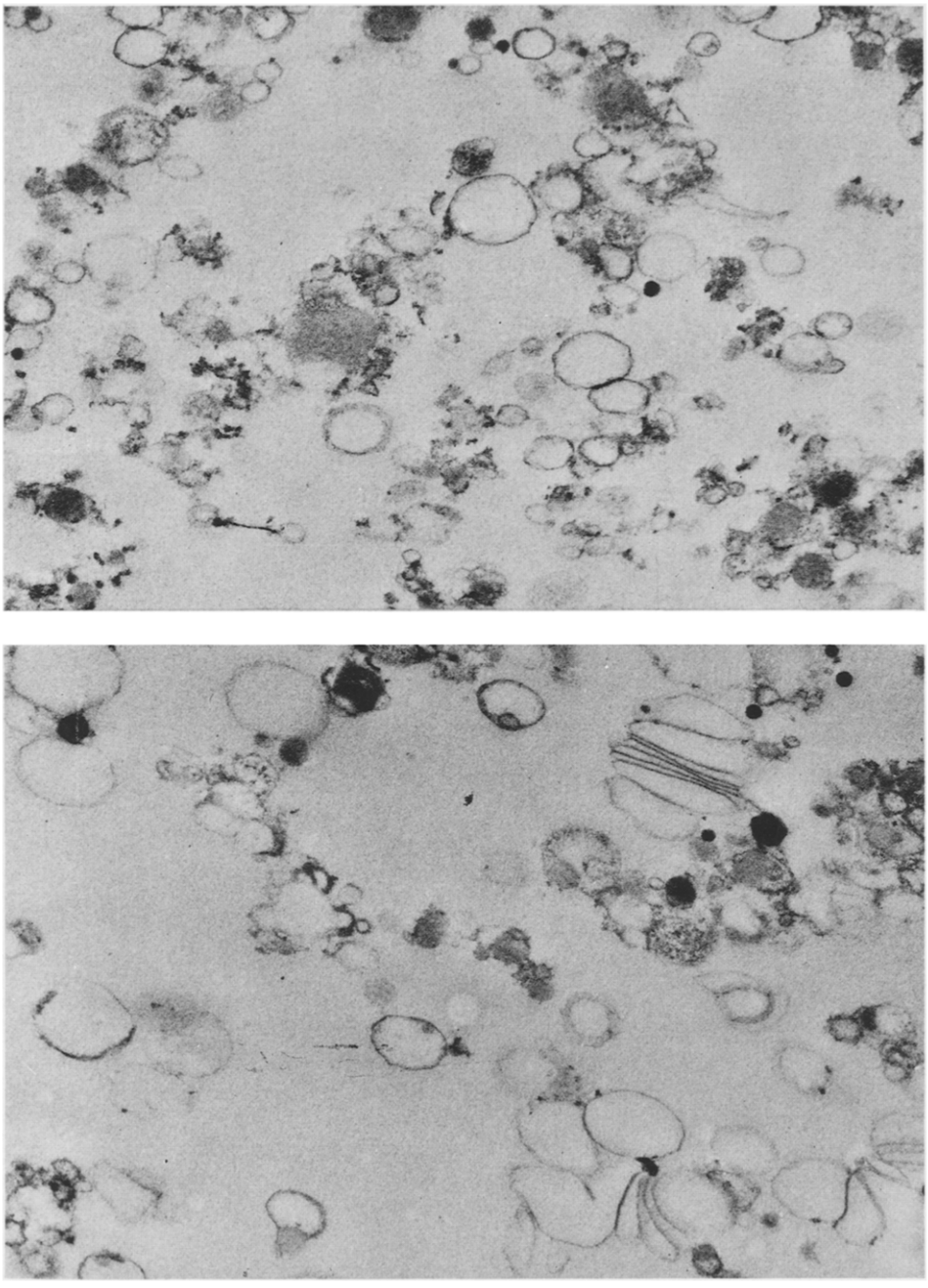


Fig. 8. Electron micrographs of two spinach small-particle fractions, separated by sucrose density gradient centrifugation. Magnification 28480 ×. Fractions 2 (top) and 4 (bottom) *cf.* Fig. 3.

shows a clear shoulder at 650 nm. Fractions 3 and 4 are intermediate, both showing a shoulder at 650 nm.

The differences between the spectra of Fractions 2 and 5 are somewhat more pronounced at —196° (Fig. 6). At this temperature a small shoulder at 650 nm shows up in Fraction 2, showing that some chlorophyll *b* is still present. The main absorption band of Fraction 2 has its maximum at about 677 nm and is broader than the corresponding band of Fraction 5, which has its maximum at about 676 nm.

(*b*) *Fluorescence.* All green fractions (2–5) show a main fluorescence maximum, at —196°, at 740 nm and two lower maxima at 685 and 695 nm. In Fraction 2 the ratio of the intensity of F740 to that of F685 and F695 is much higher than in Fraction 5 (Fig. 7). Besides, in Fraction 2 F685 is somewhat higher than F695, whereas this is reversed in Fraction 5. Fractions 3 and 4 are intermediate.

(*c*) *Electron microscopy.* Fraction 1 contains, as might be expected from its being nearly colourless, only very few thylakoids and a large mass of small particles (0.01–0.02 μ). In Fractions 2–5 thylakoids are present abundantly. The smallest ones occur in Fraction 2 (about 0.15 μ), whereas the largest ones are found in Fraction 5 (about 0.35 μ). The presence of small colourless particles in Fraction 1 is in line with the conclusion drawn in Section 5 from the protein spectrum.

Electron micrographs of Fractions 2 and 4 are shown in Fig. 8.

(*d*) *Hill reaction.* The Hill reaction in Fraction 5 particles was about as high as that in Fractions I–III, obtained from differential centrifugation. The latter fractions contained whole chloroplasts and large chloroplast fragments. The Hill reaction in Fraction 2 particles was negligible, that in Fractions 3 and 4 was intermediate, between that in Fractions 2 and 5 (Fig. 9).

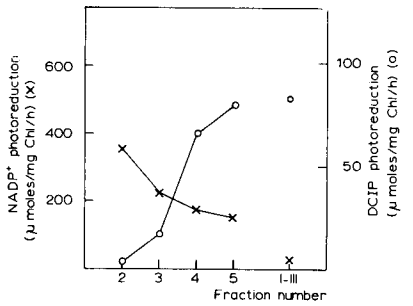


Fig. 9. Hill reaction (○) and NADP+ photoreduction (×) of spinach small-particle fractions, which sedimented between 12 000 and 105 500 × *g* and were then separated on a sucrose density gradient (Fractions 2–5). Comparison with particle fractions which sedimented between 500 and 12 000 × *g* (Fractions I–III). Hill reaction: 14.7 μg chlorophyll; illumination time 2 min. NADP+ photoreduction: 6.7 μg chlorophyll; illumination time 2.5 min.

(*e*) *NADP+ photoreduction.* Fraction 2 showed a high NADP+ photoreduction activity, whereas that measured in Fractions 4 and 5 was much lower. Fraction 3 was intermediate in this respect. NADP+ photoreduction rate of whole chloroplasts + chloroplast fragments from Fractions I–III was lower than that of Fraction 5 (Fig. 9).

DISCUSSION

Spinach Protein Factor diffuses mainly from particle Fractions IV and V, sedimenting between 12000 and 105000 × *g*. Fractionation on a sucrose density gradient yielded three types of particle fractions:

(A) Colourless particles (Fraction 1). From their high protein excretion and from electron micrographs (small particles of 0.01–0.02 μ, partly situated on small vesicles) these are assumed to be ribosomes. These particles do not excrete Spinach Protein Factor.

(B) A fraction containing mainly small green particles (mean diameter about 0.15 μ) (Fraction 2). Probably, this fraction is contaminated with particles from Fraction 1. The fraction has the following characteristics: (a) A high chlorophyll *a*/chlorophyll *b* ratio. Chlorophyll *a* absorption maximum at about 677 nm (—196°) (Fig. 6). (b) A low 685- and 695-nm fluorescence at —196°, relative to 740-nm fluorescence (Fig. 7). (c) A very low or no Hill reaction activity (Fig. 9). (d) A high $NADP^+$ photoreduction capacity as compared with that of whole chloroplasts and relatively large chloroplast fragments (Fig. 9). (e) No or only a slight diffusion of Spinach Protein Factor (Fig. 4).

(C) A fraction with mainly small green particles (mean diameter about 0.25–0.35 μ) (Fractions 3, 4, 5). This fraction has the following characteristics: (a) A lower chlorophyll *a*/chlorophyll *b* ratio as compared with that of Fraction 2 (Fig. 6). Chlorophyll *a* absorbance maximum (—196°) at about 676 nm. (b) A higher 685- and 695-nm fluorescence at —196° relative to 740-nm fluorescence, as compared with Fraction 2 (Fig. 7). (c) A high Hill reaction activity, equal to or somewhat lower than that of whole chloroplasts and relatively large chloroplast fragments (Fig. 9). (d) A low $NADP^+$ photoreduction capacity as compared with that of Fraction 2, but high as compared with that of whole chloroplasts and relatively large chloroplast fragments (Fig. 9). (e) Diffusion of Spinach Protein Factor (Fig. 4).

Characteristics a–d from Fraction 2, described under B, are similar to those for Photosystem I particles[15,19,20]. Fractions (3), 4 and 5, described under C, show some characteristics of Photosystem II. However, $NADP^+$ photoreduction is, although appreciably lower than in Fraction 2 particles, not zero. In fact, it is higher than that of intact or broken chloroplasts. A resemblance might exist between Fractions 4 and 5 and Photosystem II in the concept of KNAFF AND ARNON[21].

Particles enriched in Photosystems I or II have, as yet, only been isolated from chloroplasts treated with detergents[15,19,20,22–28] or large pressure differences[29] or from sonicated chloroplasts[30,31]. In the experiments described above spinach leaves were homogenized as prudently as possible in a buffer–sucrose medium. Fragmentation of chloroplasts by sonic treatment (Table II) does not result in particles yielding Spinach Protein Factor, indicating that these particles are different from those forming Fractions 3–5. This suggests that the small green particles isolated from spinach leaves after mild treatment do not originate from whole chloroplasts but were already present in the leaf before homogenization.

The Fractions 4 and 5, yielding Spinach Protein Factor, have a Hill reaction capacity, on a chlorophyll basis, about the same as that of whole or broken chloroplasts. However, they differ in respect to their absorption spectrum (a lower relative chlorophyll *b* content) and their fluorescence spectrum (a higher 685–695/740 nm

fluorescence ratio) (*cf.* Figs. 2 and 7). Besides, the $NADP^+$ photoreduction capacity is higher than that of whole chloroplasts or large fragments. This may indicate that $NADP^+$ is more readily accessible to Fractions 4 and 5 particles (*cf.* ref. 32).

Spinach Protein Factor diffuses mainly from Fractions 4 and 5. Fractions 4 and 5 contain, at least for the most part, green particles; this suggests that these particles are forming Spinach Protein Factor. The possiblity cannot be excluded, however, that Fractions 4 and 5 still contain some colourless particles, different from the colourless particles found in Fraction 1. This possibility will be investigated further.

If Spinach Protein Factor diffuses from the green particles, this may indicate that Spinach Protein Factor is, in some still unknown way, active in photosynthesis. In a previous paper[3] evidence was presented that Spinach Protein Factor has properties of an oxidative enzyme. A DCIPH-oxidase activity peak, found in Fraction 4 particles (Fig. 5), which also exhibit that greatest Spinach Protein Factor diffusion, is in line with the results of these previous experiments.

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REFERENCES

1 W. TERPSTRA, *Biochim. Biophys. Acta*, 143 (1967) 221.
2 W. TERPSTRA, *Biochim. Biophys. Acta*, 153 (1968) 675.
3 W. TERPSTRA, in H. METZNER, *Progress in Photosynthesis Research*, H. Laupp, Jr., Tübingen, Freudenstadt, 1968, p. 771.
4 N. E. TOLBERT, A. OESER, T. KISAKI, R. H. HAGEMAN AND R. K. YAMAZAKI, *J. Biol. Chem.*, 243 (1968) 5179.
5 N. E. TOLBERT, A. OESER, R. K. YAMAZAKI, R. H. HAGEMAN AND T. KISAKI, *Plant Physiol.*, 44 (1969) 135.
6 M. MÜLLER, J. F. HOGG AND C. DE DUVE, *J. Biol. Chem.*, 243 (1968) 5385.
7 T. G. COOPER AND H. BEEVERS, *J. Biol. Chem.*, 244 (1969) 3507.
8 R. K. YAMAZAKI AND N. E. TOLBERT, *Biochim. Biophys. Acta*, 178 (1969) 11.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
10 R. F. BEERS AND I. W. SIZER, *J. Biol. Chem.*, 195 (1952) 133.
11 J. BRUINSMA, *Biochim. Biophys. Acta*, 52 (1961) 576.
12 K. TAGAWA AND D. J. ARNON, *Nature*, 195 (1962) 537.
13 M. SHIN, K. TAGAWA AND D. I. ARNON, *Biochem. Z.*, 338 (1963) 84.
14 J. C. GOEDHEER, *Biochim. Biophys. Acta*, 88 (1964) 14.
15 C. BRIL, D. J. VAN DER HORST, S. R. POORT AND J. B. THOMAS, *Biochim. Biophys. Acta*, 172 (1969) 345.
16 A. R. SPURR, *J. Ultrastruct. Res.*, 26 (1969) 31.
17 D. H. BROWN, *Biochim. Biophys. Acta*, 162 (1968) 152.
18 I. ZELITCH AND S. OCHOA, *J. Biol. Chem.*, 201 (1953) 707.
19 N. K. BOARDMAN AND J. M. ANDERSON, *Nature*, 203 (1964) 166.
20 N. K. BOARDMAN, S. W. THORNE AND J. M. ANDERSON, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 586.
21 D. B. KNAFF AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 715.
22 J. S. C. WESSELS, *Biochim. Biophys. Acta*, 65 (1962) 561.
23 J. S. C. WESSELS, in H. METZNER, *Progress in Photosynthesis Research*, H. Laupp, Jr., Tübingen, Freudenstadt, 1968, p. 128.
24 J. M. BRIANTAIS, *Photochem. Photobiol.*, 6 (1967) 155.
25 J. M. BRIANTAIS, *Physiol. Végétale*, 7 (1969) 135.

26 J. L. BAILEY AND W. KREUTZ, in H. METZNER, *Progress in Photosynthesis Research*, Freudenstadt, 1968, p. 149.
27 L. P. VERNON, B. KE, H. H. MOLLENHAUER AND E. R. SHAW, in H. METZNER, *Progress in Photosynthesis Research*, H. Laupp, Jr., Tübingen, Freudenstadt, 1968, p. 137.
28 H. HUZIGE, H. USIYAMA, T. KIKUTI AND T. AZI, *Plant Cell Physiol. Tokyo*, 10 (1969) 441.
29 J. M. MICHEL AND M. R. MICHEL-WOLWERTZ, *Carnegie Inst. Year Book*, 67 (1967–68) 508.
30 G. JACOBI AND H. LEHMANN, *Z. Pflanzenphysiol.*, 59 (1968) 457.
31 C. F. YOCUM, J. L. KYLE AND J. A. GROSS, in H. METZNER, *Progress in Phytosynthesis Research* H. Laupp, Jr., Tübingen, Freudenstadt, 1968, p. 122.
32 Y. MATHIEU, *Biochim. Biophys. Acta*, 189 (1969) 422.
33 T. G. COOPER AND H. BEEVERS, *J. Biol. Chem.*, 244 (1969) 3514.