Biochimica et Biophysica Acta, 375 (1975) 406–421

BBA 76857

COMPARATIVE STUDIES ON THE POLYPEPTIDE COMPOSITION OF CHLOROPLAST LAMELLAE AND LAMELLAR FRACTIONS*

W. G. NOLAN** and R. B. PARK

Botany Department, University of California, Berkeley, Calif. 94720 (U.S.A.)

(Received July 9th, 1974)
(Revised manuscript received October 11th, 1974)

SUMMARY

The polypeptide composition of spinach chloroplast membranes and membrane fractions has been examined by the technique of sodium dodecylsulfate–polyacrylamide gel electrophoresis. Chloroplasts were fragmented into grana (Photosystem II enriched) and stroma lamellae (Photosystem I in character) by the French press technique. The grana lamellae were further fractionated by the use of digitonin into two fractions, one enriched in Photosystem II and the other enriched in Photosystem I. These membranes are composed of at least 15 polypeptides two of which, with approximate weights of 39 and 50 kdaltons, are observed only in granal fractions. Quantitatively the primarily Photosystem II fractions are enriched in polypeptides in the 30–23 kdalton range whereas the Photosystem I (or Photosystem I-enriched) fractions are enriched in polypeptides in the 60–54 kdalton region. The experiments reported show that contamination by soluble proteins or other membranes is negligible. The results indicate that subtle differences in composition account for the large differences in structure and function within the chloroplast membrane system.

INTRODUCTION

Although chloroplast membrane lipids have been extensively studied and are well characterised [1, 2], much less is known about the proteins from these membranes due to their insolubility under non-denaturing conditions. The most commonly used procedures for solubilizing (and denaturing) membrane proteins have employed the use of the anionic detergent sodium dodecylsulfate. A considerable amount of detergent work has been done on algal chloroplast membranes, such as the excellent work of Hoober [3–5], and recently detailed studies have been extended

* This report represents a portion of a Ph. D. thesis submitted by W. G. Nolan to the Graduate Division of the University of California, Berkeley, July 1973.

** Present address: CSIRO, Division of Food Research, Plant Physiology Unit, School of Biological Sciences, Macquarie University, North Ryde, N.S.W., 2113, Australia.

by other workers to higher plant chloroplasts and various fractions separated from them [6–10]. Anderson and Levine [8, 9] and Klein and Vernon [7] have studied the polypeptides of partially lipid-extracted spinach chloroplast lamellae and fragments of the lamellae obtained by various procedures. Both groups have indicated that higher molecular weight polypeptides predominate in Photosystem I fractions whereas lower molecular weight polypeptides predominate in Photosystem II-enriched fractions. The polypeptides associated with these fractions are termed Group I and Group II polypeptides respectively by Levine [8, 9].

The present report is an extension of a preliminary study on peptide composition of grana and stroma membranes of spinach chloroplasts which were separated by the French press technique [11]. We have studied in detail the polypeptides of lipid-extracted spinach chloroplast lamellae and lamellar fragments in order to extend our understanding of their association with the two photosystems of photosynthesis. Initially the membranes were fractionated into grana and stroma lamellae by the French pressure cell method of Sane et al. [12]. Grana lamellae have Photosystem I and II activities and large and small freeze-etch particles whereas stroma lamellae possess only Photosystem I activity and only the small particles. The detergent digitonin was then used to further fractionate grana lamellae using a modification of the method of Arntzen et al. [13] to yield two fractions, one enriched in Photosystem II and possessing both sizes of particles and a second enriched in Photosystem I and possessing only small particles (ref. 13 and Nolan, in preparation). In addition to studying lamellar fragments of chloroplasts which had been extensively washed with buffer, various extraction procedures were used in an attempt to critically evaluate which polypeptides may be truly deemed "membrane polypeptides."

MATERIALS AND METHODS

Preparation of membrane fragments and soluble proteins

Spinach chloroplasts were isolated and French press treated as outlined by Sane et al. [12] with the following exception. The chloroplasts were washed 3 times in 0.05 M potassium phosphate, pH 7.4–0.15 M KCl with 15-min centrifugations at $1500 \times g$ prior to passage through the pressure cell. The resulting French press homogenate was then treated in one of the following ways:

(*i*) *Non EDTA-washed membrane fragments.* A small portion (approx. 10 ml) of the homogenate was immediately pelleted by centrifugation at $180\,000 \times g$ for 60 min (designated the FP fraction), while the majority of the material was subjected to fractional centrifugation at $1000 \times g$ for 10 min ($1000 \times g$ fraction), $10\,000 \times$ g for 30 min ($10\,000 \times g$ fraction), $40\,000 \times g$ for 30 min ($40\,000 \times g$ fraction), and $180\,000 \times g$ for 60 min. ($180\,000 \times g$ fraction).

The $1000 \times g$ and $40\,000 \times g$ pellets were discarded.

(*ii*) *EDTA-washed membrane fragments and supernatants.* The membrane fragments (the FP, $10\,000 \times g$ and $180\,000 \times g$ fractions) were isolated as above and were resuspended and incubated for 30 min in 1 mM EDTA, pH 8.0 [14]. The fractions were pelleted in a manner similar to which they had been isolated except that the $10\,000 \times g$ fraction was centrifuged at $40\,000 \times g$ for 30 min. The resulting supernatants were made 5 % with respect to trichloroacetic acid and the resulting precipitates were collected by centrifugation at $40\,000 \times g$ for 20 min.

(*iii*) *EDTA-washed and gradient-purified membrane fragments.* The EDTA-washed membrane pellets isolated as above were resuspended in 0.05 M potassium phosphate buffer, pH 7.4. The FP and 10 000 $\times g$ pellets were resuspended to give a chlorophyll concentration of 1.5 mg per ml and the 180 000 $\times g$ pellets were resuspended to give a concentration of 0.7 mg chlorophyll per ml. Two ml of a given fraction were then layered on a buffered (0.05 M potassium phosphate, pH 7.4) linear sucrose gradient (30–60 % w/v) and centrifuged overnight at 23 500 rev./min in a SW 25 rotor. The chlorophyll bands all at the same level in the tubes were selectively removed, diluted with distilled water and pelleted by centrifugation at 180 000 $\times g$ for 1 h.

(*iv*) *Digitonin fractionation of the 10 000 $\times g$ fraction.* The 10 000 $\times g$ fraction was further fractionated by a modification of the method of Arntzen et al. [13]. The starting material was a 10 000 $\times g$ fraction which had been washed with EDTA or which had been further purified on a buffered gradient. The pellets were resuspended in 0.05 M potassium phosphate, pH 7.4–0.01 M KCl to give a chlorophyll concentration of 0.25 mg per ml. One part 2 % (w/v) twice recrystallized digitonin was then added with constant stirring to three parts of the 10 000 $\times g$ suspension. The mixture was then incubated for 30 min with constant stirring at 15$\pm$0.5°C. Five-ml aliquots were then layered over sucrose—0.05 M potassium phosphate, pH 7.4, step gradients. The sucrose concentrations and volumes used were (top to bottom) 0.7 M,7 ml; 1.0 M, 9 ml; and 1.8 M, 11 ml. Centrifugation was carried out for 1 h at 23 500 rev./min in a SW 25 rotor. The chlorophyll bands, one at the top of the tube (D-1 fraction) and the other extending through the 1.0 M sucrose portion of the gradient and concentrated at the 1.0–1.8 M sucrose interface (D-II fraction) were collected separately. The D-II fraction was diluted and recovered by centrifugation at 180 000 $\times g$ for 1 h. Not all of the chlorophyll in the D-1 fraction could be collected by a similar centrifugation and the fraction was made 10 % with respect to trichloroacetic acid. A five-ml aliquot of the digitonin incubation mixture (Dig–10 000 $\times g$ fraction) was also treated with trichloroacetic acid in a similar manner. The trichloroacetic acid-precipitated material in both the D-1 fraction and the incubation mixture was collected by centrifugation at 40 000 $\times g$ for 20 min.

Extraction, solubilization and gel electrophoresis of membrane fragments and soluble proteins

All protein fractions, including the proteins used to calibrate the molecular weight scale of the gels, were treated in the following manner. Lipids were removed by at least seven extractions (or until the supernatant was colorless) with a chloroform–methanol mixture (1:2, v/v) followed by three washings with absolute methanol. The resulting material was then dried in vacuo and the dry weight determined. The dissolving buffer 1 % dodecylsulfate and 1 % β-mercaptoethanol in 0.1 M sodium phosphate, pH 7.0, was then added in the amount of 1 ml per mg dry weight. The material was incubated overnight at 45 °C under nitrogen. The samples were homogenized and placed in a boiling water bath for approx. 20 min. After allowing the samples to cool, they were centrifuged in a clinical centrifuge and any pelleted material was saved for nitrogen analysis. The following data (for the fractions represented in Fig. 5) exemplify the degree of solubilization achieved by the methods used: EDTA–10 000 $\times g$ fraction : 99 %, Gradient–10 000 $\times g$ fraction: 97 %, Dig–

10 000×*g* fraction: 98 %, D-I fraction: 99 % and D-II fraction: 97 %. The supernatants were then dialyzed for 4 h against a large excess of 0.1 % dodecylsulfate and 0.1 % β-mercaptoethanol in 0.1 M sodium phosphate, pH 7.0. Samples were stored under nitrogen.

Samples were prepared and electrophoresis performed essentially as outlined by Weber and Osborn [15]. Samples, except standard proteins, containing 40 μg of protein (except as indicated in Results) were applied to the gels. Standard proteins were prepared so that they gave a peak absorbance which was less than 2.0 *A*. Pyronin Y was used as the tracking dye and 7 % acrylamide–0.23 % methylene–bisacrylamide gels were used. Gels 8.5 cm in length were cast in tubes of 6 mm inner diameter and 10 cm in length. Electrophoresis was performed at 8 mA per tube for 5.5 h during which time the tracking dye migrated approx. 7.5 cm. The position of the tracking dye was marked by the insertion of a small piece of nichrome wire. The gels were stained overnight using Commassie brilliant blue R made as described by Weber and Osborn [15]. Destaining was carried out for 7 h in the solution of Weber and Osborn [15], followed by placing the gels overnight in a solution of 50 % ethanol and 10 % acetic acid. Further destaining was performed in the solution of Weber and Osborn [15] until the background stain was removed. Gels were scanned at 560 mm using a Gilford linear transport accessory attached to a Beckman DU spectrophotometer using a 0.1 mm slit. The gels were first scanned at 0–1.0 *A* at a scan speed of 1 cm/min for detail and then at 0–3.0 *A* with a scan speed of 2 cm/min to record the values for peaks having greater than 1.0 *A*. The speed of the recorder was 1 inch/min. The relative mobilities of the proteins were calculated from the recorder tracings. A molecular weight scale for the gels was established by using the following proteins as standards: phosphorylase *a* (94 kdaltons), bovine serum albumin (68 kdaltons), ovalbumin (43 kdaltons), chymotrypsinogen (25.7 kdaltons), myoglobin (17.2 kdaltons), and lysozyme (14.3 kdaltons).

Assays

Chlorophylls were determined using the equations of Arnon [16]. The nitrogen content of the solubilized protein solutions and of any residues was determined by a modification of a standard micro-Kjeldahl method [17]. In the calculations it was assumed that the protein was 16 % nitrogen by weight. The protein solutions were also analyzed by the method of Lowry et al. [18] using bovine serum albumin as standard. There was excellent agreement between the values obtained by these two methods of analysis.

Data presentation

Full scale absorbance on the figures is 1.0 *A*. Absorbances and any peak splitting observed for peaks of greater than 1.0 *A* unit are indicated in the figure legends. The two vertical lines at the base of the traces indicate the top of the gel and the position of the tracking dye. The arrow indicates the direction of migration and 0.0 *A*. The upward inflection at the left hand side of the scans is due to a slight curvature at the top of the gels. For a component free from interference by surrounding components, the level of detection is probably on the order of 1 μg [19].

The components observed are referred to in terms of their weights in kdaltons. Molecular weights as low as 17 300 could be assigned by reference to a standard curve.

Peaks which have a molecular weight below 17 000 are designated by their R_m values.

RESULTS

Analysis of membrane fractions

In order to compare the polypeptides both for a given fraction within a given isolation procedure and to compare the effects of purification on a given fraction, the mean molecular weights and the mean deviations of apparently identical polypeptides for each of the same fractions obtained by the various purification procedures were calculated (Table I). The values for the D-I and D-II fractions were not included in the calculations. These values are not intended to imply a statistical analysis of the reproducibility of the method but rather to serve as a means of identifying peaks and to indicate the range of values observed for what are considered identical peaks. Samples were generally run three times (in different experiments) and in most cases the variation in R_m (or calculated apparent molecular weight) for a given peak was less than 5% (Table I). The large variability in the

TABLE I

MEAN WEIGHTS, MEAN DEVIATIONS AND AVERAGES OF THE MEANS (IN kDALTONS) OF POLYPEPTIDES PRESENT IN VARIOUS LAMELLAR PREPARATIONS OBTAINED BY VARIOUS ISOLATION PROCEDURES

[a] Non-EDTA- and EDTA-washed preparations only; [b] Non-EDTA- washed preparations only; [c] Non-EDTA- and EDTA-washed preparations and fractions in Table II; [d] Non-EDTA- washed preparations only but perhaps present as a shoulder in the buffered gradient preparations; [e] Buffered gradient preparations only, not including fractions in Table II; [f] Non-EDTA- and EDTA-washed preparations only but perhaps present as a shoulder in the buffered gradient preparation; [g] EDTA-washed preparation only.

FP fraction	10 000 × *g* fraction	180 000 × *g* fraction	Average of the means (rounded to nearest kdalton)
65.6±0.1[a]	66.0±0.4	66.1±0.2	66
59.9±0.2	59.7±0.5	59.2±0.7	60
56.3±1.2	55.9±0.8	54.0±0.2	56, 54
49.6±0.6	49.4±0.2		50
45.8±0.3	45.8±0.5	45.8±0.3	46
41.4±0.2	41.2±0.4	41.3±0.3	41
		40.2[b]	40
38.7±0.2	38.4±0.7		39
36.7[b]	36.3[b]	36.3±0.3	36
34.5±0.2	34.6±0.3	34.4±0.1	35
29.8±0.1	29.7±0.3	29.7±0.1	30
		27.4[b]	27
25.0±0.2[a]	24.9±0.3[c]	25.3[d]	25
24.1[e]	24.0[e]	24.0±0.1[f]	24
22.7±0.3	22.7±0.3	22.5±0.1	23
		20.8±0.3	21
19.3±0.1	19.3±0.2	19.1±0.2	19
17.8±0.1	17.8±0.1	17.8±0.1[g]	18

value for the peak at 56 kdaltons (in the FP and $10\,000 \times g$ fractions) is due to this peak having an extraordinarily high calculated molecular weight in one of the buffered gradient experiments. The mean deviations are generally larger for the $10\,000 \times g$ fractions than in the composites of the other two fractions; nevertheless, the means of what are considered identical peaks agree well with the means of the FP fractions as would be expected since the $10\,000 \times g$ fraction represents greater than 80% of the lamellar system. As a convenience in discussing the results, the average of the means,

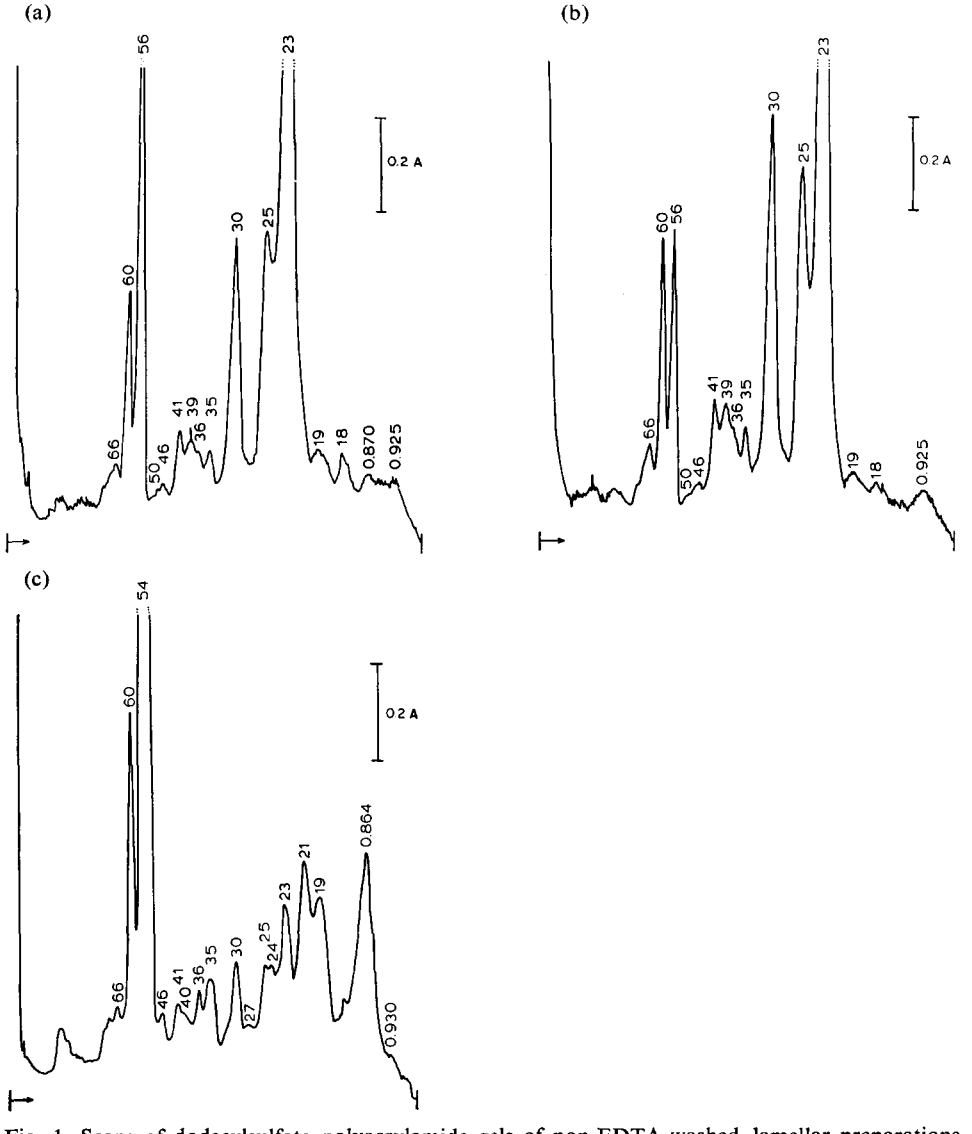


Fig. 1. Scans of dodecylsulfate–polyacrylamide gels of non-EDTA-washed lamellar preparations, (a) FP fraction, (b) $10\,000 \times g$ fraction and (c) $180\,000 \times g$ fraction. Offscale absorbances for the following molecular weight peaks are in (a) $56 \cdot 10^3$: 1.2 *A*; $23 \cdot 10^3$: 1.86 *A*, (b) $23 \cdot 10^3$: 1.96 *A* and (c) $54 \cdot 10^3$: 3.0 *A*. See text.

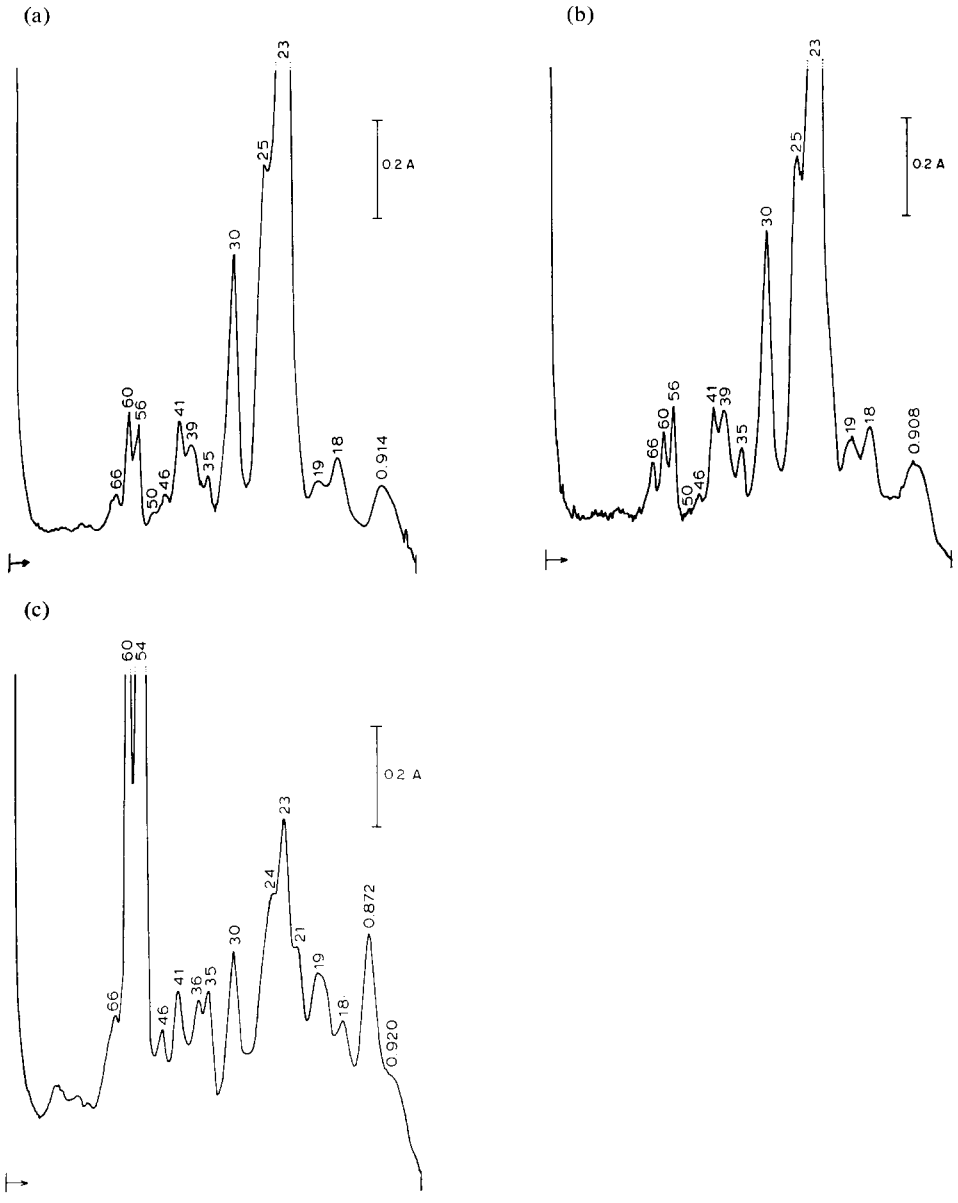


Fig. 2. Scans of dodecylsulfate–polyacrylamide gels of EDTA-washed lamellar preparations (a) FP fraction, (b) 10 000 $\times g$ fraction and (c) 180 000 $\times g$ fraction. Offscale absorbances for the following molecular weight peaks are in (a) $23 \cdot 10^3$: 2.1 *A*, (b) $23 \cdot 10^3$: 1.8 *A* and (c) $60 \cdot 10^3$: 1.96 *A*; $54 \cdot 10^3$: 2.94 *A*. See text.

rounded to the nearest kdalton, will be used with one exception. The peak at 56 kdaltons in the FP and 10 000 $\times g$ fractions will not be strictly equated with the 54 kdalton peak in the 180 000 $\times g$ fractions (see below).

For the purpose of this discussion it will be assumed that all the polypeptides stain approximately equally regardless of their extend of migration or amino acid

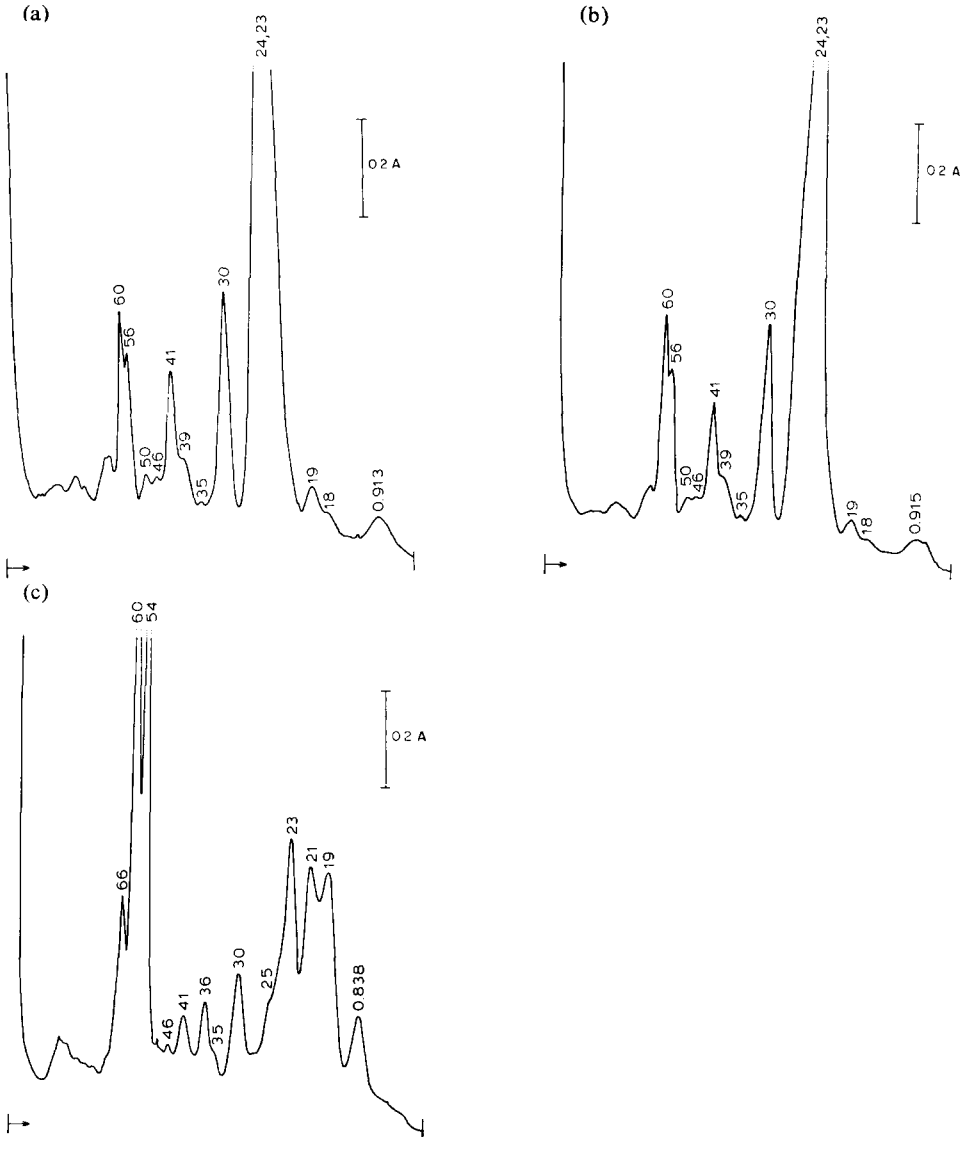


Fig. 3. Scans of dodecylsulfate –polyacrylamide gels of lamellar preparations which have been wash ed with EDTA and further purified on a buffered sucrose gradient, (a) FP fraction, (b) 10 000 × g fraction and (c) 180 000 × g fraction. The offscale peak in (a) and (b) shows peak splitting with peaks at $24 \cdot 10^3$ and $23 \cdot 10^3$ and having the following absorbances in (a) $24 \cdot 10^3$: 1.15; $23 \cdot 10^3$: 1.15 A and (b) $24 \cdot 10^3$: 1.06 A; $23 \cdot 10^3$: 1.22 A. In (c) the offscale absorbances at $60 \cdot 10^3$ and $54 \cdot 10^3$ are 1.51 and 1.35 A, respectively. See text.

composition though Fishbein [19] and Hoober [4] have shown these variables affect the intensity of staining.

(*i*) *French press fractions.* A qualitative comparison for the composites of each fraction from all the data indicates that the major differences in polypeptide composition among the FP, 10 000 × g, and 180 000 × g fractions are quantitative

rather than qualitative (Figs 1–3). A quantitative comparison of the FP and 10 000 $\times g$ and of the FP and 180 000 $\times g$ fractions isolated by a given procedure generally show the same similarities and differences irregardless of the isolation procedure. The non-EDTA fractions show that the FP fraction contained more of a 56 kdaltons component than did the 10 000 $\times g$ fraction. A scan of the 180 000 $\times g$ fraction shows the major band to be at 54 kdaltons, whereas the FP and 10 000 $\times g$ fractions have the greatest amount of polypeptide in the 25–20 kdalton range. The 180 000 $\times g$ fraction is also enriched in the 19 kdalton component and depleted in the 30 kdalton component. The same general features are observed for the EDTA, and buffered gradient fractions. An exception is that, after EDTA washing, the differences in the high molecular weight region for the FP and 10 000 $\times g$ fractions are eliminated, and there is some variability as to the major peak in this region for the 180 000 $\times g$ fractions. However, some qualitative differences do exist between the FP (Figs 1a, 2a and 3a) and the 180 000 $\times g$ fractions (Figs 1c, 2c and 3c). The FP fraction contains polypeptides of 50 and 39 kdaltons and a component(s) having an R_m value greater than 0.9 which is lacking or partially masked in the 180 000 $\times g$ fraction. The 180 000 $\times g$ fraction has polypeptides at 36 and 21 kdaltons and one (or more) having an R_m of approx. 0.86 which are generally lacking in the FP and 10 000 $\times g$ fractions. It should be noted, however, that the non-EDTA treated FP and 10 000 $\times g$ fractions (Figs 1a and 1b) do show a shoulder which might be attributed to the 36 kdalton peak. Characteristic of the 180 000 $\times g$ fractions is the presence of a peak at 54 kdaltons which is compared in Table I to a peak at 56 kdaltons present in the FP and 10 000 $\times g$ fractions. The peak at 54 kdaltons in the 180 000 $\times g$ fractions probably represent two unresolved polypeptides. In several electrophoresis runs of the EDTA -treated 180 000 $\times g$ fraction and buffered gradient 180 000 $\times g$ fractions, two bands were observed visually but were not resolved by the gel scanner.

Also, characteristic of the 180 000 $\times g$ fractions is the resolution of the peaks in the 25–20 kdalton range, when compared to the FP and 10 000 $\times g$ fractions. It should be noted that in all the fractions there is some variability in the presence of peaks at 25 and 24 kdaltons. It can be seen that other peaks are variable in their presence, particularly the 18 kdalton peak in the 180 000 $\times g$ fractions.

Several features can be observed in the FP and 10 000 $\times g$ fractions with successive purification. The amount of the polypeptides in the 25–20 kdalton range progressively increases as does that in the 50–40 kdalton range, and the amount of the polypeptides in the 60–54 kdalton region generally decreases. The same results are observed more dramatically for the 180 000 $\times g$ fractions particularly for the 54 kdalton peak. Such comparisons are somewhat complicated by the fact that visually the gradient-purified fractions appear to take up less total stain (this was particularly evident in the D-II fraction reported below).

(*ii*) *Analysis of supernatant fractions obtained upon EDTA treatment.* A comparison of supernatants from the EDTA-treated FP and 10 000 $\times g$ fractions (Figs 4a and 4b) indicates that two peaks predominate and have molecular weights of approx. 59 000 and 54 000. These two fractions also have several other peaks in common at approx. 37, 30, and 23 kdaltons and one having an R_m of approx. 0.853. The supernatant from EDTA-treated 180 000 $\times g$ fraction (Fig. 4c) shows two predominate peaks, one having a molecular weight of 53 400 and one having an R_m of 0.867. The 30 kdalton component is of interest and is considered below.

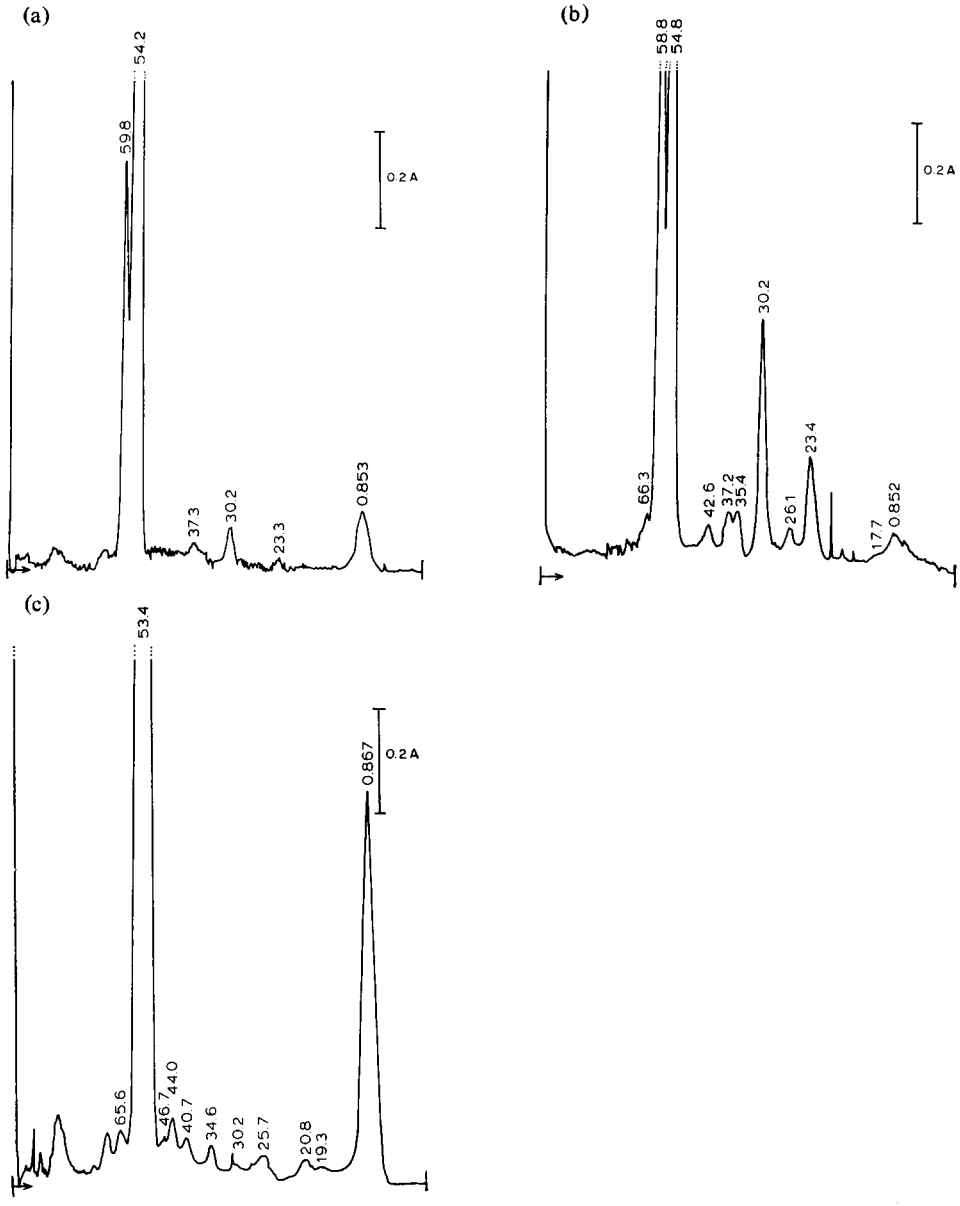


Fig. 4. Scans of dodecylsulfate–polyacrylamide gels of supernatants from fractions, (a) FP fractions, (b) 10 000 × *g* fraction and (c) 180 000 × *g* fraction. Approximate offscale absorbances, determined by the use of a reduced amount of protein per gel where necessary, for the following weight peaks are in (a) $54.2 \cdot 10^3$: 3.0 *A*, (b) $58.8 \cdot 10^3$: 1.5 *A*, $54.8 \cdot 10^3$: 2.2 *A* and (c) $53.4 \cdot 10^3$: 8.0 *A*. Note that the scans in (a) and (b) are for only 20 μg protein per gel. See text.

(*iii*) *Digitonin fractionation of grana membranes.* The extraction procedure as described under Methods, when an EDTA treatment is not employed, yields fractions which are very similar to those of Arntzen et al. [13] on the basis of chloro-

phyll *a* to chlorophyll *b* ratios, P700 content, percent chlorophyll in the fractions, structure as seen by freeze-etching, and photochemical properties. Detailed results concerning these functions will be reported elsewhere (Nolan, in preparation). EDTA washing of the 10 000 × *g* fraction does not appear to interfere with the digitonin fractionation. The digitonin derived fractions are designated D-1 (Photosystem I enriched) and D-II (Photosystem II enriched) fractions.

As would be expected from the results presented above, the use of an EDTA -treated 10 000 × *g* fraction or buffered gradient 10 000 × *g* fraction did not have an effect on the staining pattern observed. The results for the use of a buffered gradient 10 000 × *g* fraction for further fractionation are presented below in Table II and in Fig. 5. The molecular weights used in Fig. 5 are the averages of the means given in Table I.

TABLE II

CALCULATED WEIGHTS AND DESIGNATIONS, IN kDALTONS, OF POLYPEPTIDES OF VARIOUS GRANA PREPARATIONS AND FRACTIONS

All preparations and fractions are derived from the EDTA-washed grana preparation and then subjected to further purification and fractionation. See text.

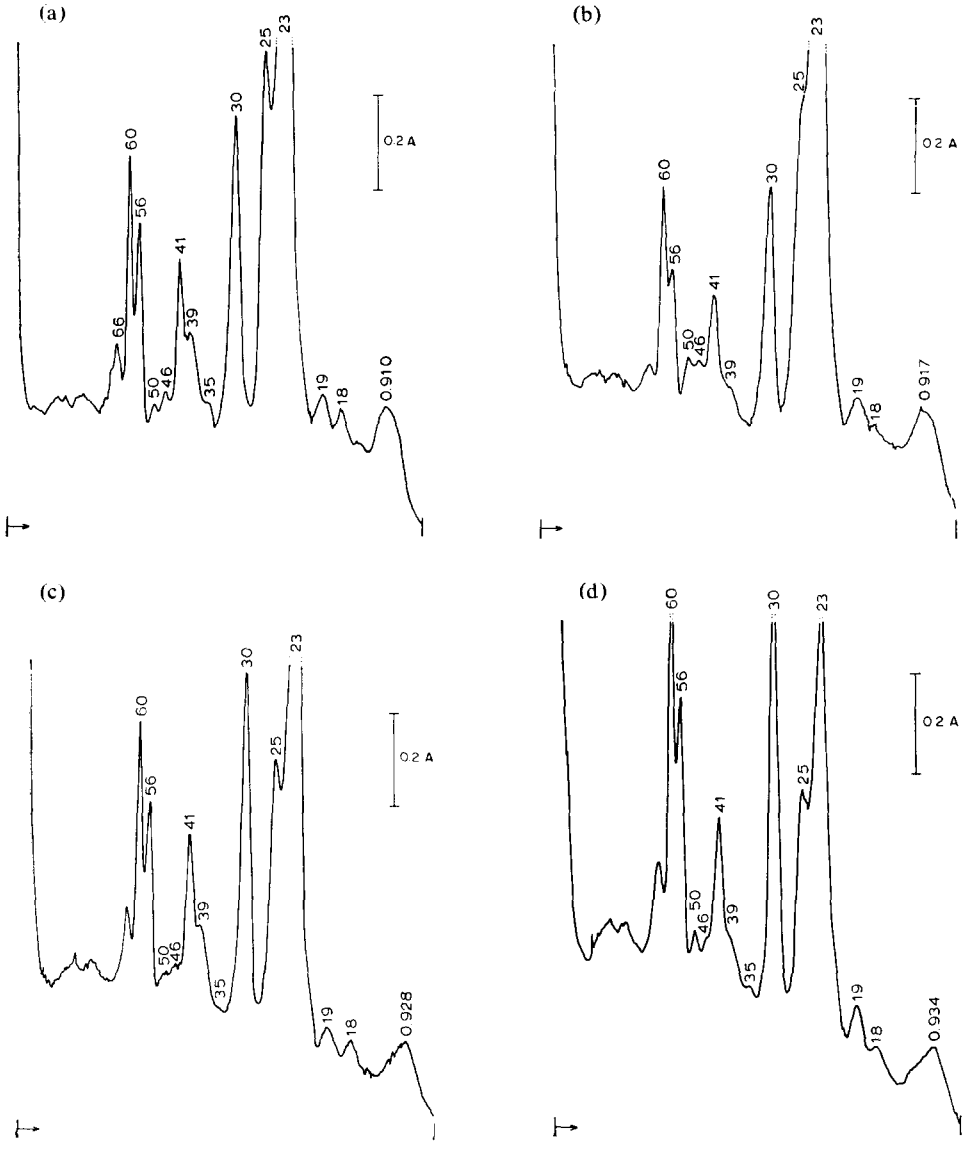
EDTA-treated 10 000 × *g* fraction	Gradient 10 000 × *g* fraction	Dig– 10 000 × *g* fraction	D-I fraction	D-II fraction	Designation[1]
65.7	—	—	—	65.6	66
59.3	59.5	60.4	60.2	60.4	60
55.5	55.5	56.2	55.6	55.5	56
49.2	49.3	49.4	50.0	49.7	50
45.5	45.6	46.2	45.5	45.5	46
41.3	41.2	41.8	42.0	41.5	41
38.8	37.6	39.5	39.7	38.7	39
34.4	—	34.9	34.8	—	35
29.6	29.6	30.1	29.9	29.6	30
25.1	24.7	25.4	25.3	25.2	25
22.7	22.8	23.1	22.8	22.6	23
19.1	19.1	19.7	19.5	19.2	19
17.7	17.8	17.8	18.0	17.7	18

[1] Average of the means in Table I.

It can be seen in Figs 5a, b and c that the EDTA-treated 10 000 × *g* fraction, gradient 10 000 × *g* fraction and incubation mixture (Dig–10 000 × *g* fraction) are essentially identical. It was essential that the Dig–10 000 × *g* fraction (isolated by trichloroacetic acid precipitation) was identical to the EDTA -treated 10 000 × *g* and gradient 10 000 × *g* fractions since the D-1 fraction could not be pelleted and was isolated by trichloroacetic acid precipitation.

Table II and Figs 5d and 5e show that there are no major qualitative differences (if any at all) between the D-1 and D-II fractions. A quantitative comparison of the two fractions is somewhat difficult since visually the D-II fraction did not appear to stain as intensely as the D-I and various 10 000 × *g* fractions. Note that the scan of the D-II fraction (Fig. 5e) is for 50 μg of protein rather than the usual

40 μg. However, when the area under the curve was calculated, it was determined that the sum of the area under the D-1 and D-II curves divided by two, equalled the area under the Dig–10 000 $\times g$ fraction curve. The calculations were performed on traces of samples which had been derived from an EDTA-treated 10 000 $\times g$ fraction and from a buffered gradient 10 000 $\times g$ fraction and a similar observation was made in both cases. The major polypeptides in the D-1 fraction appear to be in the 60–55 and 30–23 kdaltons ranges. Comparatively, the D-1 is enriched in polypeptides of 60, 56, 30 and 18 kdaltons and in a polypeptide(s) having a high R_m value. The D-II is apparently enriched in components at 66, 25 and 23 kdaltons.



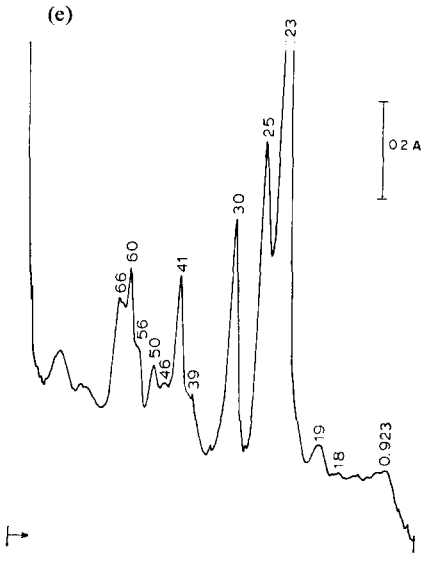


Fig. 5. Scans of dodecylsulfate–polyacrylamide gels of various grana preparations and fractions: (a) EDTA-washed grana preparation, (b) fraction in (a) after further purification on a buffered sucrose gradient, (c) fraction in (b) after incubation with digitonin ,(d) D-I fraction isolated from fraction in (b) after incubation with digitonin and (e) D-II fraction isolated from fraction in (b) after incubation with digitonin. Offscale absorbances for the following weight peaks are in (a) $23 \cdot 10^3$: 2.28 *A*, (b) $23 \cdot 10^3$: 1.50 *A*, (c) $23 \cdot 10^3$: 1.62 *A*, (c) $60 \cdot 10^3$: 1.08 *A*; $30 \cdot 10^3$: 1.08 *A*; $23 \cdot 10^3$: 1.10 *A* and (e) $23 \cdot 10^3$: 1.35 *A*. Note that the scan in (e) is for 50 μg of protein applied to the gel. See text.

DISCUSSION

With the exception of the study by Klein and Vernon [7], only superficial comparisons can be made with the data reported here to other investigations using at least partially lipid-depleted preparations. Lagoutte and Duranton [20] using thoroughly lipid-extracted lamallae of *Zea mays* reported the major polypeptide to have a M_r of approx. 25 000 and the next most abundant to have a M_r of approx. 50 000. The data of Lagoutte and Duranton (see Fig. 2, ref. 20) indicates a doublet in the 50 kdalton range, which may represent subunits of CF_1 fraction, and the 25 kdalton component is undoubtedly "structural protein"[21]. Thus, it appears that for *Zea*, the polypeptides represented in greatest amount are similar to those reported here for spinach.

Levine et al. [22] using spinach have presented results for whole lamellae and for digitonin fractions derived by the method of Anderson and Boardman [23] similar to those reported here. The whole lamellae showed the most abundant polypeptides to be present in the 30–20 kdalton range and a large amount in the 60–50 kdalton range. The digitonin-derived Photosystem II enriched fraction showed polypeptides in the 30-20 kdalton range to predominate while in the Photosystem I fraction the polypeptides in the 60–50 kdalton range were the most abundant. It would be expected, except for proteins possibly lost due to solubilization by digitonin, that the digitonin fractions would be identical to those reported here since Goodchild and Park [24] have shown that the initial action of digitonin is similar to that of French pressure cell treatment.

Klein and Vernon [7] have studied the polypeptide composition of whole chloroplasts (hypotonically shocked and washed) and fragments of chloroplasts (not shocked and washed). Fragmentation was achieved by French pressure cell treatment and by the use of Triton X-100. Klein and Vernon [7] observed approximately 14 polypeptides and assigned tentative designations to some of these on the basis of molecular weight. They obtained, by French pressure cell treatment, results very similar to those reported here. They have reported that the 10 000$\times g$ fraction resembles very closely whole lamellae (FP fraction in this report), with the bulk of the polypeptide being "structural" [21] protein. Their 160 000$\times g$ fraction resembles very closely the 180 000$\times g$ fraction reported here, with the bulk of the polypeptide being in the 60–50 kdalton range. Triton fractionation of chloroplasts yields two fractions designated TSF I fraction (Photosystem I fraction) and TSF II fraction (Photosystem II fraction). The polypeptide pattern of the TSF II fraction resembles that of the digitonin-derived Photosystem II fraction (D-II fraction) reported here, with the bulk of the polypeptide being apparently structural protein. Although the origin of the TSF I fraction is unclear (i.e., whether from grana and/or stroma lamellae), it is not unlike that of the D-1 fraction and to a lesser degree the 180 000$\times g$ fraction reported here, with the major polypeptides being in the 60–50 kdalton range.

Several of the polypeptides observed in the supernatants from EDTA-treated fractions can be tentatively designated on the basis of their apparent molecular weight as representing polypeptides of known chloroplast enzymes. The peak at approx. 54 kdaltons seen in Fig. 4c is assumed to represent the large subunit of Fraction I protein [25, 26]. Chloroplast coupling factor (CF_1 fraction) is partially extracted from lamellae by EDTA washing [27] and has five subunits of approx. 59, 56, 37, 17.5 and 13 kdaltons [28].The polypeptides in Figs 4a and 4b at approx. 59, 54 and 37 kdaltons are tentatively designated as representing several of the subunits of CF_1 fraction. In addition, Fig. 4b shows a shoulder at 17.7 kdaltons which perhaps represents another subunit of CF_1 fraction. The occurrence of the peak at 54 kdalton (Figs 4a and 4b), rather than at 56 kdalton, probably is due to the failure to resolve polypeptides representing a subunit of CF_1 fraction and the large subunit of Fraction I protein. The presence of Fraction I protein in Figs 4a and 4c may be in part due to its high molecular weight and the high centrifugal forces employed in isolating the fractions. However, Fig. 4b suggests that at least a portion is associated with the membranes and is released by EDTA treatment. It should be noted that in Fig. 4 there is no evidence of the removal of CF_1 fraction by the EDTA treatment of the 180 000$\times g$ fraction. One peak of interest in Fig. 4 is that at approx. 30 kdaltons. The amount in which it is present in the supernatant from EDTA-treated 10 000$\times g$ fraction (Fig. 4b) suggests it is removed from the membranes by the EDTA treatment. Whether it represents a different polypeptide than that present in the membrane fractions or whether, as in the case of CF_1 fraction, it is only partially extracted by the EDTA treatment is unknown. The peak having an approximate R_m of 0.85–0.87 in Fig. 4 probably represents several polypeptides since several chloroplast enzymes (soluble or known to be released by EDTA treatment) have polypeptides in the region of 14–10 kdaltons [25, 26, 28].

Though the considerable CF_1 fraction present in EDTA-washed 180 000$\times g$ fraction (Fig. 2c) could be due to fractional centrifugation of the protein, it should

be noted that the buffered gradient 180 000 × *g* fraction still contains a large amount of CF_1 fraction (Fig. 3c). Only after the drastic treatment of isolating the fractions from an unbuffered gradient is the presence of CF_1 fraction essentially eliminated from all fractions. Also of interest is the observation that the D-I fraction, which is Photosystem I in character as is the 180 000 × *g* fraction, contains most of the CF_1 fraction when compared to the D-II fraction. The results presented here indicate that there are two types of coupling factor designated either by chemical differences or by locational differences with respect to the membrane. It is known that only 50–70 % of chloroplast coupling factor is removed from lamellae by EDTA treatment [27], indicating that the protein complex is located in different environments with respect to the membrane or that there is more than one type of coupling factor. In this regard it should be noted that Livne and Racker [29] have reported a second coupling factor (CF_2 fraction) to be present in chloroplast. CF_2 fraction has not been characterized, but the work of Anderson and McCarty [30] indicates that it might be associated with Photosystem I. Uribe [31] has suggested that the residual CF_1 fraction remaining after EDTA treatment may be in a lipophilic environment in the membrane.

In conclusion, the results of experiments performed on chloroplasts fragmented into grana lamellae (Photosystem II-enriched) and stroma lamellae (Photosystem I in character) by either the French pressure cell technique or by the Anderson and Boardman digitonin technique, indicate that relatively few qualitative and some quantitative differences exist between the fractions. Although the differences are not distinct or dramatic, the similarity of the fractions is perhaps not unexpected in view of the proposed nature of stroma lamellae [32, 33]. The results obtained upon further fractionation of grana lamellae using digitonin by the method of Arntzen et al. or the fractionation of chloroplasts using Triton, appear promising in our further understanding of the photosynthetic membrane. The results presented here on the polypeptide composition and the work of Allen et al. on the lipid composition indicate that small differences in composition account for large differences in structure and function within the chloroplast membrane system.

ACKNOWLEDGEMENTS

The technical assistance of Mr A. O. Pfeifhofer is gratefully acknowledged. This work was supported in part by National Science Foundation Grant GB-25579X and by a Chancellor's Patent Fund Grant for Graduate Student Research (University of California, Berkeley). W. G. N. was supported in part by a National Science Foundation Predoctoral Traineeship.

REFERENCES

1 Allen, C. F., Good, P., Trosper, T. and Park, R. B. (1972) Biochem. Biophys. Res. Commun. 48, 907–913
2 Lichtenthaler, H. K. and Park, R. B. (1963) Nature 198, 1070–1072
3 Hoober, J. K. (1972) J. Cell Biol. 52, 84–96
4 Hoober, J. K. (1970) J. Biol. Chem. 245, 4327–4334
5 Hoober, J. K. and Stegeman, W. J. (1973) J. Cell Biol. 56, 1–12
6 Machold, O. and Aurich, O. (1972) Biochim. Biophys. Acta 281, 103–112

7 Klein, S. and Vernon, L. P. (1974) Photochem. Photobiol. 19, 43–49
8 Anderson, J. M. and Levine, R. P. (1974) Biochim. Biophys. Acta 333, 378–387
9 Anderson, J. M. and Levine, R. P. (1974) Biochim. Biophys. Acta 357, 118–126
10 Eaglesham, A. R. J. and Ellis, R. J. (1974) Biochim. Biophys. Acta 335, 496–507
11 Park, R. B. and Nolan, W. G. (1974) Ann. N.Y. Acad. Sci. 227, 580–586
12 Sane, P. V., Goodchild, D. J. and Park, R. B. (1970) Biochim. Biophys. Acta 216, 162–178
13 Arntzen, C. J., Dilley, R. A., Peters, G. A. and Shaw, E. R. (1972) Biochim. Biophys. Acta 256, 85–107
14 Howell, S. H. and Moudrianakis, E. N. (1967) J. Mol. Biol. 27, 323–333
15 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
16 Arnon, D. I. (1949) Plant Physiol. 24, 1–15
17 Ma, T. S. and Zuazaga, G. (1942) Ind. Eng. Chem. Anal. Ed. 14, 280–282
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19 Fishbein, W. N. (1972) Anal. Biochem. 46, 388–401
20 Lagoutte, B. and Duranton, J. (1971) Biochim. Biophys. Acta 253, 232–239
21 Criddle, R. S. and Park, L. (1964) Biochem. Biophys. Res. Commun. 17, 74–79
22 Levine, R. P., Burton, W. G. and Duram, H. A. (1972) Nat. New Biol. 237, 176–177
23 Anderson, J. M. and Boardman, N. K. (1966) Biochim. Biophys. Acta 112, 403–421
24 Goodchild, D. J. and Park, R. B. (1971) Biochim. Biophys. Acta 226, 393–399
25 Rutner, A. C. (1970) Biochem. Biophys. Res. Commun. 39, 923–929
26 Moon, K. E. and Thompson, E. O. P. (1971) Aust. J. Biol. Sci. 24, 755–764
27 McCarty, R. E. and Racker, E. (1967) J. Biol. Chem. 242, 3435–3439
28 Nelson, N., Deters, D. W., Nelson, H. and Racker, E. (1973) J. Biol. Chem. 248, 2049–2055
29 Livne, A. and Racker, E. (1968) Biochem. Biophys. Res. Commun. 32, 1045–1049
30 Anderson, M. M. and McCarty, R. E. (1969) Biochim. Biophys. Acta 189, 193–206
31 Uribe, E. G. (1972) Biochemistry 11, 4228–4235
32 Park, R. B. and Sane, P. V. (1971) Annu. Rev. Plant Physiol. 22, 395–430
33 Sane, P. V. and Park, R. B. (1971) Biochim. Biophys. Acta 253, 208–212