Studies on the Nature of Chloroplast Lamellae. II. Chemical Composition and Further Physical Properties of Two Chlorophyll–Protein Complexes*

J. Philip Thornber,† John C. Stewart, Mark W. C. Hatton, and J. Leggett Bailey

ABSTRACT: The amino acid and pigment composition of the two major detergent-soluble chlorophyll–proteins (complexes I and II) of green leaves has been investigated. Complex I (chlorophyll a:b, 12:1) contains mainly β-carotene and a trace of lutein, whereas complex II (chlorophyll a:b, 1.2:1) contains more xanthophylls than β-carotene; a considerable proportion of the pigments which are attached to the complexes *in vivo* is extracted from them during the isolation of the complexes. The protein moieties of the two complexes have different compositions, although they both contain a very high proportion of apolar amino acids. $s_{20,w}$ = 9 S for complex I and 2–3 S for complex II. Both complexes contain carbohydrate material. The only difference between complexes I and II prepared from a monocotyledon and those isolated from some dicotyledons was the low β-carotene content in complex II from a monocotyledon. We have contrasted our method for estimation of the pigment composition of the complexes with those used by other workers.

The lamellar membrane of the chloroplast can be rendered soluble to different extents by a variety of treatments, such as sonication, freeze thawing, or extraction with organic solvents or detergents. It is clear that the dispersion of the lamellae components which accompanies dissolution is not random, but that several significant complexes are released (Anderson and Boardman, 1966; Bril, 1965; Gross *et al.*, 1966; Kahn, 1964; Katoh and San Pietro, 1966; Ogawa *et al.*, 1966; Sironval *et al.*, 1966; Thornber *et al.*, 1966, 1967; Vernon *et al.*, 1966; Wessels, 1966a,b). The most important problem in relation to these complexes is whether they correspond to the photochemical systems involved in the collection and transfer of energy in photosynthesis. As yet, no complete and unequivocal physical separation of the two systems in an active state has been demonstrated. To this end Anderson and Boardman (1966) have obtained partial separation of the active systems, and Ogawa *et al.* (1966) and Sironval *et al.* (1966) have completely separated two inactive particles which they believe correspond to the two photochemical systems; also treatment of chloroplasts with Triton X-100, which inactivates system II, yields a particle having system I activity (Kahn, 1964; Vernon *et al.*, 1966). In Part I (Thornber *et al.*, 1967) a fractionation of two photochemically inactive particles was described, and evidence was adduced for the derivation of each of the particles from an active photochemical system.

Apart from the pigment compositions reported by Ogawa *et al.* (1966) and Ke *et al.* (1966), and evidence derived from action spectra (Clayton, 1965), little quantitative information on the individual photochemical reaction centers is available. Thus analysis of our pigmented lipoprotein complexes, even though each may have lost some of the components present *in vivo* (Ogawa *et al.*, 1966), can be expected to provide a useful approach to a description of the photoactive sites in chemical terms. We now wish to report the results of such an analysis.

Methods

Plant Material. Spinach beet (*Beta vulgaris, L. cicla*), spinach (*Spinacea oleracea*), and tobacco (*Nicotiana tabacum*) were grown in a greenhouse and the leaves were harvested immediately prior to being homogenized. Oats (*Avena sativa* var. *condor*) were grown for 17 days in a greenhouse and the leaves were stored at −20° overnight before use.

Isolation of Free-Pigment and Pigment–Protein Complexes. Full experimental details of the procedure have been given previously (Thornber *et al.*, 1967). Essentially the method was as follows. Chloroplasts were isolated by differential centrifugation of leaf homogenates, were washed once with water, and then successively extracted five times with 0.5% (w/v) SDBS[1]–0.05 M sodium borate (pH 8.0, detergent–chlorophyll, 2.5:1, w/w); this produced five detergent extracts and a small residue. The second and third detergent extracts were resolved into four pigment-containing zones by electrophoresis in polyacrylamide

* From Twyford Laboratories Ltd., London, N.W. 10, England. *Received March 7, 1967.*

† Present address: Department of Biology, Brookhaven National Laboratory, Upton, Long Island, N. Y. 11973.

[1] Abbreviations used: SDBS, sodium dodecylbenzenesulfonate; SDS, sodium dodecyl sulfate; PQ, plastoquinone.

gels (Figure 1); complexes I and II are pigment–proteins, and complexes III and IV are protein-free pigment zones; presumably detergent is attached to each complex. Disks containing each zone were subsequently dissected from the gel columns, and each pigmented complex was eluted from a slurry of the disks through a Sephadex G-200 column.

Analytical Ultracentrifugation. Complexes I and II were each eluted through a Sephadex G-200 column in order to equilibrate the lipoprotein in a known strength of detergent or in water; equilibration by dialysis took too long. Sedimentation runs were carried out in the An-E rotor of a Spinco Model E ultracentrifuge using cells of 30-mm path length. A rotor speed of 42,040 rpm was used. Samples for analysis contained about 0.5 mg of protein/ml. The viscosities and densities of the solvents were measured and used to correct the determined S values (Schachman, 1957); a partial specific volume of 0.96 was assumed (Chiba, 1960).

Amino Acid Analysis. Each of the two chlorophyll–proteins was freeze dried and extracted with 80% aqueous acetone to remove pigments and lipids. The delipidized proteins (3–5 mg) were hydrolyzed with constant-boiling HCl at 110° under N_2 in tubes sealed under reduced pressure; hydrolysis times ranged from 12 to 72 hr. The amino acid composition of the hydrolysates was obtained using an EEL-Guinness automatic analyzer. Oxidized proteins, prepared by the method of Hirs (1956), were used to determine the content of half-cystine and methionine which were estimated as cysteic acid and methionine sulfone, respectively. After removal of the pigments and lipids from solutions of each complex by treatment with butanol as described by Biggins and Park (1965), the tyrosine:tryptophan molar ratio of the resulting soluble protein was determined by the spectrophotometric method of Beaven and Holiday (1952).

Carotenoid Composition. The free-pigment and pigment–protein zones (complexes I–IV, Figure 1) were isolated from 9 ml of detergent extract, containing about 4 mg of chlorophyll and 0.5 mg of carotenoid, within 8 hr of homogenizing the leaves. To the solutions obtained were added three volumes of absolute ethanol and one volume of 10 N NaOH. The mixture was left in the dark at room temperature for 14–16 hr and then diluted with three to four volumes of water. Extraction with peroxide-free ether was carried out until no further pigment was obtained. The combined ether extracts were washed with water until the washings were neutral to litmus and then dried over anhydrous sodium sulfate (Davies, 1965). The whole procedure was carried out in subdued lighting.

In order to estimate the total carotenoid, the ether solution was evaporated to dryness and the residue was taken up in a known convenient volume of absolute ethanol. The optical density of this solution at its λ_{max} was measured on a Perkin-Elmer spectrophotometer, Model 137, adjusted so that the optical density at 600 mμ was zero. Total carotenoid was calculated using a value for $E^{1\%}_{1\,cm}$ based on the relative amounts of β-carotene and xanthophyll in the solution, this being determined subsequently.

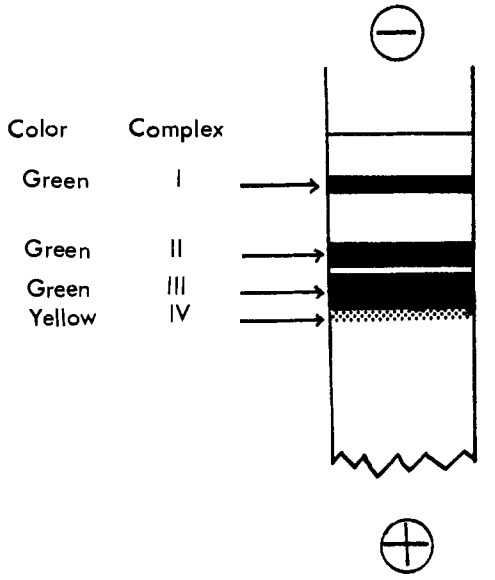

FIGURE 1: A diagrammatic representation of the major complexes separated by electrophoresis on polyacrylamide gel of an SDBS extract of chloroplasts (experimental details are given in Thornber *et al.*, 1967).

A sample of the ethanolic solution was examined by thin layer chromatography (tlc) on Kieselgel H. Components of the main yellow bands so obtained were identified by the following criteria: (a) R_F values in the two systems, ethyl acetate–carbon tetrachloride (60:40, v/v) and benzene–methanol (87:13, v/v); (b) comparison in the same solvents with β-carotene (Roche Products Ltd., London, W.1) and with the main xanthophylls of grass, isolated by the method of Davies (1965) and confirmed as lutein, violaxanthin, and neoxanthin; (c) color change on the tlc plate when exposed to HCl vapor and after elution from the tlc plate; (d) comparison of the spectra given by each band in petroleum ether (bp 30–60°), chloroform, ethanol, and carbon disulfide with the data of Davies (1965); and (e) spectral shifts on addition of 2 drops of 0.1 N HCl to the ethanolic solution. The presence or absence of hypsochromic shifts was taken as a guide to the presence or absence of neoxanthin or violaxanthin (Davies, 1965).

For the estimation of individual carotenoids a suitable amount of the ethanolic solution was run on 20 × 2.8 cm tlc plates with Kieselgel H as adsorbent and ethyl acetate–carbon tetrachloride (60:40, v/v) as developing solvent. Immediately after development the plates were scanned in a Chromoscan Model J297 (Joyce Loebl & Co. Ltd., Gateshead, England) using a violet filter (Ilford 622) with approximately level transmittance over the range of 440–456 mμ; the quantity of light reflected was recorded. The relative amounts of the main carotenoid bands were calculated by normal integration methods. Estimations were done in duplicate and the average of coincident values was taken. No correction was made for the slightly different extinction coefficients of the carotenoids involved.

Chlorophyll Content. The concentrations of chlorophylls a and b were estimated by the method of Mackinney (1941).

Nitrogen Content. Nitrogen was estimated by micro-Kjeldahl digestion followed by distillation and titration as described by Chibnall *et al.* (1943).

Carbohydrate Content. Each complex (I and II) was freeze dried and its pigments and lipids were extracted with 80% aqueous acetone. The residual material was hydrolyzed (2 N H_2SO_4 for 3 hr at 110°) and the solution was neutralized with Bio-Deminrolit (carbonate form). The concentrated hydrolysates were chromatographed on Whatman No. 1 paper in ethyl acetate–pyridine–water (8:2:1, v/v), and the monosaccharides were identified by comparison with markers after detection by the method of Trevelyan *et al.* (1950).

Identification of Other Lipids. Lipids of freeze-dried samples of detergent extracts and complexes I and II were extracted with diethyl ether–petroleum ether (bp 100–120°) (1:1, v/v) and examined on thin layer chromatograms by conventional techniques for the presence of quinones, mono- and digalactosyl diglycerides, phospholipid, and sulfolipid.

Determination of Manganese. Freeze-dried samples of detergent extracts and complexes I and II were digested with H_2SO_4 and the charring was cleared by addition of H_2O_2 and further digestion. The method of determination was based on those of Cornfield and Pollard (1950) and Yuen (1958) and communicated to us by Dr. P. C. de Kock, Macaulay Institute, Aberdeen, Scotland. The procedure differed from the published versions in that a 1:1 mixture of tetrabase (*p,p'*-tetramethyldiaminodiphenylmethane) solution and leucomalachite green solution was used to develop the color, which was read at 640 mμ, and the quantity of manganese present was calculated from a standard curve.

Results

Analysis of the Pigment–Protein Complexes

Ultracentrifugation. The SDBS extract, used as the starting solution from which the complexes were to be isolated, was diluted to bring the detergent concentration to 0.1% (w/v) and examined in the ultracentifuge. Two boundaries, one of 10 S and one of 3 S, were observed.

Examination of complexes I and II in the ultracentrifuge showed that the pigment (and presumably bound detergent) sedimented with a single protein boundary in each case. The $s_{20,w}$ values of each complex in various strengths of detergent are presented in Table I and it will be noted that the sedimentation coefficient of complex I is much greater than that of complex II. If as much of the detergent as possible is removed from the solution by equilibrating the complexes in water, then the lipoproteins aggregate. Complexes I and II isolated from the leaves of a monocotyledon have sedimentation coefficients similar to those of spinach beet (Table I).

The Protein Moieties. Several samples of the proteins of complexes I and II were obtained from different preparations of chloroplasts and were hydrolyzed for varying lengths of time as indicated in Table II. The amino acid compositions of the two proteins (Table II) are very different; the greatest variation between the best analyses occurs in the values for proline, lysine, histidine, glycine, glutamic acid, and aspartic acid. The tyrosine:tryptophan molar ratio was determined as 2.67 for complex I and 2.55 for complex II. After adjusting all the molar ratios of the amino acids so that the value for half-cystine was one, a minimum molecular weight of each protein was calculated from the best analyses. The figures obtained were 33,300 for complex I and 21,900 for complex II.

TABLE I: Sedimentation Coefficients of Complexes I and II in Various Strengths of SDBS.

Complex	SDBS Concn[a] (%) (w/v)	$s_{20,w}$
I (spinach beet)	0.2	8.3
I (spinach beet)	0.02	8.8
I (spinach beet)	Water	21.4
I (oat)	0.02	8.9
II (spinach beet)	0.2	2.5
II (spinach beet)	0.02	3.1
II (spinach beet)	Water	5.7
II (oat)	0.02	2.6

[a] Solutions of SDBS were made up in 0.05 M sodium borate (pH 8.0).

The amino acid compositions of the proteins of the two equivalent complexes isolated from chloroplasts of other plant species (spinach, tobacco, and a monocotyledon, oat) are very similar to those of spinach beet chloroplasts (Table III). The amino acid composition of the material which remains after five successive extractions of the chloroplasts with SDBS solution was also determined but the results were not very consistent. They did, however, indicate that the amino acid composition resembled that of complex II more closely than that of complex I (*i.e.*, the values for proline and lysine were high while for histidine they were low).

Chlorophyll a:b and Chlorophyll:Protein Ratios of the Pigment–Protein Complexes. The chlorophyll a:b ratio in the two complexes has been reported previously (Thornber *et al.*, 1967); complex I had a mean value of 12:1 and complex II of 1.2:1. These ratios have now been confirmed by direct estimation of the two chlorophylls after separation by tlc following the procedure of Bacon (1965); traces of pheophytins a and b were observed in each complex.

The chlorophyll:protein ratio was determined by estimation of the weight of chlorophyll and of nitrogen in samples of each complex, from which soluble acrylamide contaminants had been removed by gel filtration. The weight of nitrogen was corrected for that contributed by the chlorophyll; no other corrections were made, and all the remaining nitrogen was assumed to be derived from

TABLE II: Amino Acid Analysis of Complexes I and II.[a]

Amino Acid	Length of Time of Hydrolysis (hr)[b]						Best Anal.[c]	Residues[d]
					Oxidized[e]			
	12	24	48	72	18	24		
				A. Complex I				
Half-cystine[f]	—	—	—	—	0.03	0.03	0.03	1
Aspartic acid	0.79	0.78	0.76	0.71	0.84	0.74	0.77	25.7
Methionine	—	—	—	—	0.15	0.12	0.12	4
Threonine	0.40	0.48	0.30	0.12	0.46	0.37	0.48	16
Serine	0.33	0.51	0.19	0.04	0.44	0.38	0.51	17
Glutamic acid	0.82	0.73	0.67	0.63	0.74	0.72	0.72	24
Proline	0.47	0.44	0.40	0.40	0.44	0.52	0.44	14.7
Glycine	0.98	1.02	0.94	0.91	1.10	1.03	1.02	34
Alanine	0.87	0.92	0.88	0.88	0.95	0.88	0.90	30
Valine	0.64	0.56	0.49	0.48	0.58	0.53	0.57	19
Isoleucine	0.58	0.59	0.62	0.57	0.62	0.59	0.60	20
Leucine	1.06	1.06	1.06	1.06	1.06	1.06	1.06	35.3
Tyrosine	0.27	0.22	0.25	0.23	0.13	0.01	0.24	8
Phenylalanine	0.65	0.62	0.62	0.62	0.63	0.59	0.62	20.7
Lysine	0.30	0.25	0.21	0.27	0.28	0.30	0.27	9
Histidine	0.24	0.33	0.45	0.44	0.38	0.26	0.45	15
Arginine	0.35	0.24	0.34	0.31	0.26	0.27	0.33	11
Tryptophan	—	—	—	—	—	—	0.09	3
				B. Complex II				
Half-cystine[f]	—	—	—	—	0.06	0.05	0.053	1
Aspartic acid	0.94	0.99	0.98	0.99	1.04	1.04	1.01	19.1
Methionine	—	—	—	—	0.17	0.17	0.17	3.2
Threonine	0.45	0.33	0.24	0.11	0.37	0.34	0.57	10.8
Serine	0.33	0.47	0.12	0.05	0.37	0.43	0.47	8.9
Glutamic acid	1.06	1.00	1.00	—	1.02	1.00	1.00	18.9
Proline	0.59	0.86	0.76	0.79	0.73	0.82	0.80	15.1
Glycine	1.15	1.40	1.31	—	1.31	1.53	1.41	26.4
Alanine	0.97	1.10	1.12	—	1.16	1.14	1.15	21.8
Valine	0.79	0.79	0.69	0.73	0.64	0.68	0.72	13.5
Isoleucine	0.59	0.47	0.49	0.45	0.52	0.51	0.49	9.3
Leucine	1.06	1.06	1.06	1.06	1.06	1.06	1.06	20.0
Tyrosine	0.30	0.02	0.28	0.25	0.08	—	0.28	5.3
Phenylalanine	0.60	0.64	0.66	0.62	0.62	0.59	0.63	11.9
Lysine	0.49	0.56	0.52	0.58	0.56	0.61	0.57	10.8
Histidine	0.08	0.12	0.13	0.13	0.06	0.05	0.13	2.5
Arginine	0.44	0.34	0.33	0.38	0.31	0.27	0.32	6.0
Tryptophan	—	—	—	—	—	—	0.11	2.1

[a] Expressed as molar ratios after each value (not corrected for losses) had been adjusted so that the value for leucine was constant. [b] Two samples were used for each set of figures except those of 12 and 18 hr (oxidized) when a single sample was used. [c] Based on all analyses except the 12-hr values. [d] Calculated on the basis of one half-cystine residue. [e] Oxidized proteins were prepared by the method of Hirs (1956). [f] A small peak in the position of cysteic acid was also found with unoxidized samples.

protein. The weight of nitrogen was converted to weight of protein by multiplying by 6.23 (complex I) and 6.48 (complex II), these figures having been calculated from the amino acid analyses. The mean value of the chlorophyll: protein weight ratio for complex I was 0.114 (range 0.100–0.133) and for complex II it was 0.075 (range 0.050–0.082). From these values and a knowledge of the percentages of complex I and complex II in whole lamellae (Thornber *et al.*, 1967) it is possible to calculate an approximate ratio of the weight of chlorophyll in the two complexes; a value of 0.87 (complex I: complex II) is obtained. Thus if each complex has lost the same per-

TABLE III: Amino Acid Composition[a] of Complexes I and II in Different Plant Species.

	Complex I				Complex II			
	B. vulgaris[b]	*S. oleracea*[c]	Tobacco[c]	Oat[c]	*B. vulgaris*[b]	*S. oleracea*[c]	Tobacco[c]	Oat[c]
Aspartic acid	0.77	0.77	—	0.76	1.01	0.93	0.94	0.96
Threonine	0.48	0.43	0.42	0.45	0.33	0.25	0.30	0.37
Serine	0.51	0.38	0.44	0.35	0.47	0.33	0.42	0.32
Glutamic acid	0.72	0.66	0.66	0.66	1.00	0.85	0.91	0.94
Proline	0.44	0.48	0.49	0.36	0.79	0.76	0.78	0.69
Glycine	1.02	0.99	1.01	0.99	1.39	1.32	1.40	1.29
Alanine	0.90	0.90	0.90	0.92	1.13	1.00	1.19	1.13
Valine	0.57	0.39	0.56	0.48	0.72	0.68	0.66	0.70
Isoleucine	0.60	0.57	0.62	0.66	0.49	0.40	0.47	0.46
Leucine	1.06	1.06	1.06	1.06	1.06	1.06	1.06	1.06
Tyrosine	0.24	0.18	0.18	0.22	0.28	0.21	0.23	0.27
Phenylalanine	0.62	0.60	0.68	0.62	0.62	0.69	0.67	0.62
Lysine	0.27	0.30	0.25	0.27	0.57	0.60	0.53	0.56
Histidine	0.33	0.36	0.34	0.36	0.12	0.13	0.09	0.10
Arginine	0.33	0.29	0.24	0.33	0.33	0.35	0.32	0.34

[a] Expressed as molar ratio after each value had been adjusted so that the value of leucine was constant. [b] Best analysis except for histidine, serine, and threonine, which are the values after 24 hr. [c] Values for single analysis after 24-hr hydrolysis.

TABLE IV: Distribution of Carotenoids[a] in Fractions Obtained from SDBS Extracts of Lamellae of Spinach Beet and Oat.

		Spinach Beet				Oat			
	Proportion of Total Carotenoid Obtained in Each Isolated Complex (%)	β-Carotene (+ β-carotene epoxide)	Lutein	Violaxanthin	Neoxanthin	β-Carotene (+ β-carotene epoxide)	Lutein	Violaxanthin	Neoxanthin
Detergent extract	—	44.0	37.0	13.1	5.9	36.0	38.7	15.7	10.0
Complex I	13	92.5	7.0	0	<1	94.0	6.0	0	0
Complex II	30	30.8	51.7	3.2	13.1	7.0	70.5	2.0	21.0
Free-pigment zone (complex III)	52	32.2	49.4	12.0	6.4	21.5	52.7	13.7	12.0
Free-pigment zone (complex IV)	5	3.9	60.9	25.4	10.5	<1	62.0	29.5	8.0
Residue	—	11.1	55.0	21.2	12.5	10.0	54.0	23.0	13.0

[a] Expressed as percentages; each value is the mean of those obtained in three experiments.

centage of the total chlorophyll present in the complex *in vivo*, which seems possible since the chlorophyll a:b ratio (1.8–1.9:1, second detergent extract) and carotenoid composition of the free-pigment zones do not reflect particularly either complex I or II, then it may be inferred that the ratio of the weights of chlorophyll in the two photochemical systems will also be near unity.

Carotenoid Content. The carotenoid compositions of the SDBS extract from which the pigment–proteins were fractionated, complexes I and II, the free-pigment zones (complexes III and IV), and the material remaining after five successive detergent extractions (residue) are given in Table IV.

Four major carotenoid components were found in the

TABLE V: Composition of Protein–Pigment Particles[a] Isolated from Spinach Chloroplasts after Treatment with Various Detergents.

	SDBS		Digitonin[b]		Triton X-100[b]	
	I	II	I	II	I	II
Chlorophyll a	120	45	71	40	18	84
Chlorophyll b	10	38	12.5	19	3.2	42
Chlorophyll a:b	12	1.2	5.7	2	5.7	2
β-Carotene	8.8 (90)	3.6 (29)	11.7 (52)	4.0 (20)	3.4 (62)	7.5 (22)
Lutein	0.9 (9)	6.7 (54)	5.0 (22)	9.0 (46)	1.5 (27)	20.0 (59)
Neoxanthin	0.1 (1)	1.7 (14)	1.7 (7.5)	3.0 (15)	0.2 (4)	2.5 (7)
Violaxanthin	0	0.3 (2.5)	4.2 (19)	3.5 (18)	0.4 (7)	3.8 (11)
Total carotenoid	9.8	12.3	22.6	19.5	5.5	33.8
Protein	1	1	1	1	1	1
Total chlorophyll	125	83	83.5	59	21.2	126

[a] Values for pigments are expressed in millimicromoles per milligram of protein. [b] Calculated from the results of Ke *et al.* (1966). [c] Figures in parentheses are percentages of the total carotenoid in the fraction.

detergent extract, namely β-carotene, lutein, violaxanthin, and neoxanthin. Small quantities of β-carotene epoxide were noted, and traces of a yellow band running on the thin layer chromatogram between violaxanthin and lutein were sometimes observed.

In complex I the chief carotenoid was β-carotene; lutein was present in very minor quantities and violaxanthin and neoxanthin only occasionally and then in trace amounts. Complex II, on the other hand, had lutein as its chief component with the other three carotenoids in lesser amounts. The residue is characterized by its low β-carotene content in comparison with the detergent extract. The free-pigment complexes (III and IV) reflect the over-all carotenoid composition of the pigment–protein complexes. No qualitative differences were noted between *B. vulgaris* (a dicotyledon) and *A. sativa* (a monocotyledon) (Table IV). However, there are some quantitative differences; the detergent extract of oat lamellae contains a lower proportion of β-carotene and more xanthophylls than the beet preparation; complex I, the free-pigment zones, and the residues are not significantly different in the two species, but complex II of oat lamellae reflects the same differences as the detergent extract.

If a disk of gel containing either complex I or II is re-electrophoresed through another polyacrylamide gel column within a few hours of having been removed from the first gel, some of the pigments of the complex appear in the free-pigment zone. In this case, the composition of the free-pigment zone is the same as that of the parent complex.

Carbohydrate Content. Both proteins of the complexes contained the following monosaccharides in decreasing amount: glucose, galactose, mannose, arabinose, and xylose; complex I contained a greater proportion of arabinose than did complex II. If the complexes were not extracted with lipid solvents then galactose was the predominant monosaccharide.

Lipid Content. Only traces (less than 2 μg) of PQ-A and α-tocopherolquinone were observed in either complex I or II, which were obtained by electrophoresis of 60 1-ml samples of detergent extract (containing 6 μg of PQ-A/ml). Successive detergent extracts were also examined. The first supernatant (70 μg of PQ-A/mg of chlorophyll) contained PQ-A, PQ-C, and α-tocopherolquinone in measurable quantities and traces of PQ-B and PQ-D, the second extract (20 μg of PQ-A/mg of chlorophyll) contained measurable quantities of PQ-A and α-tocopherolquinone only, and the third supernatant (3 μg of PQ-A/mg of chlorophyll) contained traces of PQ-A and α-tocopherolquinone. The amounts of mono- and digalactosyl diglyceride and of phospholipid in complexes I and II were too small to permit specific identification. No trace of sulfolipid could be detected in either complex.

Manganese Content. Over 75% of the manganese in lamellae was obtained in the first of the five successive detergent extracts. Complexes I and II were isolated from 16 polyacrylamide gel columns, on to which detergent extract containing a total of 1 μg of manganese had been loaded; there was no significant amount of manganese in either complex.

Discussion

Estimation of Carotenoids. Comparison of the content of total and individual carotenoids of our complexes (Tables IV and V) with the values obtained for equivalent entities by other investigators (Ke *et al.*, 1966; Ogawa *et al.*, 1966), shows that the carotenoid:chlorophyll ratio is lower in our complexes, as are the values for violaxanthin. It is necessary to show whether this discrepancy arises from the methods employed for estimation of the pigments or whether it represents a meaningful difference between our complexes and those obtained by different techniques.

We have been unable to discover any substantial difference in the recovery of either the total amount of carotenoid or of each individual carotenoid between our isolation procedure for the complexes and Procedure A (electrophoresis of complexes from gel segments into alumina paste) of Ogawa *et al.* (1966), or in the estimation of the carotenoids between our methods and those of Ke *et al.* (1966). There are, however, certain other possible sources of error in our procedure which are discussed below.

We have found that the recovery of chlorophyll and the carotenoids after electrophoretic separation is very variable, both as regards individual pigments and preparations; in most experiments it was 40–50% of the total carotenoid loaded onto the gels, and was always less than the recovery of chlorophyll; Ogawa *et al.* (1966) did not report their recovery figure. In spite of the difference in total recovery from preparation to preparation, the values obtained for the pigment composition are reasonably consistent, so for the purposes of the present discussion this variation may be ignored. The efficiency of recovery of the pigments is in order: chlorophyll > lutein > neoxanthin > β-carotene > violaxanthin and this variation is more serious. There are two main causes of loss of pigments: decomposition, owing largely to the action of light and oxygen, and incomplete recovery from the gel. The latter effect is likely to apply fairly equally, and the order of recovery quoted above probably reflects the stabilities of the compounds concerned. As a first consideration it might be thought that it is for this reason alone that the violaxanthin values are low compared to those quoted by Ke *et al.* (1966). However, the situation is complicated by two observations. (1) The residue still contains a high proportion of violaxanthin, in spite of being subjected to treatment more drastic than that given to complexes I and II; and (2) the free-pigment zones (III and IV) which are exposed to the same conditions as complexes I and II also have a high violaxanthin content.

The manner in which we treated our samples immediately prior to estimation of their chlorophyll and carotenoid content differed from that of other investigators (Ke *et al.*, 1966; Ogawa *et al.*, 1966). The chlorophyll content of the complex was estimated directly and within 8 hr of cutting the leaves; the complexes were then treated with alkali which saponifies any esters present and yields the carotenoids free from the other pigments (Davies, 1965). No measurable decomposition of the carotenoids occurred during storage in alkali in the dark.

We have favored estimation of carotenoids from densitometer tracings of chromatograms on account of the greater convenience and speed involved, but it should be noted that the results are not exactly the same as those obtained when the elution method is used. The main objection to our method is that light of a range of wavelengths is used and the carotenoids have different λ_{max} values; comparison of the two methods suggests that this error is in the range, ±10%. On the other hand, the elution method is slower and in some cases involves an evaporation to dryness, allowing decomposition, which certainly does not take place at the same rate for each carotenoid. On balance, we feel that the Chromoscan method is superior. For the reasons given above, we conclude that our methods are preferable and that there is insufficient discrepancy between the methods to account for the variations in carotenoid composition observed between our results and those of other workers; hence the differences are real.

The Composition of Chloroplast Lamellae. The nature of the lamellar macromolecular structure is such that the membrane cannot be solubilized by a known technique mild enough to ensure no alteration of individual components. Those techniques which have so far been used have ruptured the membrane to differing extents; with some the resulting particles are large and can only be partially resolved by differential centrifugation, whilst with others much smaller particles are obtained and although these particles may then exist as single entities, they have almost certainly lost many of the compounds with which they were associated *in vivo*. We prefer the second of these two states, since the products are more amenable to fractionation by classical protein techniques and the purity of fractions can be examined directly.

Thornber *et al.* (1967) showed that two chlorophyll–protein complexes could be purified from chloroplasts dissociated by the anionic detergents, SDBS and SDS; it was also shown that these two components contained over 75% of the protein of whole lamellae. After isolation, complex I is larger than complex II. This is demonstrated by their sedimentation coefficients (Table I) and their movement in polyacrylamide gel during electrophoresis (Figure 1); the difference in electrophoretic mobility is almost certainly due to a difference in size rather than in net charge, since no electrophoretic separation of the two complexes occurs on a solid support that does not have the fine molecular-sieving properties of polyacrylamide gel.

Previous reports of the amino acid composition of chloroplast lamellae have been limited to studies by Bailey *et al.* (1966), Criddle (1966), and Weber (1962). From an acetone powder of lamellae Criddle (1966) has isolated a structural protein which represents over 40% of the total protein of the chloroplast. Since this protein constitutes such a great proportion of the lamellae protein, it is likely to be related to one or possibly both of our proteins; however, there is little correspondence between the amino acid composition of the structural protein and that of either of our proteins. However this does not completely eliminate a relation, since Criddle (1966) reported only one analysis of his protein and that was after 12-hr hydrolysis; it might, therefore, be possible to relate the structural protein with our components after further investigation of its amino acid composition; complex I has a minimum molecular weight similar to that of the structural protein. The combined analyses of the proteins of our two complexes show a mean molar ratio for each amino acid residue corresponding very closely to the values reported for whole lamellae protein (Bailey *et al.*, 1966; Weber, 1962). This very probably confirms our earlier observation that the proteins of complexes I and II make up the bulk of the lamellae pro-

tein. It is clear (Table II) that the two proteins are of a different composition and minimum molecular weight. Hatch (1965) has shown that the physical characteristics of a protein can be correlated with its amino acid composition; the properties of the proteins of complexes I and II are in accord with this hypothesis. If the polar:apolar ratio of the amino acid residues in each protein is calculated as described by Hatch (1965), the resulting values (complex I, 0.91; complex II, 1.02) indicate an unusually high proportion of apolar amino acids. The behavior of the two proteins is typical of proteins containing a high proportion of apolar amino acids, for example, they aggregate when their lipid is removed, and they interact with other apolar material giving rise to lipoprotein membranes.

Van Wyk (1966) and Weber (1963) have shown that chloroplast lamellae contain a structural glycoprotein which represents 25–35% of the lamellae protein. We detect monosaccharides in hydrolysates of both of our delipidized proteins, but in addition to the sugars observed by Van Wyk (1966), our proteins contain arabinose; this is mainly in complex I.

The function of the carotenoid pigments in photosynthesis continues to be a matter for speculation. They have been considered suitable for protection of the chlorophylls against photooxidation (Stanier and Cohen-Bazire, 1957) and as photoreceptors in an accessory role (Whittingham, 1965). The xanthophylls specifically have been implicated in the Hill reaction (Sapozhnikov *et al.*, 1964) but no satisfactory explanation of this involvement has been provided, and in fact Yamamoto *et al.* (1962) were unable to demonstrate any direct connection. The present work can make only a limited contribution to any hypothesis of carotenoid function, since the particles examined were inactive, but two results are of interest. First, complex I contains very little xanthophyll (Table IV), whereas complex II, derived from the system responsible for the Hill reaction, contains considerably more xanthophyll than β-carotene. Second, a consideration of the results of Ke *et al.* (1966) suggests that high values for the chlorophyll a:b ratio for photochemical system I are accompanied by high values for the β-carotene:xanthophyll ratio, and our results lend support to this generalization.

Electrophoresis on polyacrylamide gel of any detergent extract yields, in addition to the pigment–protein zones, two free-pigment zones (Figure 1). It can be seen from Table IV that these zones contain a large amount of the carotenoid of the detergent extract; furthermore, their carotenoid composition is as definite as that of any other fraction, and the carotenoids do not appear to have been released from one particular pigment–protein. These free-pigment zones may be present because the pigments they contain are not bound to any protein *in vivo* or because their binding to protein is weak or their position relatively "external." Continued exposure of the isolated complexes I and II to detergent, which extracts more pigment, may eventually produce chlorophyll–proteins from which no further pigments can be extracted by detergent.

Lastly there is the problem of the nature of the residual material after detergent extractions of chloroplasts; the least readily extracted of the complexes is complex II (Thornber *et al.*, 1967) and, therefore, it would be expected that unless another detergent-insoluble pigment–protein is present, the amino acid and pigment composition of the residue (containing some 1.3% of the lamellar protein and 4% of the chlorophyll) would reflect that of complex II; the results do not substantiate this and thus the residual material must contain other minor components, the nature and function of which requires further investigation.

The major conclusions about the two pigment–proteins studied in this work can be summarized as follows. (a) The major portion (over 75%) of the lamellae protein is present in the two complexes. (b) The complexes are different with respect to their protein composition and pigment content. (c) Complexes I and II are very probably derived from photochemical systems I and II, respectively. (d) Complex I is more readily extracted from the lamellar matrix. (e) Complex I is larger than complex II. (f) Some protein-poor residual material remains after detergent extractions. Hence these observations support a concept of two distinct photochemical systems containing different protein and pigment assemblies.

How the two complexes might be accommodated into the models of the lamellae proposed by many investigators is very largely a matter of conjecture. It is tempting to postulate that each complex is a dissociated form of one of the two types of particle observed on different sides of the lamellar membrane (Branton and Park, 1965; Park, 1965, 1966; Muhlethaler *et al.*, 1965); the residue may be some extracted derivative of the supporting matrix. An investigation by electron microscopy of lamellae treated with detergent should indicate whether this postulation is plausible.

Acknowledgments

We thank Miss Carol A. Smith for her skillful technical assistance. We also wish to thank Dr. J. F. Pennock, Biochemistry Department, University of Liverpool, England, for samples of ubiquinone 10, plastoquinone 9, α-tocopherolquinone, and vitamin k_1; Roche Products Ltd., London, England, for samples of plastoquinone 9 and ubiquinone 10; and Professor A. A. Benson, Scripps Institute of Oceanography, La Jolla, Calif., for a sample of sulfolipid. We are greatly indebted to Professor T. W. Goodwin and his colleagues at the University College of Wales, Aberstwyth, Wales, for valuable discussions about estimation of carotenoids.

References

Anderson, J. M., and Boardman, N. K. (1966), *Biochim. Biophys. Acta 112*, 403.

Bacon, M. F. (1965), *J. Chromatog. 17*, 322.

Bailey, J. L., Thornber, J. P., and Whyborn, A. G. (1966), *in* Biochemistry of Chloroplasts, Vol. 1, Goodwin, T. W., Ed., London, Academic, p 243.

Beaven, G. H., and Holiday, E. R. (1952), *Advan.*

Protein Chem. 7, 319.
Biggins, J., and Park, R. B. (1965), *Plant Physiol. 40*, 1109.
Branton, D., and Park, R. B. (1965), *Plant Physiol.*, xxviii.
Bril, C. (1965), *Carnegie Inst. Washington Yearbook 64*, 370.
Chiba, Y. (1960), *Arch. Biochem. Biophys. 90*, 294.
Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943), *Biochem. J. 37*, 354.
Clayton, R. K. (1965), Molecular Physics in Photosynthesis, New York, N. Y., Blaisdell.
Cornfield, A. H., and Pollard, A. G. (1950), *J. Sci. Food Agr. 1*, 107.
Criddle, R. S. (1966), *in* Biochemistry of Chloroplasts, Vol. 1, Goodwin, T. W., Ed., London, Academic, p 203.
Davies, B. H. (1965), *in* Chemistry and Biochemistry of Plant Pigments, Goodwin, T. W., Ed., London, Academic, p 489.
Gross, J., Shefner, A., and Becker, M. (1966), *Nature 209*, 615.
Hatch, F. (1965), *Nature 206*, 777.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Kahn, J. S. (1964), *Biochim. Biophys. Acta 79*, 234.
Katoh, S., and San Pietro, A. (1966), *J. Biol. Chem. 241*, 3575.
Ke, B., Seliskar, C., and Breeze, R. (1966), *Plant Physiol. 41*, 1081.
Mackinney, G. (1941), *J. Biol. Chem. 140*, 315.
Muhlethaler, K., Moor, H., and Szarkowski, J. W. (1965), *Planta 67*, 305.
Ogawa, T., Obata, F., and Shibata, K. (1966), *Biochim. Biophys. Acta 112*, 223.
Park, R. B. (1965), *J. Cell Biol. 27*, 151.
Park, R. B. (1966), *in* The Chlorophylls, Vernon, L. P., and Seely, G. R., Ed., London, Academic, p 283.
Sapozhnikov, D. I., Alkhazov, D. G., Eidelman, Z. M., Bazhanova, N. V., Lemberg, I. K., Maslova, T. G., Girshin, A. B., Popova, I. A., Saakov, V. S., Popova, O. F., and Shiryaeva, G. A. (1964), *Dokl. Akad. Nauk. SSSR Botan. Sci. Sect. 154*, 20.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Sironval, C., Clijsters, H., Michel, J.-M., Bronchart, R., and Michel-Wolwertz, R. M. (1966), *Proc. 2nd Western Europe Conf. Photosyn. Zeist*, Netherlands, Rotterdam, Donker, Ad., p 111.
Stanier, R. Y., and Cohen-Bazire, G. (1957), *7th Symp. Soc. Gen. Microbiol.*, London, Cambridge University, p 56.
Thornber, J. P., Gregory, R. P. F., Smith, C. A., and Bailey, J. L. (1967), *Biochemistry* (in press).
Thornber, J. P., Smith, C. A., and Bailey, J. L. (1966), *Biochem. J. 100*, 14P.
Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.
Van Wyk, D. (1966), *Z. Naturforsch. 21b*, 700.
Vernon, L. P., Shaw, E., and Ke, B. (1966), *J. Biol. Chem. 241*, 4101.
Weber, P. (1962), *Z. Naturforsch. 17b*, 683.
Weber, P. (1963), *Z. Naturforsch. 18b*, 1105.
Wessels, J. S. C. (1966a), *Proc. 2nd Western Europe Conf. Photosyn.* Zeist, Netherlands, Rotterdam, Donker, Ad., p 129.
Wessels, J. S. C. (1966b), *Biochim. Biophys. Acta 126*, 581.
Whittingham, C. P. (1965), *in* Chemistry and Biochemistry of Plant Pigments, Goodwin, T. W., Ed., London, Academic, p 360.
Yamamoto, H. Y., Chichester, C. O., and Nakayama, T. O. M. (1962), *Photochem. Photobiol. 1*, 53.
Yuen, S. H. (1958), *Analyst 83*, 350.