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STUDIES ON CHLOROPLAST MEMBRANE STRUCTURE

I. ASSOCIATION OF PIGMENTS WITH CHLOROPLAST LAMELLAR PROTEIN

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SUMMARY

1. Lamellar protein was prepared by extraction of sonicated lamellae with acetone. Although homogeneous in the ultracentrifuge, the preparation contained several electrophoretically distinct components. Large quantities of this protein are readily obtained and are suitable for reassociation studies. The physical properties of this protein mixture resemble those of more purified preparations.

2. Extraction of all pigments from chloroplast lamellae except β-carotene yields a red, carotene–protein complex with unique absorption maxima at 538 nm, 498 nm, 460 nm, and 418 nm. A complex with these absorption properties is not present in intact lamellae. Extraction of β-carotene removes all absorption except that at 418 nm which is caused by cytochromes. Using a double-dialysis system, β-carotene can be reassociated with lamellar protein to produce a complex with absorption maxima similar to those of the isolated carotene–protein complex.

3. Spectral studies and selective extractions of chloroplast lamellae with organic solvents were used to examine the interactions of lamellar components. Apparently β-carotene in lamellae absorbs light maximally at 468 nm and 493 nm. Results suggest that this spectrum depends on the polarizing effects of the lamellar protein and the solvating effects of the phytol portion of chlorophyll.

4. The data appear more consistent with a lipoprotein monolayer model for membrane structure, than with the simple lipid bilayer model.

INTRODUCTION

Several models for the chloroplast lamellar membrane have been proposed; these are based upon electronmicroscopic studies, X-ray-scattering analyses, and chemical composition[1–3]. Lamellar protein fractions have been isolated and characterized[4–8], but only recently have several laboratories successfully isolated pigment–protein complexes which function in some typical photosynthetic reactions[9–11]. The ability to isolate these active complexes, which have rather defined pigment compositions, either by electrophoresis[11,12] or differential centrifugation[10] implicates specific associations between pigments and lamellar protein. An intimate and perhaps

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specific lipid–protein interaction is also supported by optical rotatory dispersion studies[13] and circular dichroism data.[14]

Carotenoid–protein complexes are found in bacteria[15], and in marine organisms as crustacyanin[16] and ovorubin[17]. JENCKS AND BUTEN[18] clearly demonstrated the influence of protein conformation on the light absorption properties of crustacyanin. Phospholipids are required for optimal activity of cytochrome oxidase systems from mitochondria[19]. KRINSKY, CORNWELL AND ONCLEY[20] have shown that vitamin A is bound to a specific fraction of high-density lipoprotein in the blood. These studies certainly support specific interactions between pigments, lipids and proteins.

Using conditions which cause the formation of β-carotene–protein complexes[21,22], we attempt to evaluate the environment which surrounds β-carotene in chloroplast lamellae. Our results from spectral measurements, extractions and reconstitution studies support the lipoprotein monolayer model for chloroplast lamellar membrane structure.

METHODS

Preparation of lamellar protein

Chloroplasts were prepared from spinach obtained from a local market. The deribbed leaf tissue was macerated in a Waring Blendor in a volume of buffer, 0.8 M sucrose, 0.01 M NaCl, and 0.02 M tricine at pH 7.9, equal to the weight of tissue. After 15 sec at maximum grinding speed, the homogenate was filtered twice through

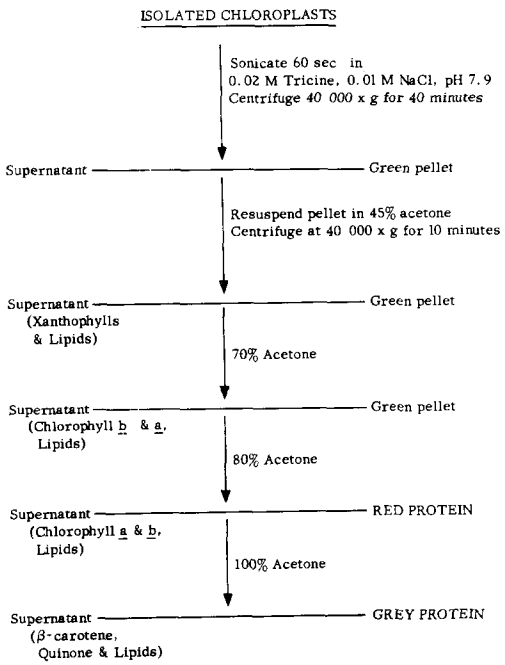

Fig. 1. Sequential extraction procedure for the preparation of β-carotene–protein complex (red protein) and lamellar protein (grey protein). All operations were performed in an ice bath and residues were resuspended by sonication or by a glass–Teflon homogenizer. Approx. 10 vol. of aqueous acetone were used in these extractions.

4 layers of cheese cloth. The chloroplasts which sedimented between 250 × *g* and 650 × *g* for 10 min were resuspended in 0.01 M NaCl, 0.02 M tricine at pH 7.9, and sonicated at 20 kcycles/sec for 60 sec from 0°–5°. After removal of whole chloroplasts, the lamellae were centrifuged for 40 min at 35 000 × *g*. The chloroplast fragments were then treated as outlined in Fig. 1 in order to obtain the red β-carotene–protein complex.

Protein characterization

Amino acid composition of the lamellar protein was obtained from the hydrolysate of 0.4 mg protein in 0.5 ml of 6 M HCl contained in a sealed ampul at 107° for 24 h. The amino acids were analyzed using the Beckman Automatic Amino Acid Analyzer according to the method of SPACKMAN, STEIN AND MOORE[23].

Lamellar protein was solubilized in either 0.2 % sodium dodecyl sulfate or 0.5 % sodium dodecylbenzyl sulfate (4 mg protein/ml detergent solution). This solubilized protein was analyzed in the ultracentrifuge as described by BIGGINS AND PARK[5]. Disc-gel electrophoresis of the solubilized protein was performed according to the method of THORNBER *et al.*[11]. Acrylamide (5%) gel separations were performed at 3 mA per gel with 0.05 M sodium borate buffer at pH 8.5 containing 0.5 % sodium dodecylbenzyl sulfate.

Spectrophotometry

For spectral measurements, lamellar particles approx. 0.5 mg dry wt./ml buffer were suspended by sonication for 60 sec or less at 5°–10° in 0.01 M NaCl, 0.02 M tricine at pH 7.9. Particles that were sequentially extracted with different solvents were always lyophilized prior to the addition of solvent. Many of these protein suspensions had scattering properties which interfered with spectral measurements. Hence, we measured all spectra with the Shimadzu Multipurpose Recording Spectrophotometer Model MPS-50, the optics of which minimize scattering effects. All spectra were measured at room temperature using a tungsten lamp source between 340 nm and 800 nm.

Analysis of pigments and lipids

Extracted pigments were assayed spectrophotometrically using the absorption coefficients of MACKINNEY[24] for chlorophyll, and DAVIES[25] for carotenoids. Pigments were purified on thin-layer plates of silica gel G using a solvent system of hexane–acetone (80:30, v/v).

Thin-layer chromatography on silica gel G in a solvent of chloroform–methanol–water (75:25:2, v/v/v) provided good separation of the lipids from chloroplast extracts. After development of thin-layer plates, phospholipids were initially detected with the molybdate spray reagent of DITTMER AND LESTER[26]. The plates were then sprayed with a 5 % dichromate–sulfuric acid solution and heated for 15 min at 100°. In this manner, the principal chloroplast lipids were detected. The positions of mono- and digalactosyldiglycerides were determined independently with diphenylamine spray[27]. The R_F values are: monogalactolipid 0.72, digalactolipid 0.54, phosphatidylglycerol 0.49, sulfolipid 0.32, and chlorophyll 0.94. Quantitative assays for these glycolipids were based on the determination of sugar contents in lipids[28]. Phosphorous assay according to the method of YANG, FREER AND BENSON[29] was used for phospholipid determination.

Reassociation of lipoprotein

The reassociation of protein with lipophilic components demands an experimental process which permits sufficient solubilization of both lipid and protein. We approached this requirement by using a double-dialysis system. A uniform suspension of lamellar protein in methanol (0.1 mg/ml) was put into a small diameter dialysis bag. The membrane material for this bag was acetylated (anhydrous pyridine–acetic anhydride, 2:1 v/v) for 1 h in order to enhance lipid permeability. This bag was sealed and put into a larger dialysis bag containing 0.6–8.5 μg of β-carotene per ml of methanol. After 9–10 h, equilibrium between the two solutions was interrupted by the addition of aqueous methanol to the exterior of the large dialysis bag. When the system approached 60% methanol in water, the protein was recovered and analyzed for β-carotene content as were the supernatant solutions. From this technique we have been able to evaluate the association of β-carotene with the lamellar protein and the properties of the reassociated complex.

RESULTS

Protein characterization

Table I presents the amino acid composition of the lamellar protein fraction prepared as described above. The protein contained a large fraction of neutral and hydrophobic amino acids; the composition agrees with the data of WEBER[6] and BAILEY, THORNBER AND WHYBORN[4]. This protein appeared homogeneous in the ultracentrifuge, having a 2.2-S value in agreement with BIGGINS AND PARK[5]. However, disc-gel electrophoresis of detergent-solubilized protein (see METHODS) demonstrated several components. The two major protein bands may be related to the two fractions described by THORNBER *et al.*[11].

TABLE I

AMINO ACID COMPOSITION OF LAMELLAR PROTEIN

Protein was hydrolyzed in 6 M HCl for 24 h at 107° and analyzed according to the method of SPACKMAN, STEIN AND MOORE[23].

Amino acid	*Mole %*
Asp	8.8
Thr	4.7
Ser	5.7
Glu	9.2
Pro	5.9
Gly	10.5
Ala	9.6
Cys	0.7
Val	6.5
Met	1.7
Ile	5.3
Leu	11.0
Tyr	3.8
Phe	6.5
Lys	5.5
His	1.4
Arg	4.2
NH_3	7.0

Selective extraction of lipids and difference spectroscopy

Fig. 2 presents spectra from particles initially extracted with 70% acetone (curve C), and extracted with heptane after lyophilization (curve D). Absorption maxima in the difference spectrum (C–D) occur at 680 nm, 538 nm, 498 nm, 460 nm, and 438 nm. From the quantitative data in Table II, we conclude that maxima at

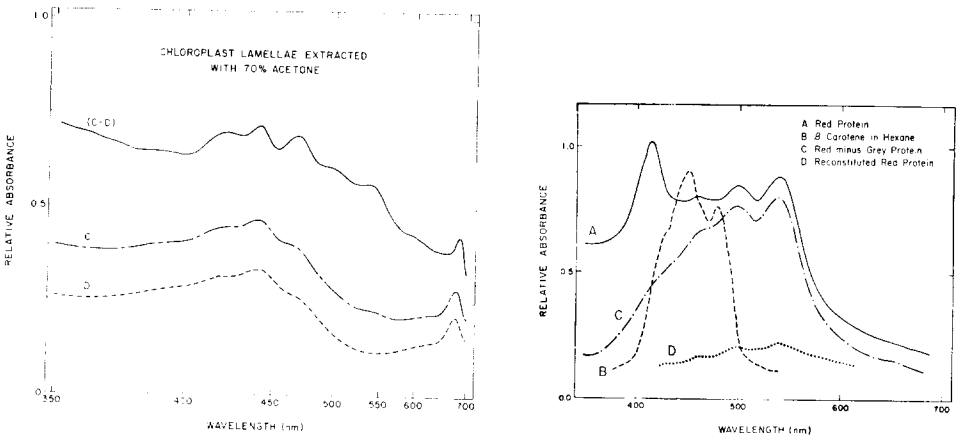

Fig. 2. Absorption spectra of chloroplast lamellae suspensions. Chloroplast lamellae were extracted with 70% acetone, divided into 2 equal portions and lyophilized. Half of the lamellae were further extracted with heptane. Both portions were suspended in buffer and spectra measured. C, lamellae extracted with 70% acetone; D, lamellae extracted with 70% acetone and heptane; C–D, difference spectrum – C was sample and D was reference – scale expanded 5 times.

Fig. 3. Absorption of chloroplast lamellae protein suspensions. A, spectrum of β-carotene–protein complex isolated from lamellae. B, spectrum of β-carotene in hexane solution. C, difference spectrum, red protein was sample; an equal quantity of grey protein was the reference sample – scale expended 5 times. D, difference spectrum, reassociated red protein (see METHODS) was the sample and an equal quantity of grey protein was the reference sample – scale expanded 5 times.

TABLE II

LIPIDS OBTAINED BY SEQUENTIAL EXTRACTION OF CHLOROPLAST LAMELLAE

Lamellae were prepared as described in METHODS, and initially extracted with either 45% or 70% aqueous acetone. After centrifugation, the particles were lyophilized and then extracted with heptane. The particles were again lyophilized and finally extracted with 100% acetone. Each of the extracts were analyzed for pigment and lipid composition as described in the text. Extracts were performed at 0°–5°.

Lipid	*Analyses of lamellae extracts* (%)					
	Initial extraction with 45% acetone			*Initial extraction with 70% acetone*		
	45% acetone	*Heptane*	*100% acetone*	*70% acetone*	*Heptane*	*100% acetone*
Monogalactolipid	59.5	15.8	24.7	61.7	16.1	22.2
Digalactolipid	48.9	19.3	31.8	53.0	19.5	27.5
Phosphatidylglycerol	37.2	20.8	42.0	39.5	23.1	37.4
Chlorophyll *a*	1.1	9.2	89.7	15.1	8.9	76.0
Chlorophyll *b*	14.6	4.7	80.7	73.0	8.7	18.3
Chlorophyll *a* + *b*	4.5	8.0	87.5	32.1	8.8	59.1
Xanthophylls	100	—	—	100	—	—
β-Carotene	—	99.4	0.6	—	97.2	2.8

680 nm, 460 nm, and 438 nm result from the additional extraction of chlorophylls *a* and *b* by heptane. The 498-nm and, in particular, the 538-nm maxima are related to the absorbance of β-carotene. These maxima do not appear in preparations of untreated lamellae or lamellae extracted with 45 % acetone. The percentage of lipids extracted by either 45 % or 70 % aqueous acetone is similar (Table II). Significantly, however, 70 % acetone extracted almost 10 times more chlorophyll than did 45 % acetone.

Treatment of lamellae with 80 % acetone removes most lipids and all pigments except β-carotene and quinones; the β-carotene can be removed with 100 % acetone or heptane to yield "grey" lamellar protein. The absorbance (Fig. 3) at 418 nm is due to cytochromes; difference spectroscopy at −196° also revealed the presence of cytochromes (low temperature spectra measured by W. CRAMER in W. L. BUTLER's laboratory). The absorption maxima at 538 nm, 498 nm, and 460 nm in the difference spectrum (Fig. 3) are caused by a shifted β-carotene absorption.

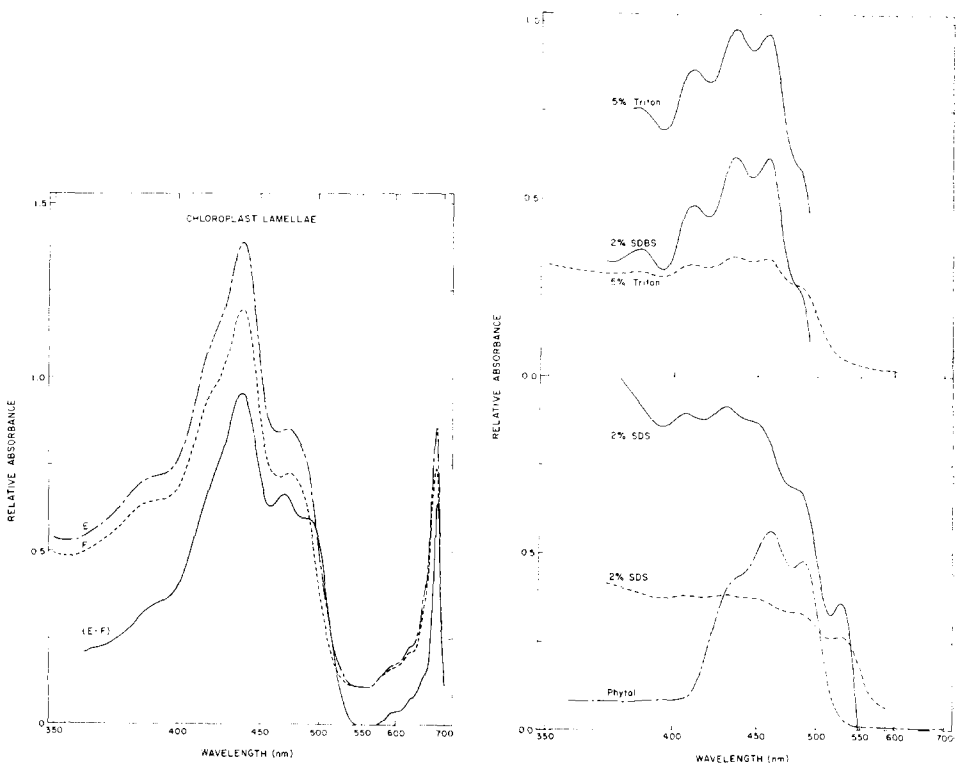

Fig. 4. Absorption spectra of chloroplast lamellae suspensions. Lamellae were lyophilized and extracted with either heptane or isooctane. After suspension of particles in equivalent quantities of buffer, spectra were measured. E, untreated lamellae; F, lamellae extracted with heptane or isooctane; E–F, difference spectrum, E was sample, F was reference—scale expanded 5 times.

Fig. 5. Absorption spectra of various β-carotene solutions. β-carotene was dissolved in 5 % Triton X-100, 2 % sodium dodecylbenzyl sulfate (SDBS), 2 % sodium dodecyl sulfate (SDS) or phytol. Crystalline β-carotene was shaken with the solvent for at least 4 h; then the solution was centrifuged for 20 min at 30000 × *g*. Absolute quantities of β-carotene were not determined. Solid lines indicate a 5-fold scale expansion.

Lyophilized chloroplast lamellae were extracted with isooctane or heptane directly. Analyses of these extracts by thin-layer chromatography revealed β-carotene as the major component with small amounts of chlorophylls *a* and *b*. The absorption maxima in the difference spectrum (Fig. 4), curve (E–F), at 680 nm and 438 nm are due to chlorophyll *a*. The maximum at 468 nm is caused, in part, by chlorophyll *b*. However, the 468-nm and 493-nm maxima are caused by the β-carotene in the lamellar protein.

Our spectra of the β-carotene–protein complex (Fig. 3) differ from those reported by SHIBATA[30] for β-carotene crystal suspensions in water. Spectral properties of the red protein remain unchanged even after solubilization of the complex in 5 % sodium dodecylbenzyl sulfate. However, 5 % Triton X-100 or sodium dodecylbenzyl sulfate solutions of free β-carotene reveal absorption spectra markedly different from the spectrum of the red protein (contrast Figs. 3 and 5). The spectrum of a detergent solution of "grey" lamellar protein added to a detergent solution of free β-carotene maintained light absorption properties of the free β-carotene solution.

Since the removal of phytol from the lamellar system alters the spectral properties of β-carotene (see above), we measured the spectrum of β-carotene dissolved in phytol. The maxima at 460 nm and 487 nm (Fig. 5), absorb light at an approx. 10-nm longer wavelength than in hexane. Thus, the absorption maxima of β-carotene in lamellae at 468 nm and 493 nm resemble those of the phytol solution more than the maxima of a hydrocarbon solution of β-carotene.

β-carotene could be reassociated with lamellar protein as demonstrated in Fig. 6. The protein binds approx. 1 mole β-carotene per mole of lamellar protein assuming a molecular weight of 23000. The spectral properties of this complex appeared similar to those of the red protein isolated directly from lamellae (Fig. 3). Formation of this

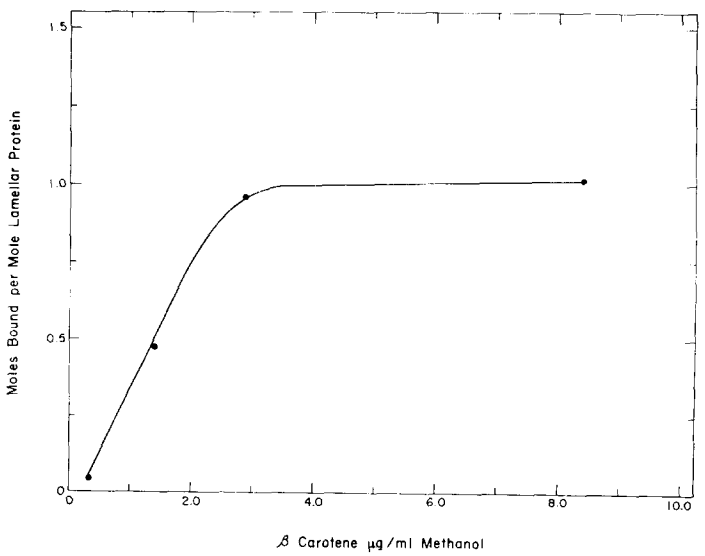

Fig. 6. Binding of β-carotene with chloroplast lamellar protein. The association of β-carotene with lamellar protein was measured as a function of the initial concentration of β-carotene in the methanol solution. This solution was used in the double-dialysis system described in METHODS for the reformation of the protein–carotene complex.

complex appears to be specific; similar association was not observed with bovine serum albumin.

DISCUSSION

All attempts to purify lamellar protein depend upon the initial solubilization of chloroplast lamellae with detergent[4,5]. However, detergents displace lipids from lipoprotein[12] and their use is not suitable for a study of the nature of the lipid–protein association. Thus, we chose to utilize total lamellar protein, which gel electrophoresis revealed as heterogeneous, rather than a specific membrane fraction.

The amino acid composition and sedimentation behavior of this lamellar protein are similar to those already characterized by other laboratories[4,6,7]. The protein is apparently hydrophobic in properties, and thus is suitable for investigating its lipid absorption or association properties.

We attempted to evaluate the nature of β-carotene's environment in chloroplast lamellae from the selective extraction of lipids and the spectral properties of different lamellae particles. 45 % acetone extracted lipids but did not alter the absorption spectrum of β-carotene in lamellae. However, the additional extraction of 30 % of the chlorophyll (Table II) resulted in an altered β-carotene environment as reflected by its spectral properties (Fig. 2). The extensive extraction of lipid and pigments could result in a conformational change in the lamellar protein. As is evident with cytochrome oxidase, however, acetone extraction does not eliminate the ability for phospholipid to restore enzymatic activity[19].

The properties of crustacyanin and ovorubin can be restored after acetone extraction simply by their reassociation with the carotenoid, astaxanthin[16,17]. Thus, the removal of chlorophyll does not necessarily result in denaturation. The branched phytol chains may associate closely with the isoprenoid structure of β-carotene. BUTLER has shown structural requirements for energy transfer from carotenoids to chlorophyll, and has suggested phytol may be in direct contact with carotene[31].

The ease of obtaining reproducible preparations of the carotene–protein complex from different sources, the reassociation of β-carotene with lamellar protein, and the spectral data suggest that the red-protein complex forms as a specific complex rather than a simple product of co-precipitation. Even solubilization by detergent does not affect spectrum of red protein. PLATT[32] has predicted such a spectral red shift for long-chain conjugated polyenes for which charged excited states may be stabilized by complementary charge polarization of surrounding medium. The red shift of β-carotene absorbance from 493 nm to 538 nm is a result of polarization effects of either lamellar protein or ionic lipids. From the extraction data (Table II) it is apparent that removal of charged lipids had little effect on the absorption spectrum of lamellar β-carotene. Charged groups within protein must be responsible for the observed polarization effects. Unless β-carotene were associated with lamellar protein, neither the altered polarization effects of lamellar protein nor the persistence of the characteristic absorption spectrum of red protein in detergent solutions would be readily explainable. We suggest that the *in vivo* environment of β-carotene is neither purely hydrophobic nor extremely polar, since we observe neither the absorption maxima of β-carotene dissolved in hydrophobic solvents nor the red-protein absorption maxima resulting from association of β-carotene with polarizing groups within the protein. The maxima

at 468 nm and 493 nm (Fig 4.), probably the *in vivo* absorption maxima for β-carotene, result from phytol's solvating effects and its ability to "insulate" β-carotene from the direct effects of the polar groups of the lamellar protein. Significantly, absorption spectra of synthetic mixed monolayers of β-carotene and chlorophylls containing no protein have lower absorbance between 490–500 nm than do *in vivo* systems even though the chlorophyll absorptions of the two systems are nearly identical[33].

Lipid extraction experiments (Table II) demonstrated that lipid solubility did not correlate with lipid extractability. Extractability may depend upon two parameters: (1) the lipoprotein may assume different conformations related to the solvent system; (2) various hydrophobic associations, exerted by lipids and lamellar protein, may minimize the solubilizing effects of polar groups. Apparently, phosphatidylglycerol maintains stronger hydrophobic associations with lipoprotein than do the galactolipids, since it is extracted poorly by aqueous acetone and more easily with heptane. The fatty acids of galactolipids are probably more polar, because of unsaturation, than fatty acids of phosphatidylglycerol[34]. The π orbital systems of the lamellar protein chain may interact directly with the three olefinic π electron systems in α-linoleneate, the major fatty acid in the galactolipids. This and similar association between lipid and protein are related to extractability properties of lamellar components.

The hydrophobic regions of lipoproteins may bind micelles of lipids and pigments in addition to binding individual lipid molecules at specific individual sites. The membrane probably has various binding sites which interact with particular types of micelles, since the flexibility and dynamic nature of lamellar membrane systems require the accommodation of varied lipid and pigment compositions. Our data emphasize the importance of hydrophobic lipid–protein associations in the structure of the chloroplast. The simple lipid bilayer model for membrane structure accommodates the results of this investigation less effectively than does the lipoprotein monolayer model[1,35].

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