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Properties of Protein–Chlorophyll Complexes from Pea (*Pisum sativum* L.) Leaves

THE ORGANIZATION OF CHLOROPHYLL

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Chlorophyll–protein–detergent complexes were prepared from pea chloroplasts by using sodium dodecylbenzenesulphonate and polyacrylamide-gel electrophoresis. Circular-dichroism spectra showed that complex CPI has a dimeric arrangement of chlorophyll *a*, with additional weaker interactions. Ellipticities were determined for both complexes and for purified chlorophylls in solution, and it is argued that the circular dichroism of complex CPII is derived from chlorophyll–protein interaction rather than from interaction between chlorophylls *a* and *b*. The detergent could be removed from the complexes by using urea and gel filtration, leaving the chlorophyll–protein in solution, although in each case a diminished ellipticity indicated some loss of organization. Three-peaked circular-dichroism spectra of chloroplast fragments before and after addition of detergent were compared with a curve obtained by summing graphically the spectra of complexes CPI, CPII and the free-pigment fraction. There was good correspondence at 650nm, and the longer-wavelength peaks agreed in form and magnitude, but with discrepancies in position. It was concluded that complexes CPI and CPII pre-exist in the original material, but that there is an environmental effect which is destroyed when the complexes are extracted.

Sodium dodecyl sulphate and the related detergent sodium dodecylbenzenesulphonate have been extensively used for disintegrating thylakoid material from chloroplasts into particles small enough to be resolved by polyacrylamide-gel electrophoresis. By this means Ogawa *et al.* (1966) and Thornber *et al.* (1967) identified two predominating chlorophyll–protein–detergent complexes, which (with the 'free' pigment) accounted for most of the chlorophyll in the original material. The complex known as CPI has since been found in all groups of oxygen-evolving photosynthetic organisms (Thornber, 1969); it has a molecular-weight range of 150000–160000 and some 20 molecules of chlorophyll *a* (with traces of chlorophyll *b* in higher plants). There is evidence (for example, Gregory *et al.*, 1972) that complex CPI has an essential role in photosystem I of photosynthesis, and carries the photoactive chlorophyll form P700 (Dietrich & Thornber, 1971). The complex CPII, which as usually prepared contains one molecule each of chlorophylls *a* and *b*, has been shown to be absent from a photosynthetically competent mutant of barley (Thornber & Highkin, 1974) and is therefore not an essential component for photosynthesis. It is regarded as an accessory pigment complex.

In the present paper we present additional evidence that the complexes CPI and CPII exist in chloroplast fragments that are photosynthetically active, since the first step in their isolation, treatment with detergent, renders the chloroplast completely inactive, and might allow the possibility that the complexes may represent spurious combinations of detergent-solubilized chlorophyll and protein. We make use of the technique of circular dichroism (c.d., differential absorption of right- and left-handed circularly polarized light), which selectively responds to those chlorophyll molecules in which the electronic transition associated with the absorption of light is influenced by an asymmetric environment. It was previously shown (Gregory *et al.*, 1972) that the CP complexes possessed characteristic c.d. spectra, and these can be perceived in unresolved systems. C.d. also gives some indication as to the type of interaction in each case.

A common problem in the investigation of membrane proteins is that they tend to be of a hydrophobic nature and to resist solubilization except in the presence of detergents. The proteins of the chloroplast thylakoids were regarded as typical in this respect [see, e.g., Criddle (1966), and Thornber (1969)]. In the present paper we show that the detergent can be removed, leaving the chlorophyll–protein complex in solution. The method may be applicable to proteins generally.

The ultimate goal of studies such as this is the elucidation of the structure of the thylakoid membrane in which chlorophylls in a greater or lesser degree of

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complexing with protein form light-harvesting and reaction-centre pigment arrays with so high an efficiency for the capture of light and the initiation of electron-transport reactions.

Materials and Methods

Peas (*Pisum sativum* L., var. Meteor) were grown in soil for 16 days and the leaves harvested. Leaves (150g) were blended for 15s in an Atomix blender (M.S.E.) at 1°C in 500ml of a buffer containing 50mM-Tris and 30mM-HCl (pH approx. 8.0 at 25°C). The resulting brei was filtered through nylon gauze (25μm mesh; Henry Simon Ltd., Cheadle, Stockport, U.K.) and the chloroplast suspension ['Type E' of Hall (1972)] precipitated by centrifugation at 10000*g* at 4°C for 5min. The green lamellar precipitate was suspended in 50ml of the above buffer in a hand homogenizer, and resedimented by centrifugation at 100000*g* for 20min. This washing process was repeated twice to remove soluble proteins.

Lamellae were solubilized by the following method, based on that of Thornber *et al.* (1967). A washed suspension of lamellar material, typically containing 30mg of chlorophyll in 50ml of the above buffer, at 4°C and in dim light, was homogenized by hand with the addition of a solution of sodium dodecylbenzenesulphonate (Koch–Light, Colnbrook, Bucks., U.K.; 10g/litre in the Tris–HCl buffer) so that the ratio of detergent to chlorophyll was 10:1 (w/w). The homogenate was centrifuged at 100000*g* for 25min, the supernatant reserved and the pellet resuspended in the minimum volume of Tris–HCl buffer (typically 30ml) and treated again with sodium dodecylbenzenesulphonate by the above procedure. In this way a series of supernatant extracts were obtained. In most cases the residue after three extractions was negligible.

Although Kung & Thornber (1971) have described a chromatographic separation of complexes CPI and CPII by using hydroxyapatite, in the present study it was found easier to prepare them by means of large-diameter (1.8cm) polyacrylamide gels, as described by Thornber *et al.* (1967), by using dodecylbenzenesulphonate (1g/litre) in the Tris–HCl buffer in the reservoir vessels. The extracts (obtained above) were applied to the gels (approx. 0.6mg of chlorophyll each) and typically 16 gels were run in one preparation. After slicing the gels and disrupting the appropriate slices by extrusion through nylon gauze, the chlorophyll-containing material was eluted by stirring with a small volume of the Tris–HCl buffer containing 1g of dodecylbenzenesulphonate/litre and filtering through filter paper (Whatman no. 1): this extraction was repeated twice and the combined extracts were, where necessary, concentrated by ultrafiltration in an Amicon Centriflo unit with a CF 50 cone. The purity of the complexes isolated in this way was checked by analytical gel electrophoresis, which would reveal inaccurate sectioning of the preparative gels; however, any contaminant which was superimposed on a CP complex would not be detected by this check.

C.d. measurements were performed in a Cary 61 CD spectropolarimeter (Varian Associates, Monrovia, Calif., U.S.A.) in a cuvette of path-length 1cm and a band-width of 2nm. D-Camphor-10-sulphonic acid was used as a standard, taking $\theta^{0.1\%}_{292nm} = 310m^\circ \cdot cm^{-1}$.

In this paper, measurements of c.d. are given in terms of ellipticity (m°), which is the most common parameter used. Both the principle of operation of the Cary 61 CD spectropolarimeter and the interpretation of the phenomenon, however, depend on the difference in the absorption of circularly polarized light, alternately left- and right-handed. An ellipticity of 1.0m° corresponds to a difference in extinction $(E_L - E_R)$ of 3×10^{-5}.

The detergent was removed from the complexes by incubating samples (eluted from the polyacrylamide gel) with 8M-urea in the Tris–HCl buffer. The volume was decreased as necessary to give a chlorophyll concentration of approx. 3mg/ml, and 3ml samples were applied to a column (45cm×2.5cm diam.) of Sephadex G-50 (Pharmacia, Uppsala, Sweden), previously equilibrated with 8M-urea in the Tris–HCl buffer. Gel filtration was performed by elution of the column with the same urea–Tris solution, at a rate of 0.8ml/min. The green material emerged in the void volume, and the detergent was eluted subsequently. The effectiveness of the separation was checked by using sodium dodecylbenzene[^{35}S]sulphonate, and

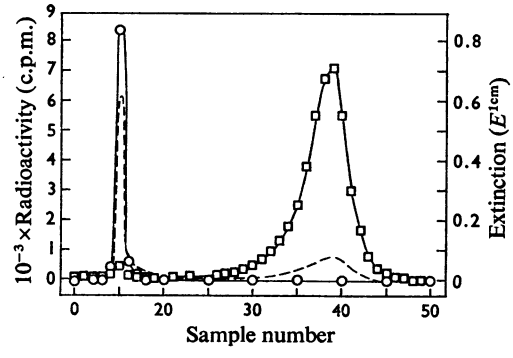

Fig. 1. *Removal of detergent from chlorophyll–protein complexes*

Complex CPII (prepared in sodium dodecylbenzene[^{35}S]-sulphonate) was applied to a column of Sephadex G-50 equilibrated with 8M-urea in 50mM-Tris–acetate buffer, pH8.6. The Figure shows the elution of radioactivity (□), chlorophyll (○; in terms of E^{1cm}_{670nm}) and protein (----; in terms of E^{1cm}_{260nm}). The abscissa shows the elution volume in terms of 20ml samples.

under the conditions described 98.8–99.5% of the radioactivity was separated from the chlorophyll-containing fraction (see Fig. 1). The green material was freed from urea by exhaustive dialysis against 0.01 M-Tris–0.005 M-acetic acid buffer, pH 8.1, at 4°C in the dark.

Concentrations of chlorophyll-containing samples are presented in terms of chlorophyll content (but see Fig. 1). Chlorophyll was measured spectrophotometrically by using the data of Mackinney (1941). In some cases this method failed, owing to the formation of phaeophytin, and recourse was made to the method of Hill (1963), in which phaeophytin formation is carried to completion with oxalic acid. Chlorophylls *a* and *b* were purified for comparative studies by the chromatographic method of Deroche & Carrayol (1971).

Results and Discussion

Circular dichroism

C.d. spectra of the complexes and of the free-pigment zone of the gel are shown in Fig. 2. Also shown are purified chlorophylls *a* and *b* in 1% sodium dodecylbenzenesulphonate. It was found that purified chlorophyll *a* in detergent solution tends to aggregate, forming microcrystals with a prominent c.d. feature at 705–747 nm ($\theta_{705nm} - \theta_{747nm} > 15.4 m^{\circ} \cdot$ litre$\cdot$mg$^{-1}\cdot$cm^{-1}) (see Scott, 1974). Traces of materials such as β-carotene (which do not absorb appreciably at the chlorophyll *a* wavelength) or chlorophyll *b* delay the crystallization (see Fig. 2). There is no such problem with purified chlorophyll *b* in this detergent. In the discussion of the data attention is concentrated on the red region of the spectrum.

In the CPI complex, a substantial part of the c.d. curve can be fitted to a curve obtained by subtracting two Gaussian curves of equal magnitude and S.D. (6.4 nm) but with peak positions differing by 1.9 nm, as shown in Fig. 3. The centre of the fitted curve is at 685 nm. The fitting is consistent with an explanation of the c.d. in terms of a dimer (split-exciton) interaction between pairs of chlorophyll molecules, an argument set out by Dratz *et al.* (1966). However, the absorption maximum of the complex CPI is at 674–676 nm, so that if the split-exciton hypothesis is valid then most of the chlorophyll of complex CPI is not taking part in the dimeric organization. Of the 20 chlorophyll molecules reported to be present (Thornber, 1969), only one or two pairs per molecule would be generating the split-exciton signal. The remainder, either as substantially 'free' chlorophyll, or by virtue of weak interactions, might account for the discrepancies in the fitting in Fig. 3.

The magnitude of the c.d. signal of complex CPI (+0.74 m° · litre · mg of chlorophyll$^{-1}\cdot$cm^{-1} at 678 nm, −0.57 m° · litre · mg$^{-1}\cdot$cm^{-1} at 690 nm) is greater than

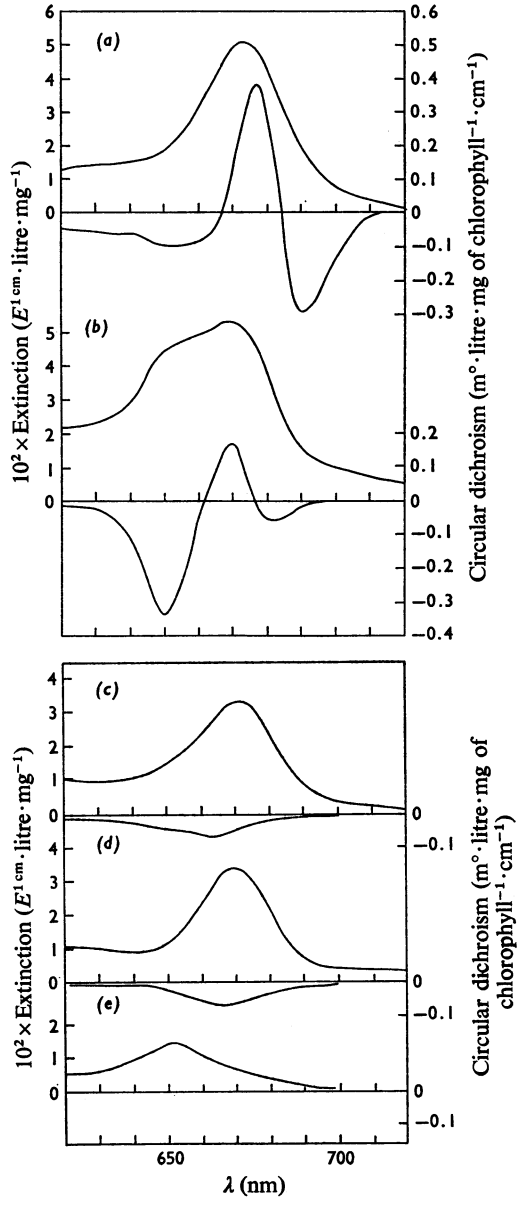

Fig. 2. *Comparison of absorption and c.d. spectra of the complexes (a) CPI and (b) CPII, together with (c) the free-pigment material and (d,e) purified chlorophylls a and b in solution in sodium dodecylbenzenesulphonate*

C.d. is shown as ellipticity. All concentrations refer to chlorophyll. The free-pigment fraction contained 57% chlorophyll *a*, 43% chlorophyll *b* (both included in the calculation of chlorophyll concentration). The solvent was 50 mM-Tris–acetate buffer, pH 8.1, containing 0.1% detergent (*a*, *b*, *c*) or 1% detergent (*d*, *e*). The sample of chlorophyll *a* was mixed with approx. 10% (w/w) of chlorophyll *b* to delay crystallization (not included in the calculated concentration).

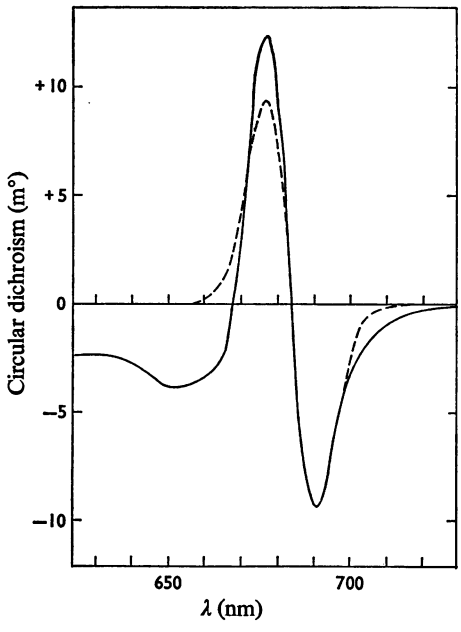

Fig. 3. *Resolution of the c.d. spectrum of complex CPI (——) into Gaussian components*

Curve ---- was obtained by subtracting a Gaussian curve with a standard deviation of 6.4nm, height of 52.2m°, centred at (685.0+0.95nm), from an identical curve centred at (685.0−0.95nm). The concentration of complex CPI represented here is 16.5μg of chlorophyll/ml.

that of the c.d. signal of complex CPII (discussed below).

The c.d. spectra of complex CPII appear superficially similar to that of complex CPI insofar as the principal component seems to be related to a difference between two Gaussian curves. However, an explanation based on a split-exciton interaction is not offered in this case, since each extremum of the c.d. spectrum agrees reasonably well with the chlorophyll *a* and *b* maxima visible in the absorption spectrum. Also, although the c.d. properties of a chlorophyll *a*–*b* complex are hard to predict in general terms, it might be expected that each absorption maximum would be split, yielding four c.d. components. A preferred explanation is that the c.d. contribution from each chlorophyll is separately enhanced by pigment–protein interaction. Thus the ellipticity of chlorophyll *a* is $-0.18\,m^{\circ}\cdot litre\cdot mg^{-1}\cdot cm^{-1}$ at 662–663nm in 80% (v/v) acetone, and $-0.12\,m^{\circ}\cdot litre\cdot mg^{-1}\cdot cm^{-1}$ at 667nm in 1% dodecylbenzenesulphonate; chlorophyll *b* has ellipticities of $-0.14\,m^{\circ}\cdot litre\cdot mg^{-1}\cdot cm^{-1}$ at 643nm and $-0.04\,m^{\circ}\cdot litre\cdot mg^{-1}\cdot cm^{-1}$ at 650nm in the same solutions, whereas the extrema of complex CPII are +0.19 and $-0.46\,m^{\circ}\cdot litre\cdot mg^{-1}\cdot cm^{-1}$ at 671 and 650nm respectively. (Since the comparison in this paper is only semi-quantitative, data are given in terms of the ellipticity values at the extrema. For precise calculations it is better to calculate the rotational strengths, based on the integrated areas under the c.d. curves.) Possible pigment–protein interactions could arise either by the effect on the chlorophyll transition of the asymmetric fields of the L-amino acid peptide chain and its higher-order structures, or by physical deformation of the chlorin ring, thus increasing its inherent asymmetry. Such an explanation avoids the difficulties of the split-exciton hypothesis, and since the contribution of each chlorophyll could still be basically Gaussian, the resultant c.d. of complex CPII which is close to a difference spectrum of two Gaussian curves, is explained. It has to be regarded as fortuitous that the c.d. contribution of chlorophyll *a* in complex CPII is of positive sign, whereas monomeric chlorophyll *a* in solution, and chlorophyll *b* both in solution and in complex CPII, give c.d. spectra of negative sign.

Removal of detergent

Hydrophobic proteins are often extractable only in detergents [e.g. structural protein from bean and wheat chloroplasts (Mani & Zalik, 1970) and structural protein from mitochondria of *Euglena* (Rogers & Lees, 1971)] and subsequent analysis is thereby complicated. We offer a method for removing such detergent and a procedure for testing the completeness of the separation. The success in obtaining detergent-free chloroplast proteins in aqueous solution was unexpected, since previous work (Criddle, 1966) yielded material soluble only in sodium dodecyl sulphate, and in the cholic acid detergents. It should be noted, however, that water-soluble chlorophyll–protein complexes have been obtained before, such as those from *Chenopodium album* (Yakushiji *et al.*, 1963) and *Brassica oleracea* (Murata *et al.*, 1968). There is a question whether these complexes represent native chloroplast protein–chlorophyll complexes or whether they represent artificial complexes between chlorophyll and soluble proteins, aided by possible surface-active agents in the leaves.

In the case of soluble proteins such as aspartate transcarbamylase, Weber & Kuter (1971) showed that inactivation caused by addition of detergent (sodium dodecyl sulphate) could be considerably reversed by its removal. This was achieved by adding urea to the detergent-treated enzyme followed by removal of the detergent on an anion-exchange resin and dialysis to remove the urea. They noted that when intermediate operations such as polyacrylamide-gel electrophoresis had been carried out, there was less recovery. Chlorophyll–protein complexes, however, bind strongly to ion-exchange materials even in the presence of urea,

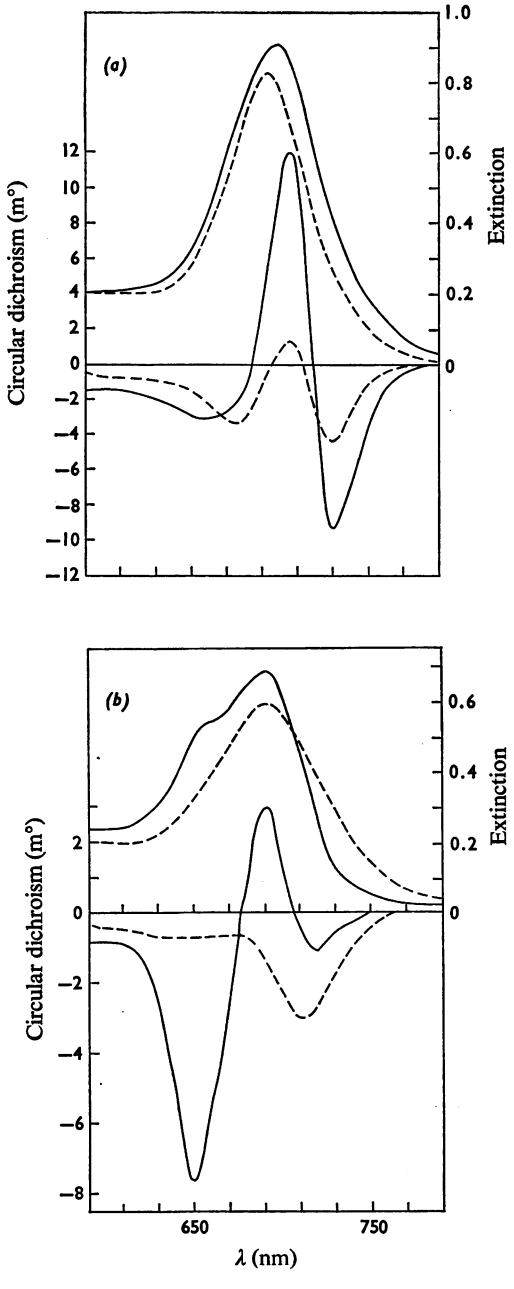

Fig. 4. *Effect of the removal of detergent on the c.d. and absorption spectra of protein–chlorophyll complexes*

The spectra of the complexes (*a*) CPI and (*b*) CPII are shown, first as they are prepared in sodium dodecylbenzenesulphonate (see the Materials and Methods section) (——) and secondly after removal of detergent (----). In both cases the buffer solution used was 50mM-Tris–acetate, pH8.1.

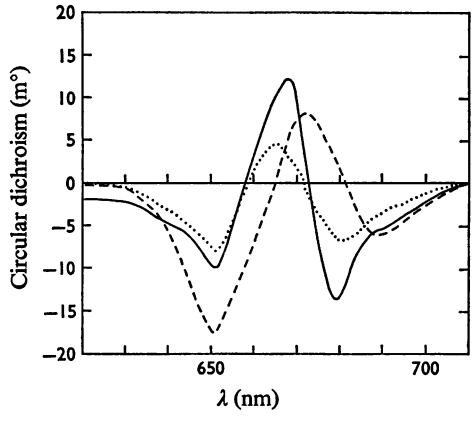

Fig. 5. *Effect of sodium dodecylbenzenesulphonate on c.d. spectra of chloroplast fragments*

Comparison of the c.d. spectra of chloroplast fragments, before (——) and after (····) addition of detergent, and of the supernatant extract from which complexes CPI and CPII were prepared (----). The concentration of the first two samples was 19 μg of chlorophyll/ml, and that of the third was 25 μg of chlorophyll/ml. The solvent was 50mM-Tris–acetate buffer, pH8.1.

and the somewhat simpler method of gel-filtration was found adequate in the present study. This finding confirms the suggestion of Weber & Kuter (1971) that urea weakens the detergent–protein interaction facilitating the removal of the detergent. In the present case there was a loss in structure, shown by the c.d. spectra (Fig. 4), when the detergent was removed; this took place on the addition of urea and neither the passage of time nor the readdition of detergent has been observed so far to bring about recovery. Fig. 6 shows that the electrophoretic step did not itself change the c.d. spectra.

In the present study we conclude that the complexes possess tertiary structures which allow them to be dissolved in water. The structures are weakly bound and are easily opened allowing the predominantly hydrophobic nature of the protein to take effect. On removal of detergent, complex CPI retains its c.d. spectrum to a greater degree than does complex CPII, (see Fig. 4), and may be judged to have either the more strongly bound protein structure, or a lesser dependence on the protein for the stability of the chlorophyll arrangement.

The 'free pigment'

The diffuse band of chlorophyll that moves fastest in polyacrylamide-gel electrophoresis (referred to as 'free pigment'; Thornber *et al.*, 1967) can be matched moderately well in its absorption and c.d. spectra by solutions of purified chlorophylls *a* and *b* in sodium

dodecylbenzenesulphonate (see Fig. 2). Further, these solutions of purified chlorophylls (made with precautions against microcrystal formation) migrate in gels at the same velocity as the 'free pigment' band in electrophoreses of thylakoid extracts. This supports the general consensus that that band does indeed contain a simple mixture of monomeric pigments dissolved in the detergent solution, and is correctly named. Such mixtures of chlorophylls and carotenoids do not show the tendency to form microcrystals that complicates the study of pure chlorophyll *a*.

Pre-existence of the complexes CPI and CPII

Fig. 5 shows the c.d. of chloroplast fragments before and after the addition of sodium dodecylbenzenesulphonate. It is apparent that there is little initial change in the wavelengths of the extrema, although in the experiment shown there was a diminution of some 50% in the magnitudes of the 668 and 680nm bands. This diminution varies considerably, and was not seen with spinach (*Spinacea oleracea*) (Gregory *et al.*, 1972).

Also shown in Fig. 5 is the c.d. of the first supernatant extract obtained from the detergent-treated fragments by centrifugation (see the Materials and Methods section). There are changes in the position of the extrema, and a further diminution in intensity. The c.d. spectrum of a similar extract was found to fit closely a curve obtained by adding together the c.d. spectra of complexes CPI, CPII and free pigment (shown in Fig. 6*a*) isolated from the extract by electrophoresis. The good fit (Fig. 6*b*) is evidence that the complexes CPI and CPII exist in the extract, before electrophoresis.

The comparison of c.d. spectra in Fig. 5 shows that the three-peaked form is continuously present from the stage of thylakoid fragments to the application of the extract to the polyacrylamide gels. If we conclude that complexes CPI and CPII pre-exist in the fragments (before the addition of detergent) then we have to explain the changes in wavelength and magnitude that are shown in Fig. 5. (It is noteworthy that the peak at 650nm attributed to chlorophyll *b* is the most resistant to change.) Three possible explanations can be offered. First, there may be interacting groups of complex CPI molecules which are progressively broken down, with loss of c.d., by the action of detergent. Secondly, each CPI unit may be associated in the original thylakoid with chlorophyll *a* molecules which are dissolved away by the detergent (becoming free pigment) and again losing the c.d. that arose from the interaction. Thirdly, there may be a change, such as a conformational change in the protein part of the CPI molecule, that disrupts the interaction between some of the chlorophylls within the complex. This last possibility is in accord with the observation that not all of the chlorophylls of complex CPI take part in the

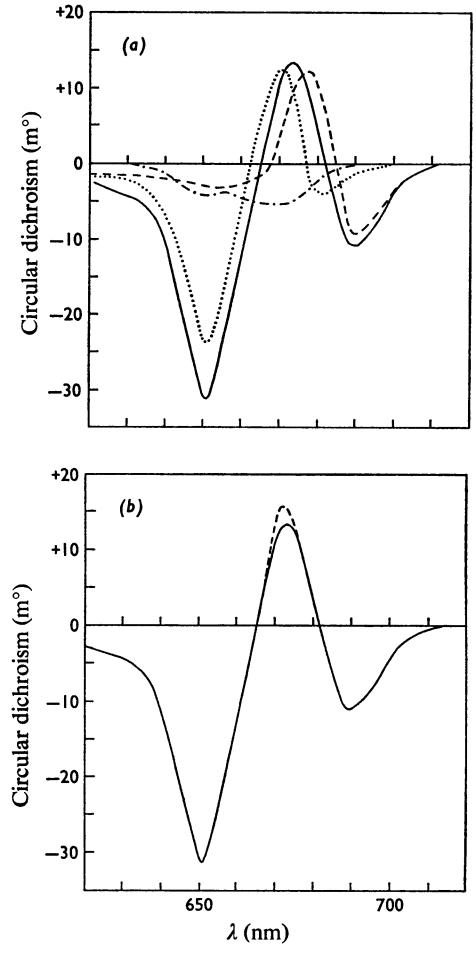

Fig. 6. *Relationship between c.d. spectra of the complexes CPI and CPII and the detergent extracts of chloroplast fragments*

(*a*) C.d. spectra of CPI (----), CPII (····) and free pigment (-·-·), and a curve (——) representing their algebraic sum. (*b*) Comparison of the above sum (——) and the c.d. spectrum of the supernatant extract from which the above fractions were isolated.

split-exciton interaction revealed by the c.d. referred to earlier in this discussion.

There is a question of the significance of the free pigment fraction of chlorophyll seen in the polyacrylamide-gel analysis: whether it is in fact a breakdown product of, say, complex CPI, during its isolation. In the present study such a breakdown would appear to be indicated. However, complex CPI is present in relatively small quantities, and the change in the

longer-wavelength c.d. peaks with the detergent does not account for all the free pigment observed. Secondly, we find appreciable quantities of chlorophyll *b*, in the free pigment fraction, which could not originate from complex CPII, since there is little loss of the 650nm (chlorophyll *b*) peak on treatment with dodecylbenzenesulphonate. We suggest that, of the chlorophyll in the chloroplast fragments, that which is eventually isolated as complex CPI or CPII is more tightly bound to protein than that which forms the free pigment zone. Presumably the latter fraction is organized so as to be closely interacting with the chlorophyll of the chlorophyll–protein complexes, since otherwise the efficiency of the light-harvesting and energy-transmitting system would be impaired.

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