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DETERGENT EFFECTS ON AN ALBUMIN-CHLOROPHYLL COMPLEX MODEL OF PHOTOSYNTHETIC PROTEIN–PIGMENT COMPLEXES*

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The absorption, fluorescence, fluorescence polarization and circular dichroism spectra of an artificial complex human serum albumin–chlorophyll-a were measured at 20 °C in buffer solution (pH 7.2) and the effect of detergents (digitonin, sodium lauryl sulfate, above critical micelle concentration) was studied. A strong chlorophyll–chlorophyll interaction found in the complex is removed by detergent treatment. The ionic detergent sodium lauryl sulphate induces pheophytinization and conformational changes in the protein part of the complex leading to changes in the absolute configuration of the transition moments of the pigment. Profound changes in the fluorescence polarization spectra are caused by both detergents. The effects of detergents suggest that the possibility of similar phenomena in treating plant material should be rigorously considered.

Introduction

Molecules involved in the primary processes of photosynthesis, absorption of light, migration of excitation energy to the reaction centre, and charge separation at the centre can be grouped as follows: *lipids* (forming the membranes), *pigments* (chlorophyll-a and accessory pigments, constituents of the light harvesting system, reaction centre and the chain of the electron excitation energy) and *proteins* (metalloproteins and complexes with chlorophylls, constituents of the oxidation-reduction electron transfer chain).

According to recent knowledge pigment–pigment and pigment–protein interactions play the major roles in photosynthetic processes. The growing interest in pigment–protein complexes is well documented in two recent reviews [1] and in the Proceedings of the recent International Photosynthesis Congress with over one hundred relevant papers [2].

* Dedicated to Prof. I. Tarján on his 70th birthday.

An interesting point in all studies of these *complexes isolated from different algae and higher plants* is the universality in apparent molar weights and spectroscopical properties [3]. These complexes have different molar weights above 20 kD, absorption maxima around 436 and 673 nm (chlorophyll-a bands), and at about 280 nm (protein band). *Different complexes isolated from the same plant* (maize) *have very similar absorption spectra* though the apparent molar weights differ widely (from 20 to 250 kD). It is worth mentioning that no significant amounts of free chlorophylls were found in this plant [4], which shows that practically all the chlorophyll exists as chlorophyll–protein complexes demonstrating the importance of these complexes in photosynthetizing systems.

Owing to the universality of chlorophyll–protein complexes artificial model complexes with well-defined composition can be prepared. These complexes are very similar to those existing in vivo, and can therefore be used for studying environmental effects and for determining roles existing under in vivo conditions. The modeling of pigment–protein complexes is not new; milk proteins [5], casein [6], bovine [7] and human serum albumin have been used to study the interactions between pigment and protein molecules, bonding states, photochemical activities and the aggregation of pigments in the complexes. A recently prepared chlorophyll–bovine serum albumin complex has been investigated spectroscopically [8] and its luminescence has been studied [9]. An improved method [10] resulted in a human serum albumin complex with an apparent molar weight of about 230 kD, containing 3 albumin molecules each carrying about 3 chlorophyll-a molecules. This stoichiometry is remarkably similar to that of a recently isolated complex [1, 4] with a molecular weight of 250 kD and composed of two or more subunits, each probably containing three chlorophyll-a molecules. It is remarkable that all of these artificial complexes show spectroscopic properties very similar to one another as well as to those isolated from plant material, i.e. to the in vivo complexes. There are models in which the chlorophyll–chlorophyll interactions have been eliminated by providing complexes of one molecule protein (apomyoglobin) with one molecule chlorophyll [11]. In another model these interactions do exist, but can be regulated [12]. These models are very useful to find the consequences of the association of protein and chlorophyll molecules. If, however, we are interested in the interaction of the complex molecule as a whole with its environment, then models containing more subunits and several chlorophyll molecules which are reminiscent of in vivo complexes seem more reliable.

In order to study the protein–pigment complexes present in plant material, the complexes first need to be isolated. An important step in the isolation process is the solubilization of the complex using detergent (digitonin, triton, sodium dodecyl sulphate, etc.). This is followed by fractionation (gel filtration, electrophoresis). With this procedure complexes of different apparent molar weights may be separated. However, the presence of detergent molecules may bring about new interactions and in addition to the above-mentioned molecular interactions detergent–pigment, detergent–protein and detergent–lipid interactions may appear and produce artifacts

in studies performed on the isolated medium. The importance of this possibility has already been recognized by several authors and very recently Argyroudi-Akoyunoglou [13] found that the binding of chlorophyll to protein in the complexes is specific and not an artifact due to sodium lauryl sulphate treatment. However, depending on the detergent concentration, certain changes occur in the absorption and fluorescence spectra of the light-harvesting pigment-protein complex isolated from pea chloroplasts [14]. Among the complexes isolated in some laboratories, there is an unstable one, named CPa (a complex of chlorophyll-a), which is considered to be the reaction centre of the second photochemical system (PS-2) of photosynthesis. This statement has been confirmed recently by several authors [13]. However, this complex is labile to detergent treatment therefore it cannot be safely isolated [15]. All these reports on chlorophyll-a-protein complexes, and on the effect of detergent treatments point out the importance of obtaining more information about chlorophyll-a-protein complexes and about the effects of different environments, particularly those created by detergent molecules. We have already experimented on the effects of detergents on chlorophylls and on other dyestuffs [16] and on proteins [17]. We decided to investigate an artificial human serum albumin-chlorophyll-a complex recently prepared in our laboratory and already used successfully in spectroscopic studies [10]. The following main problems were studied: a) the condition of the pigment in the complex; b) the effect of detergent treatment on a pigment-protein complex; c) the relevance of these effects on in vivo complexes extracted by a similar treatment.

Materials and methods

A chlorophyll-a-human serum albumin (Chl-HSA) complex prepared in our laboratory was used. The details of the preparation are published elsewhere [10]. The main steps are the following. Lecithin dissolved in chloroform and Chl dissolved in diethyl ether are mixed and evaporated to dryness. The dry material is dissolved in Britton-Robinson buffer and is sonicated to produce liposomes containing Chl. HSA is added to the suspension and after shaking and waiting for the complexation of Chl and HSA a Sephadex column is used for separating the complex. The fractions may have different absorption coefficient ratios $k(280\,\mathrm{nm})/k(680\,\mathrm{nm})$ depending on the number of Chl molecules per molecule of HSA. If fractions with ratios from 0.95 to 1.10 are collected, the apparent molar weight of the complex is about 230 kD and the number of Chl molecules per molecule HSA is 3 to 1 depending on the concentration of the solutions. (If the ratio is lower or higher than 1:1, more or less Chl-s, respectively, are attached to one HSA molecule.)

For detergent treatment sodium lauryl sulphate (SLS, an ionic detergent, product of Fluka AG, Buchs SG, Switzerland) and digitonin (D, a non-ionic detergent, product of Reanal, Hungary) were used. These detergents are both very often applied in the separation of pigment-protein complexes. Above a critical detergent concentration

micelles are formed (in the case of SLS this concentration is about $2.5 \cdot 10^{-3}$ M, in non-ionic detergents it is lower). In order to follow the procedures used in solubilizing in vivo complexes their concentrations were well above the critical concentration; in all cases $6.6 \cdot 10^{-3}$ M detergent solutions were used.

The absorption spectra were measured with a Specord UV-VIS (Zeiss) or (especially in the Chl absorption region) with an SF-18 recording spectrophotometer (USSR). The fluorescence spectra and fluorescence polarization spectra were recorded with a Perkin-Elmer (Type MPF-44A) spectrofluorimeter. Correcting for instrument error the degree of polarization was calculated from the formula

$$p = \frac{I_{\parallel} - \rho I_{\perp}}{I_{\parallel} + \rho I_{\perp}},$$

where $I_{\parallel}$ is the intensity of fluorescence measured with both the observing and the exciting polarizers vertically oriented, and $I_{\perp}$ is the intensity measured with the exciting polarizer vertically oriented and the observing polarizer horizontally oriented. The correction factor is the ratio of fluorescence intensities obtained with the exciting polarizer horizontally oriented and with the observing polarizer both vertically and horizontally oriented. The ratio $\rho = I_{\text{vertical}}/I_{\text{horizontal}}$ takes the systematic error of the instrument into account.

The circular dichroism (CD) spectra were recorded with a JASCO 40 C spectropolarimeter.

All measurements were carried out at room temperature. All manipulations with chlorophyll-containing materials were carried out in dim light; the preparations were kept under air in the dark and in a refrigerator at 4 °C. In these circumstances the optical properties remained reasonably constant for 3—4 days.

Results

Absorption spectra

The absorption spectra were recorded from 220 nm to the red end at about 700 nm. The spectra are shown in Fig. 1 from 350 nm only, because the absorption peak at 274 nm (belonging to HSA absorption) published in [10] did not change except for a slight shift towards longer waves and the change in the absorption intensity was less than 5 per cent. The detergent treatment in the absorption region of Chl (Fig. 1) causes considerable change when the ionic detergent SLS is used. The peak of the absorption of the complex at 440 nm disappears and a new peak at 418 nm appears together with the maxima at 512 and 550 nm, which should be attributed to an extensive pheophytinization of chlorophylls. In the red band a shift of the maximum from 674 to 677 nm is observed. The non-ionic detergent D does not induce such spectral changes, but causes a decrease of the extinction, $E(\lambda)$. (By using proper sample thicknesses the

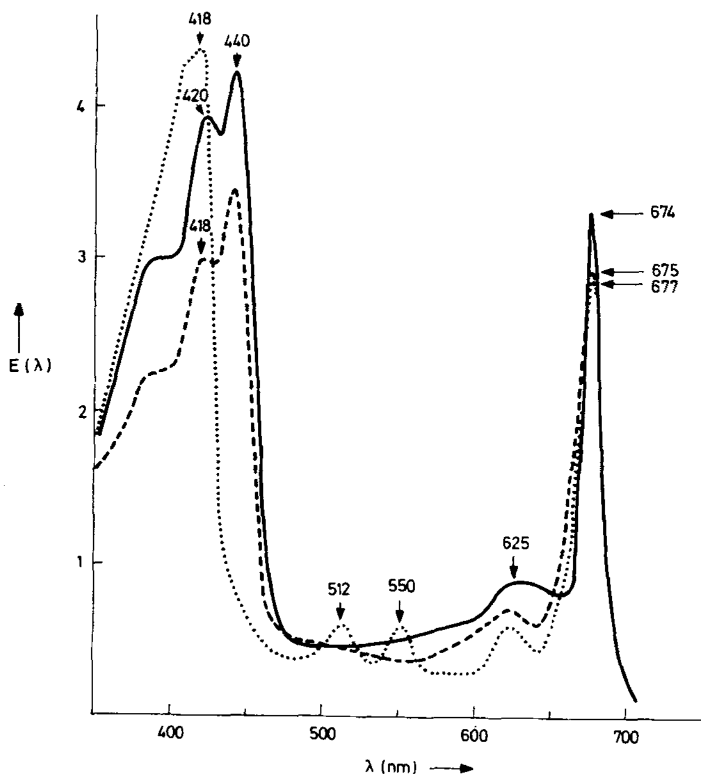

Fig. 1. Absorption spectra of the complex human serum albumin–chlorophyll-a in Britton–Robinson buffer (pH 7.2; solid line), complex $+6.6 \cdot 10^{-3}$ M sodium sulphate (dotted line), complex $+6.6 \cdot 10^{-3}$ M digitonin (broken line)

actual measured values of extinction never exceeded 0.8. In Fig. 1 all $E(\lambda)$-values refer to 1 cm layer-thickness.)

Since the incubation time with detergent has been found to be an important factor of the detergent effect, the absorption spectra after 1 and 24-hour incubation were recorded (Fig. 2). (Note the different scales for SLS- and D-treated solutions.) The effect of detergents on the absorption spectrum of the complex is apparent after 1-hour incubation.

Fluorescence spectra

The spectral distribution of the intensity of protein fluorescence excited at 280 nm and that of the chlorophyll-a fluorescence excited at 435 nm are shown in Fig. 3. Relative intensities $F_{rel} = F/F_{max}$ are plotted; $F_{max} = 1$ is taken arbitrarily for both the HSA and the Chl part of the spectrum. The intensity scale, F_{rel}, is different for the fluorescence of HSA-Chl complex solutions and for the solutions treated with D and

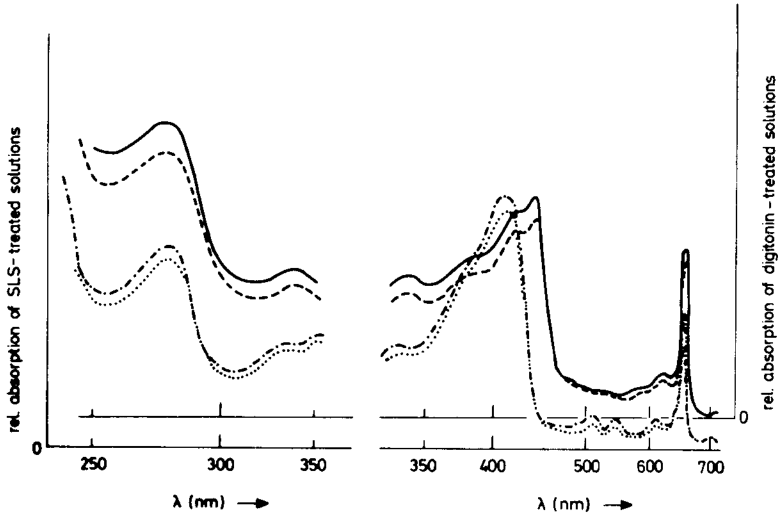

Fig. 2. Relative absorption spectra of the complex human serum albumin–chlorophyll-a treated with $6.6 \cdot 10^{-3}$ M sodium lauryl sulphate for 1 hour (dotted line) and for 1 day (dash-dotted line), and treated with $6.6 \cdot 10^{-3}$ M digitonin for 1 hour (solid line) and for 1 day (broken line)

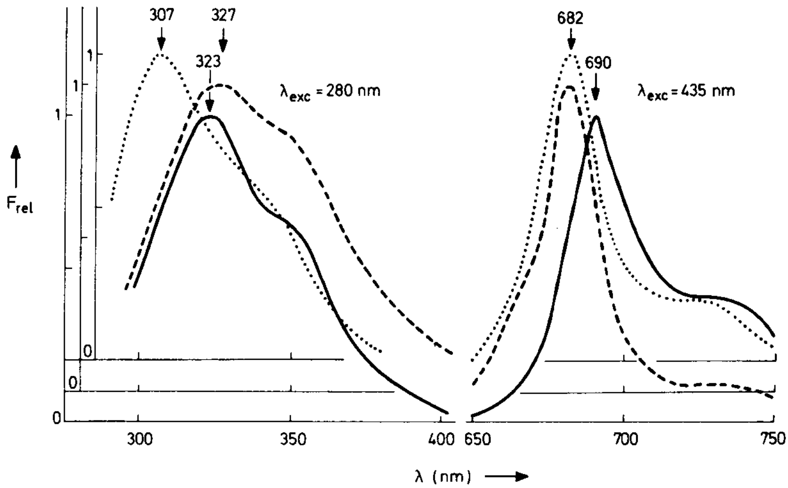

Fig. 3. Relative fluorescence spectra of the complex human serum albumin–chlorophyll-a and the spectra of detergent-treated samples (notation as in Fig. 1)

SLS detergents. The protein fluorescence peak of SLS-treated solution is shifted far to the ultraviolet part (to 307 nm). The peak of chlorophyll fluorescence is shifted from 690 to 682 nm after detergent treatment. The absolute intensities of the fluorescence are greatly increased in the Chl band after detergent treatment: relative to the untreated sample the maximum height of the peaks at 682 nm is about 50 times higher. With excitation at 280 nm (HSA absorption peak) the fluorescence of chlorophyll-a appears and the spectral distribution is similar to that shown in Fig. 3.

Circular dichroism spectra

These spectra exhibit very pronounced changes after detergent treatment (Fig. 4). The CD-spectrum of the HSA-Chl complex in the red band of absorption $E(\lambda)$ (shown in Fig. 1) has a typical shape attributed to the presence of Chl dimers. The positive and negative peaks of almost equal heights and the intersection at 694 nm on the wavelength axis are characteristic of the CD spectrum of the complex.

After D treatment a single negative band appears with maximum at 677 nm. After SLS treatment a single positive band is obtained with peak at 683 nm.

Fluorescence polarization spectra

Relative fluorescence polarization spectra are shown in Fig. 5, where p_{max} denotes the maximum value of p in the appropriate spectral range. For comparison the absorption spectrum $E(\lambda)$ is shown (right scale). This presentation gives information on

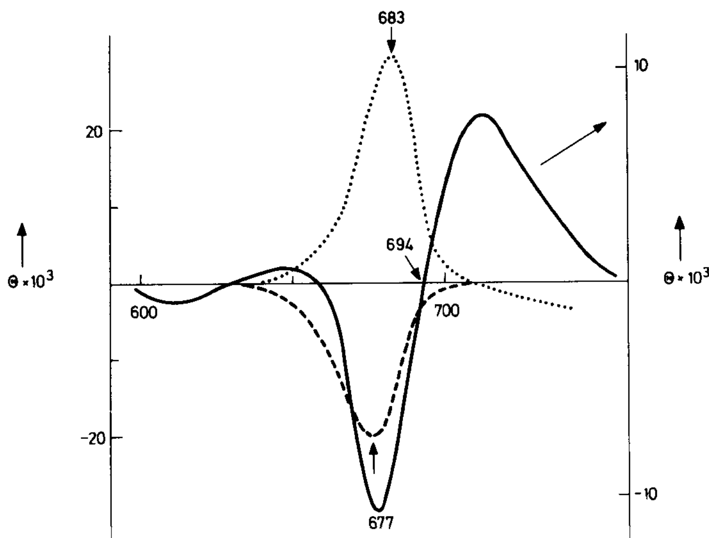

Fig. 4. Circular dichroism spectra of the complex human serum albumin–chlorophyll-a and the spectra of detergent-treated samples (notation as in Fig. 1)

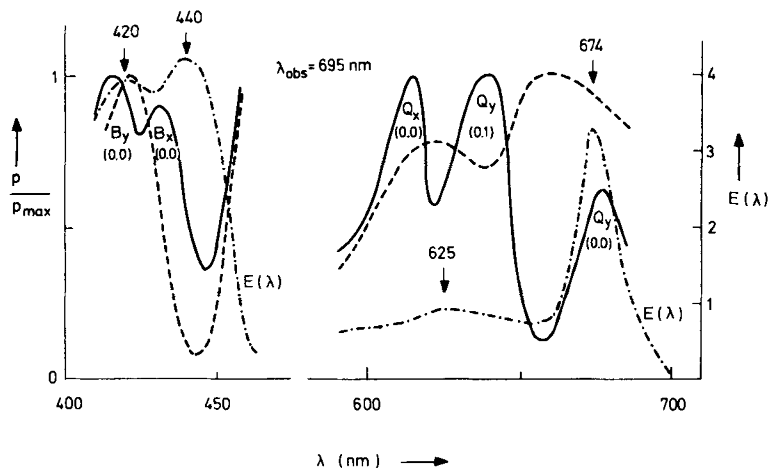

Fig.5. Relative fluorescence polarization spectra of the complex human serum albumin–chlorophyll-a and the spectra of detergent-treated samples (notation as in Fig. 1)

the spectral distribution of p. The distribution was reproducible: from 3 samples prepared independently the peaks were at 415, 436, 615, 640 and 677 nm within ± 3 nm accuracy. However, the absolute values of p varied for some peaks by over 100 per cent.

Discussion

From the experimental results conclusions can be drawn: a) on the composition (and partly on the structure) of the complex prepared for modeling plant pigment–protein complexes, especially for that of CPa, which cannot be easily isolated from plants; b) on the effect of detergents on the structure of our artificial pigment–protein complex; c) on the points which should be considered in the evaluations of spectroscopic data of plant pigment–protein complexes obtained with detergent treatment.

In [9] the evaluation of absorption and fluorescence spectroscopic data leads to the conclusion that HSA does not induce essential changes in the spectral and energetic parameters of the pigment part of the complex. This statement is valid only if spectroscopy means strictly fluorescence spectroscopy, and the red shift of the absorption (10—15 nm) and the increase of the band width (by about 5 nm and the appearance of long-wave fluorescence) are neglected. In this sense our absorption and fluorescence spectra of the HSA–Chl complex are similar to those of Chl-solutions, too. Low-temperature spectra, however, are more sensitive to the changes of pigment entity after complexation and they suggest the presence of Chl associates in the complexes. In [12] casein–Chl complexes with a peak of associated Chl molecules appeared at 672 nm if the number of Chl molecules per complex molecule ≈ 5—6. Though the

absorption and fluorescence spectra, especially at ambient temperatures, do not show definite pigment–pigment interactions in all cases, the CD spectra reveal the presence of associates without any doubt. As Fig. 4 indicates, the CD spectrum of HSA–Chl has an exciton signal with extrema at 677 (−) and 715 nm (+) in the main red absorption band of Chl, which means that dimeric Chl molecules are present in our HSA–Chl complex. The prerequisite of pigment association is given, since from earlier evidence [10] we know that about 9 Chl molecules are bound to each HSA unit (of 3 HSA molecules). Since, however, the pigment is non-covalently linked to protein, only an average number of chlorophyll molecules per protein molecule can be given [21].

In D-treated complex solutions the absorption spectra do not change much in accordance with the above statement about the low sensitivity of these spectra (Fig. 1). In the CD spectrum a negative band appears with a maximum at 658 nm indicating the presence of monomeric Chl. (A similar negative band is seen in diethyl ether solution, where only monomeric forms can be present.) D is a non-ionic detergent having a slight effect, but still strong enough to break the Chl associates, most probably by incorporating Chl molecules into D micelles.

In SLS-treated complex solutions the absorption spectrum (Fig. 1) exhibits very pronounced changes: the shift of the blue band (from 440 to 418 nm) and the appearance of new peaks between 500 and 600 nm indicate a pheophytinization of Chl. A profound change in the CD spectrum (Fig. 4) is the reversal of the single band with maximum at 662 nm from a negative (obtained with D-treatment) to a positive band. One should not forget, however, that this CD-band belongs to pheophytinized Chl.

This behaviour has been found very recently in bacteriochlorophyllides [11]. The association of protein and chlorophyll in defined (1:1) structures was studied using apomyoglobin (as protein) and different chlorophyllides (as chlorophyll analogs). In this system the heme group of the protein is replaced by a single pigment molecule. If bacteriochlorophyllide was used for replacement, several transitions in the CD spectrum became entirely reversed. The reversal can be explained by the change of the absolute configuration of the transition moments. In our case, the SLS treatment should induce a conformational change of the HSA matrix. This change transfers a monomer linked to a given site into another orientation. In micellar solutions the concentration of monomeric detergent molecules is a saturation concentration therefore the number of monomeric detergent molecules is always sufficiently high to interact with proteins.

According to [18] monomeric forms can only interact with proteins and they often induce conformational changes or even disruption of the protein macrostructure. The conformational change of HSA is corroborated by the great shift of the fluorescence maximum from 327 to 307 nm, which can be explained by conformational changes leading to tyrosine fluorescence (hindering the excitation transfer from tyrosines to tryptophan) or to changes of the environment of tryptophan.

Detergents are able to solubilize Chl and can easily form micellar Chl solutions. The effect seems to take place comparatively quickly. After one hour of incubation the

effect on the absorption spectrum appears (Fig. 2); not much further change can be seen after one day of incubation. This might be different when chloroplast fragments of larger size are treated [14].

The general features of the relative fluorescence polarization spectrum of the complex (Fig. 5) are similar to those obtained earlier for Chl-s or Chl analogues. The polarization spectrum of Chl-a in castor oil showed a strong overlap of two bands around 400 nm and a further overlap around 650 nm [23]. The potassium chlorophyllide fluorescence polarization spectrum in glycol had maxima at 408 and 415 nm and an increase beyond 430 nm [24]. The Chl-a fluorescence polarization spectrum in ethanol at 77 K shows three smaller peaks from about 400 to 480 nm, a sharp peak at about 630 nm and an increase toward longer waves [20].

In connection with our spectra two statements can be made: a) the reproducibility of the absolute values of the degree of fluorescence polarization was very bad; b) the D treatment induced profound changes in the spectral distribution of the degree of polarization. The first statement can be easily understood. Since Chl molecules are non-covalently linked to different amino acid residues, in spite of their similar spectroscopic properties and energetically similar build-up, the fluorescence polarization may be different in different samples. The degree of polarization reflects the mobility of the pigment molecule in its microenvironment, which is highly sensitive to minor changes.

The second statement cannot be easily explained. The peaks at around 430 and 640 nm disappear in D-treated samples. Two possible explanations can be suggested: a) The electronic transitions with perpendicular transition moments Q_x and Q_y [24] interact differently with detergent molecules. The transitions of the moments Q_y and Q_x lie around 674 ($Q_y(0,0)$), 625 nm ($Q_y(0,1)$) and around 615 nm ($Q_x(0,0)$); the transitions of the moments B_x and B_y are at about 440 ($B_x(0,0)$) and 240 nm ($B_y(0,0)$). The detergent molecules affect the transitions $B_x(0,0)$ and $Q_y(0,1)$ only. This picture is not clear however. It is generally known that Q_y is perturbed more strongly by environmental effects [24], but one cannot accept that $Q_y(0,0)$ and $B_y(0,0)$ are not concerned. b) Two equilibrating ionic forms of the complex [19] interact with the HSA–Chl complex differently. At pH 7.2 there exist two forms, a cationic form with a more pronounced absorption peak at about 420 nm and a neutral form with higher maximum at around 440 nm. Since the peak of the fluorescence polarization spectrum at longer wavelength in this region (at 432 nm) disappears after D treatment, the neutral form of the complex seems to be influenced by D molecules. In the red band of Chl absorption, however, this explanation cannot be satisfactorily applied either, because in this region the bands of the neutral and cationic forms strongly overlap, just as the fluorescence originating from the excitation of these forms [19].

The conclusions can be briefly summarized as follows. a) The artificial HSA–Chl complex incorporates Chl dimers and can therefore be a candidate for modeling CPa. b) The detergent treatment of this complex removes the dimers. The ionic detergent SLS induces pheophytinization and conformational changes of the complex; further, it

causes changes in the absolute configuration of the transition moments. Non-ionic detergents, such as D, may change the spectral distribution of fluorescence polarization both by removing some characteristic bands and by shifting the maxima. c) Under in vivo conditions, using the same types of detergents in similar concentration, one should consider the possibility of detergent–complex interactions which were found with this artificial complex. SLS treatment needs careful consideration, and the data of polarization should be very carefully analysed. The effect of detergent on the protein part of the complex deserves special attention, because it is believed that detergent micelle formation diminishes the concentration of monomers and prevents protein denaturation.

References

1. J. P. Thornber, T. P. Markwell and S. Reinman, Photochem. Photobiol., **29**, 1205, 1979.; J. Barber, ibid., **30**, 203, 1980.
2. Proceedings of the Vth International Congress on Photosynthesis, Kallithea, Greece, Sept. 1980. Ed. G. Akoyunoglou, Balaban International Science Service, Philadelphia, 1981.
3. R. G. Hiller, in Proc. Vth International Congress on Photosynthesis, see Ref. [2].
4. J. P. Markwell, J. P. Thornber and R. T. Boggs, Proc. Natl. Acad. Sci. USA, **76**, 1233, 1979.
5. Yu. E. Giller, G. B. Krasichoa and D. I. Saposhnikov, Biofizika (Moscow) **15**, 38, 1970; Yu. E. Giller, L. N. Yuhanova and S. K. Abdullaeva, Dokl. Akad. Nauk SSSR, **207**, 1475, 1972; M. Dinant and J. Aghion, Photochem. Photobiol., **17**, 25, 1973; M. Widart, M. Dinant, F. Téchy and J. Aghion, Photobiochem. Photobiophys., **1**, 103, 1980.
6. A. Meister and E. Brecht, Biochem. Physiol. Pflanzen, **174**, 305, 1979.
7. G. M. Sherman, Nature, **224**, 1108, 1963.
8. E. Tombácz and E. Vozáry, Acta Biol. Univ. Szeged, **26**, 33, 1980.
9. E. I. Zenkevich, G. A. Kochubeev and A. M. Kulba, Biofizika (Moscow) **26**, 389, 1981.
10. E. Tombácz, Z. Várkonyi and L. Szalay, Zh. Prikl. Spectr. (Minsk) **36**, 64, 1982.
11. S. G. Boxer, 9th Ann. Meeting. Amer. Soc. Photobiol. Program and Abstract. June 14—18, p. 61. 1981.
12. Yu. E. Giller, Mol. Biol. (Moscow), **14**, 1057, 1980.
13. A. Wild, Z. Pflanzenphysiol., **100**, 1, 1980; J. M. Anderson, FEBS Lett., **117**, 327, 1980; J. H. Argyroudi-Akoyunoglou, A. Castorinis, Arch. Biochem. Biophys., **200**, 326, 1980.
14. M. D. Illina, E. A. Kotova and A. Yu. Borisov, Biochim. Biophys. Acta, **636**, 193, 1981.
15. A. C. Stewart, FEBS Lett., **114**, 67, 1980.
16. D. B. Knaff, R. Malkin, J. C. Myron and M. Stoller, Biochim. Biophys. Acta, **459**, 402, 1977; T. Szitó, J. Hevesi and E. Bálint, Zh. Prikl. Spectr. (Minsk) **33**, 751, 1980; E. Lehoczki and K. Csatorday, Biochim. Biophys. Acta, **396**, 86, 1975; E. Lehoczki, ibid. **408**, 223, 1975.
17. E. Vozáry and L. Szalay, Acta Phys. Chem. Szeged, **23**, 17, 1976.
18. A. Helenius and K. Simons, Biochim, Biophys. Acta, **415**, 29, 1975.
19. L. Szalay, Z. Várkonyi and E. Tombácz, Biofizika (Moscow), **26**, 628, 1981.
20. P. Koka and S. Pill-Soon, Biochim. Biophys. Acta, **495**, 220, 1977.
21. I. N. Stadnichuk and M. V. Guseva, Photosynthetica, **15**, 221, 1981.
22. G. P. Gurinovich, A. N. Sevchenko and K. N. Solovjev, Spectroscopy of Chlorophylls and Related Compounds. Translated from Russian AEC-tr-7199, Chemistry (TID-4500) US Atomic Energy Commission, Div. Techn. Inform. p. 241, 1971.
23. D. Frackowiak and Z. Kojro, Proc. Int. Conf. Luminescence, Budapest, Akadémiai Kiadó, 301, 1966.
24. R. K. Clayton, Photosynthesis: Physical Mechanisms and Chemical Patterns. IUPAB Biophysics Series; 4. Cambridge University Press, p. 147, 1980.