DIRECT AND INDIRECT MECHANISMS OF DEAGGREGATION BY FATTY ACIDS IN CHLOROPHYLL-CONTAINING SYSTEMS

M. BRODY *and* B. NATHANSON

From the Department of Biological Sciences, Hunter College of the City University of New York, New York 10021

ABSTRACT The ability of an exogenous long-chain unsaturated fatty acid (linolenic acid) to induce changes in the circular dichroism (C.D.) spectra of chlorophyllous systems of various levels of organization is demonstrated and attributed to its deaggregating influence. In the case of chlorophyll in solution (CCl_4 or CCl_4-hexane), deaggregation is by direct action on the chromophore. Evidence is also given for an indirect mechanism when chlorophyll is attached to protein (e.g., in HP-700 complexes); in this case, deaggregation results from a conformational change in the protein. Interpretations are given for the differences in C.D. spectra of nonmembranous and membranous chlorophyll-containing systems. (The latter include "digitonin-isolated" system I particles, subchloroplast particles obtained by means of sonication, and specially prepared intact chloroplasts.)

INTRODUCTION

In previous publications (1–4), we have reported that an extract ("RLE") from the leaves of *Ricinus communis* can induce changes in fluorescence of chloroplasts (from algae and higher plants) incubated in it. The major changes, as observed at 77°K, consisted of an increase in intensity of emission of the chlorophyll band with maximum at 698 nm, and a concomitant decrease of the band with maximum at 735 nm. As these changes in emission occurred, it was observed that in the (77°K) action spectrum for exciting fluorescence at 698 nm, the contribution by the excitation band with maximum at 674 nm increased, while the contribution of the band with maximum at 682 nm decreased; in the low temperature excitation spectrum for sensitizing fluorescence at 735 nm, a decrease in contribution of the 705 nm band was additionally observed. Associated with the fluorescence changes (1, 2) are absorption changes, measured at 77°K as well as at room temperature (5), changes in fluorescence induction patterns (3, 6), light-induced absorption changes in chlorophylls a_I and a_{II} (7), and changes in chloroplast ultrastructure (2, 3). A protein fraction of RLE was found to be responsible for generating these changes (1).

That long-chain, unsaturated fatty acids can serve as models for the action of RLE on chloroplasts was reported earlier (3, 4, 6). Incubation of either intact algal cells or chloroplasts of higher plants or algae in the presence of fatty acids results in spectral changes comparable to those which occur in the presence of RLE; e.g., in room temperature absorption spectra there is an increase at 674 nm, and decreases at 682 and 705 nm (5).[1] Since at these latter wavelengths absorption is predominantly by aggregated forms of chlorophyll (8, 9), the changes induced by RLE or fatty acids were postulated to result from a deaggregation to monomeric chlorophyll (5).[1] Furthermore, with various chlorophyllous systems, such as chloroplasts, subchloroplast particles, chlorophyll-protein complexes, chlorophyll in solution, etc., these spectral changes were found to be alike,[1] suggesting that the (postulated) deaggregation process occurs at all levels of organization.

While direct action on the chlorophyll chromophore could exclusively be invoked to explain the deaggregation process, some of our earlier observations bring at least one additional option to mind. In reference 3, electron micrographs reveal configurational changes to be occurring in spinach chloroplasts incubated in fatty acids. Since such configurational changes reflect conformational changes (10), the possibility exists that the spectral conversions under consideration arise from an indirectly induced deaggregation in which the exogenous fatty acids act on the protein and/or lipid environment of the chlorophyll molecule, resulting in conformational changes unfavorable to the maintenance of chlorophyll aggregates.

Since Dratz (11, 12), and Houssier and Sauer (13), have shown that aggregate and monomer bands of chlorophyll (both in vivo and in vitro) may be resolved separately in C.D. spectra, we have used C.D. as a tool to investigate the possible occurrence of conformational changes. Our studies do, indeed, indicate deaggregation by such indirect means.

Also included in the present work is an investigation of the changes in infrared (I.R.) spectra which occur when deaggregation is brought about in concentrated solutions of chlorophyll upon addition of ethanol or linolenic acid.

MATERIALS AND METHODS

Chloroplasts from market spinach, isolated according to Spencer (14), were largely intact; i.e., ~85% had chloroplast envelopes (3). Subchloroplast particles were prepared from spinach by means of sonication, according to the method of Anderson and McCarty (15). Particles containing either system I or systems I and II were also isolated from spinach, using digitonin, according to the method of Anderson and Boardman (16). System I chlorophyll-protein complex, isolated from *Phormidium* (and prepared according to the method of Thornber [17]) was very kindly given to us by Dr. R. Gregory (Department of Biochemistry, University of Manchester, Manchester, England). HP-700 chlorophyll-protein complex was isolated from spinach according to the method of Yamamoto and Vernon (18). Crystalline chlorophyll *a* was obtained from Dr. S. S. Brody; its isolation and purification is described

[1] Nathanson, B., and M. Brody. Manuscript in preparation.

in reference 19. The chlorophyll concentrations of chloroplasts and fractions thereof were determined according to the method of Arnon (20). Concentrations of chlorophyll in CCl_4 and in the solvent pair 40% CCl_4–60% hexane were determined by weighing out samples and also, in the case of chlorophyll in CCl_4, from its molar extinction coefficient (21).

The Durrum-Jasco spectropolarimeter (Durrum Instrument Corp., Palo Alto, Calif.) Model J-20 (equipped with a red-sensitive S-20 response photomultiplier tube), used in the present work for C.D. measurements, was made available by the Chemistry Department of Hunter College, as was the Perkin-Elmer Infracord recording I.R. spectrophotometer (Model 137, Perkin-Elmer Corp., Norwalk, Conn.). Visible and UV absorption spectra were made with a Cary model 14-R recording spectrophotometer (Cary Instruments, Monrovia, Calif.). The silica spectrophotometer cells used in conjunction with both the Cary 14-R recording spectrophotometer and the Durrum-Jasco spectropolarimeter were equipped with inserts which permitted the 1 cm path length cell to be reduced to 0.1 or 0.05 mm. In those C.D. experiments in which wavelengths between 350 and 200 nm were monitored, quartz cells of 1 mm path length were used.

Reagent grade linolenic acid was obtained from Sigma Chemical Co., St. Louis, Mo., and was dissolved in absolute ethanol (spectral grade, J. T. Baker Chemical Co., Phillipsburg, N. J.) immediately before each set of experiments. In the series involving crystalline chlorophyll *a*, however, in which the solvent was CCl_4 (spectral grade, J. T. Baker Chemical Co.), stock solutions of linolenic acid were made in CCl_4. In those experiments in which a mixture of hexane and CCl_4 was the solvent used for chlorophyll, the chlorophyll was first dissolved in CCl_4, and then hexane was added so that its final concentration was 60%.

RESULTS

Influence of Linolenic Acid on the States of Chlorophyll in Systems Having a Low Order of Organization

Effect of Ethanol on the State of Aggregation of Chlorophyll a in Solution. In Fig. 1 is seen a C.D. spectrum of 10^{-3} M chlorophyll *a* in carbon tetrachloride (curve given by solid line). With this control there is seen, in the red region of the spectrum, a large positive band with maximum at 676 nm and a small negative band with minimum at 656 nm. Both bands have been attributed to the dimeric form of chlorophyll *a* and indicate the asymmetric interaction of the two chlorophylls of the dimer with one another (11–13); such degenerate interactions (in aggregates) result in a C.D. doublet that crosses zero in the region of the absorption maximum (12). At this chlorophyll concentration, up to ~90% of the total chlorophyll may be in the dimeric form (22). If the same amount of chlorophyll *a* is dissolved in a mixture of 60% hexane–40% CCl_4, additional bands are found in the C.D. spectrum (see Fig. 2, curve given by solid line). On the basis of their work with I.R. and nuclear magnetic resonance (NMR), Katz et al. (23, 24) concluded that solvents such as hexane-CCl_4 favor the formation of an aggregate of higher order than dimers. In Fig. 2 it can be seen that this second, more highly aggregated form of chlorophyll gives rise to a small positive band at 760 nm and a very large negative band at 740 nm. Since there is a reversal in sign close to the center of the absorption band of this highly aggregated form of chlorophyll (see below), it is tentatively

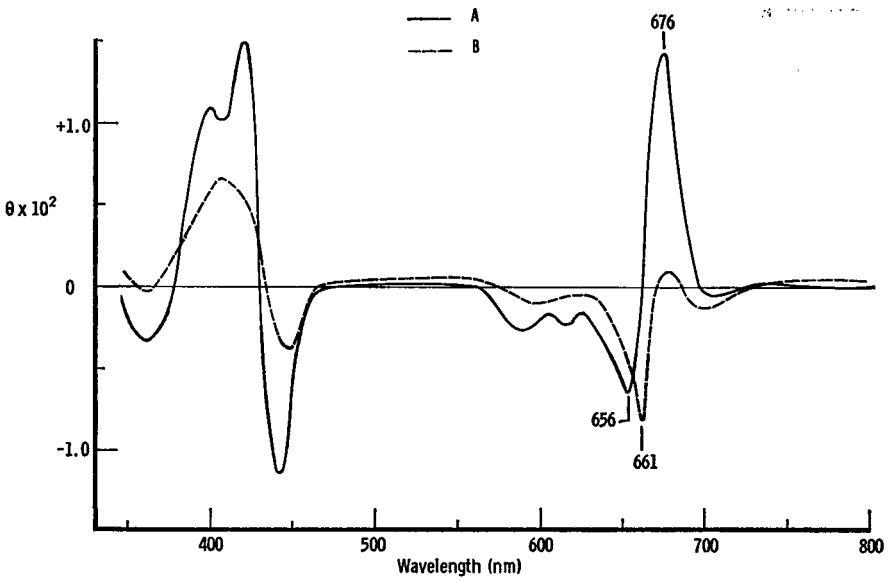

FIGURE 1 (A) C.D. spectrum of chlorophyll *a* dissolved in CCl_4 (solid line), and (B) C.D. spectrum of chlorophyll *a* dissolved in CCl_4, to which had been added 0.02% ethanol (broken line). Chlorophyll concentration = 3×10^{-3} M; path length = 0.005 cm.

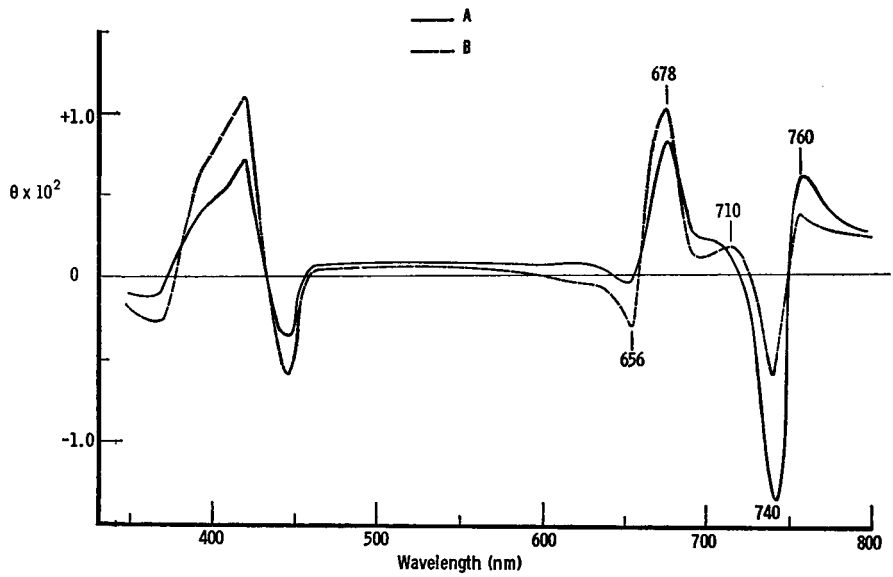

FIGURE 2 (A) C.D. spectrum of chlorophyll *a* dissolved in 40% CCl_4–60% hexane (solid line), and (B) C.D. spectrum of chlorophyll *a* dissolved in 40% CCl_4–60% hexane, to which had been added 0.02% ethanol (broken line). Chlorophyll concentration = 2.5×10^{-3} M; path length = 0.005 cm.

assumed (in analogy with the situation of the chlorophyll *a* dimer in CCl_4) that these bands represent a doublet; it will be seen that constituent bands of such doublets respond in parallel fashion to a variety of situations.

A comparison for the two solvent systems can be made between C.D. data and data for absorption in the visible region of the spectrum. In the case of chlorophyll *a* dissolved in CCl_4 there is observed in the absorption spectrum a large band with maximum at 668 nm. In the absorption spectrum of the same amount of chlorophyll dissolved in a mixture of hexane-CCl_4, the peak (at 667 nm) is much reduced (OD down ~50%) and an additional (small) absorption band with maximum at ~745 nm is present. Introduction of ethanol (at a final concentration of 0.02%) to the chlorophyll in the solvent pair results in the disappearance of the 745 nm peak; this disappearance is accompanied by marked increase in the absorption peak at 666 nm. If one examines the corresponding C.D. spectrum (Fig. 2, curve given by dashes) one notes that upon addition of 0.02% ethanol to the chlorophyll in 40% CCl_4–60% hexane, there is a decrease (less positive) in θ (ellipticity) at 760 nm of 4 m° (millidegrees) and a large decrease (less negative) in θ of 12 m° in the 740 nm negative band; these decreases are accompanied by increases (in the dimeric form of chlorophyll) at 656 and 678 nm. Thus, in the presence of ethanol, which is known to deaggregate chlorophyll (12, 13, 22) there is conversion of highly aggregated chlorophyll to chlorophyll dimers.

As shown earlier in the C.D. work of Houssier and Sauer (13), at a chlorophyll *a* concentration of 2×10^{-5} M in CCl_4, addition of 0.5% ethanol (final concentration) results in deaggregation of the dimeric form of chlorophyll *a* to its monomeric form. From Fig. 1 (curve given by dashes) it may be seen that even[2] addition of ethanol at a final concentration of 0.02% yields a marked reduction in the intensity of the positive C.D. band that has its maximum at 676 nm; the negative band at 661 nm (corresponding to the monomeric form of chlorophyll *a*) is now predominant. In the absorption spectrum which results when 0.02% ethanol is added to chlorophyll in CCl_4, there is evident an increase of the monomer band and a diminution of absorption in the region around 682 nm. We will assume that reagents which bring about effects on C.D. similar to those brought about by ethanol are causing deaggregation.

Effect of Linolenic Acid on Chlorophyll a in Solution. The change in C.D. which results from addition of fatty acid to chlorophyll in CCl_4 is similar to that occurring upon addition of ethanol, insofar as there is strong decrease of the C.D. band at 678 nm; however, there is only a small decrease in the negative band at 656 nm. A slight inflection on this latter band at ~660 nm may possibly represent mon-

[2] The concentration of ethanol used here to cause deaggregation of chlorophyll *a* in CCl_4 was much lower than that used by Houssier and Sauer (13). We did not take special steps to dry our reagents, and it is possible that trace amounts of water permitted deaggregation to occur at lower concentrations of ethanol. (See, for example, J. J. Katz, K. Ballschmiter, M. Garcia-Morin, H. H. Strain, and R. A. Uphaus. 1968. *Proc. Natl. Acad. Sci. U.S.A.* **56**:1377.)

omer. In the visible absorption spectrum of chlorophyll *a* in CCl_4, the addition of linolenic acid results in a maximal decrease in absorption at 682 nm (the peak of aggregated chlorophyll), a decrease in absorption of the band with maximum at 667 nm, and the appearance of two discrete bands with maxima at 508 and 538 nm; the latter two bands correspond to absorption by pheophytin. Deaggregation by ethanol does not result in pheophytinization of chlorophyll *a*. Our examination of infrared spectra of (*a*) chlorophyll *a* in CCl_4, (*b*) chlorophyll *a* in CCl_4 + 0.02% ethanol, and (*c*) chlorophyll *a* in CCl_4 + 1.3×10^{-3} M linolenic acid reveals that the 1650 cm^{-1} band of dimeric chlorophyll (23) disappears upon addition of either linolenic acid or ethanol. This disappearance is associated with an increase in the I.R. band at 1700 cm^{-1}. The 1700 cm^{-1} band, assigned to the ketone of ring V in chlorophyll (23), increases in polar solvents and also when deaggregation occurs (23).

From the experiments made with chlorophyll *a* dissolved in CCl_4 (in which there exist no aggregates of higher order than dimers), it is not possible to determine with certainty whether linolenic acid, in addition to bringing about conversion into pheophytin, also brings about deaggregation. The ability of linolenic acid to cause deaggregation of chlorophyll can be seen, however, in the solvent pair CCl_4-hexane (see Fig. 3, curve given by dashes). A comparison of this C.D. figure with the control

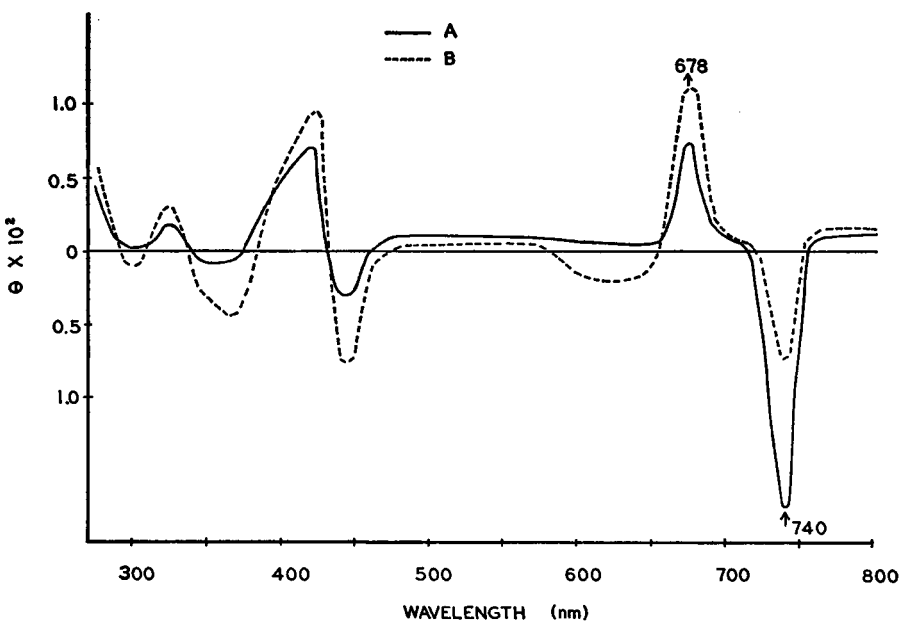

FIGURE 3 (A) C.D. spectrum of chlorophyll *a* dissolved in 40% CCl_4–60% hexane (solid line), and (B) C.D. spectrum of chlorophyll *a* dissolved in 40% CCl_4–60% hexane to which had been added 1.3×10^{-3} M linolenic acid (broken line). Chlorophyll concentration = 2.5×10^{-3} M; path length = 0.005 cm.

(Fig. 3, curve given by solid line) reveals that linolenic acid is capable of changing the highly aggregated species of chlorophyll (absorbing at 740 and 760 nm) into dimers absorbing at 678 and 656 nm (some of the absorption in the latter band may be due to monomers). Although the C.D. spectra which result from addition of either linolenic acid or ethanol to chlorophyll in CCl_4-hexane are essentially identical, visible absorption spectra reveal an important difference between the two. The similar deaggregation (decreases at 745 nm and increases at 668 nm) is accompanied, in the case of linolenic acid, by the appearance of a pheophytin band at 535 nm.

Effect of Linolenic Acid on the State of Aggregation of Chlorophyll in System I Chlorophyll-Protein Complexes. In the case of these chlorophyll-protein complexes, the chlorophyll concentration was calculated to be 1.5×10^{-5} M using the extinction coefficient given by Thornber (17). In Fig. 4 are the C.D. spectra of system I chlorophyll-protein complexes, (*a*) suspended in 0.05 M Tris buffer, pH 8.0, to which was added ethanol at a final concentration of 0.5% (solid line), and (*b*) suspended in buffer to which was added linolenic acid (in ethanol) to yield final concentrations of 1.5×10^{-4} M fatty acid and 0.5% ethanol (curve given by dashes). Upon addition of linolenic acid, one observes a disappearance of the (presumably doublet) bands at 662 and 676 nm, as well as a marked decrease of the band at 690 nm; a negative band appears at 671 nm.

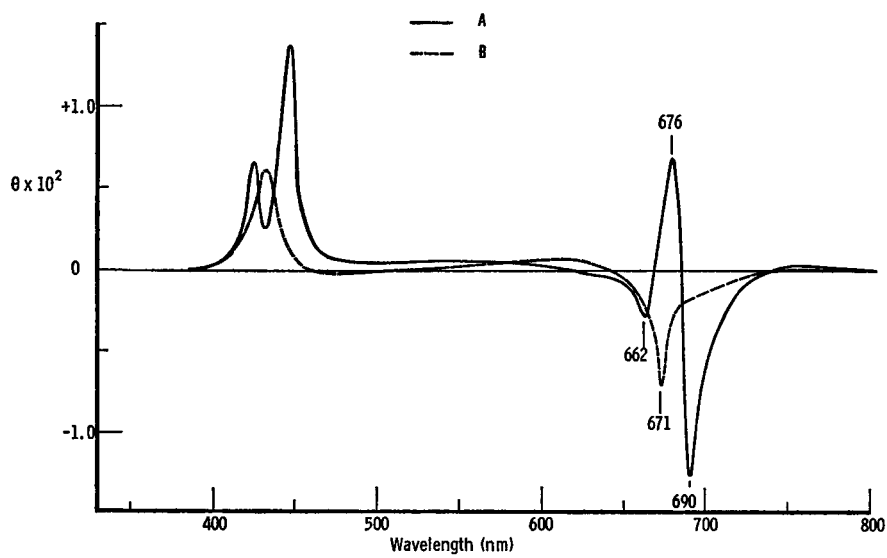

FIGURE 4 C.D. spectra of (A) system I chlorophyll-protein complexes (17) suspended in 0.05 M Tris-HCl buffer, pH 8.0 (solid line) to which had been added 0.5% ethanol, and (B) of chlorophyll-protein complexes suspended in Tris-HCl buffer to which had been added 1.5×10^{-4} M linolenic acid (broken line). Chlorophyll concentration = 2.5×10^{-5} M; path length = 1 cm.

Effect of Linolenic Acid on the State of Aggregation in HP-700 Chlorophyll-Protein Complexes. In Fig. 5 are seen the C.D. spectra of control HP-700 complexes suspended in 0.40 M sucrose + 0.02 M Tricine buffer, pH 8.0 + 0.01 M NaCl (STN solution) (solid line), and HP-700 complexes suspended in STN buffer to which linolenic acid had been added (curve given by dashes) at a final concentration of 2.5×10^{-5} M (equimolar with the chlorophyll concentration). In the control, four bands in the red region of the spectrum are evident. As in the case of chlorophyll *a* in CCl_4-hexane, we assume that the small positive band at 755 nm and the large negative band at 736 nm constitute a doublet. (We have observed that when our HP-700 preparations are lyophilized and then resuspended in buffer, both these bands are absent. A similar lability of these bands is noted if HP-700 is prepared from lyophilized chloroplasts.) The remaining two bands consist of a small, rather broad, positive band with maximum at about 695–700 nm and a large negative band with minimum at 660 nm. The negative band at 660 nm could either be characteristic of HP-700 monomer or be due to contamination by solubilized chlorophyll. In the presence of linolenic acid (Fig. 5, curve given by dashes) there are seen decreases in the 695–700, 705, 736, and 755 nm bands.

HP-700 has been reported to be a relatively pure (and lipid-free) preparation of reaction center chlorophyll-protein complex (18). Therefore, we used HP-700 to determine whether deaggregation can occur indirectly, from conformational change of the protein. In the presence of 8 M urea, as in the presence of linolenic acid,

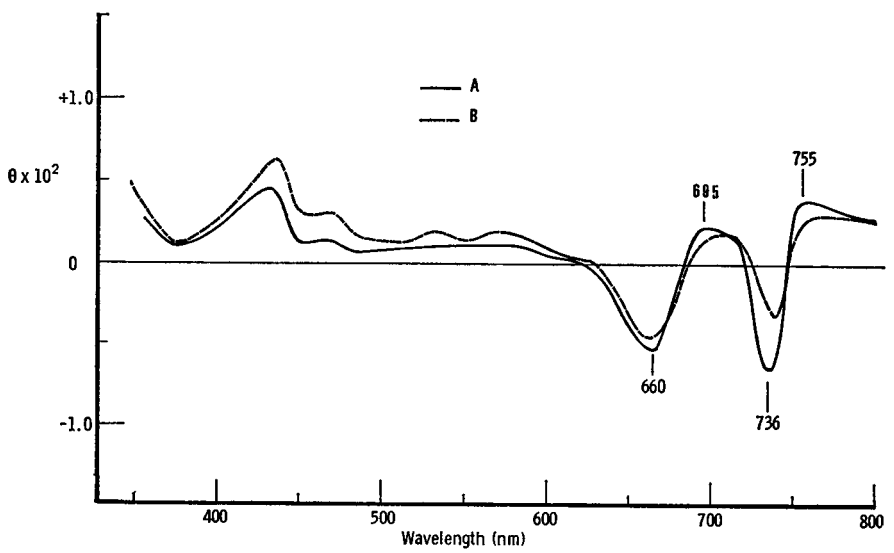

FIGURE 5 C.D. spectra (A) of HP-700 chlorophyll-protein complexes suspended in STN buffer, pH 8.0 (solid line), and (B) of HP-700 complexes in STN buffer, to which had been added 2.5×10^{-5} M linolenic acid (broken line). Chlorophyll concentration = 2.5×10^{-5} M; path length = 1 cm.

there occurs a very large decrease at 736 nm and also decreases at 755 and 695–700 nm, resulting in the production of a C.D. spectrum characteristic of the monomeric form of chlorophyll. In the presence of urea, as in the presence of linolenic acid, there are increases on the intensities of the near ultraviolet C.D. bands; such increases, in the case of other proteins, have been attributed to conformational changes (25). It was noted with both linolenic acid and urea that the changes in C.D. in the ultraviolet begin at lower concentrations than do the changes in the red region. Partial removal of the urea (by cellulose sac dialysis or Sephadex chromatography) did not always result in a return to the control spectrum, for reasons not apparent at the present time. Pheophytin formation was not detected when fatty acids were added to chlorophyll-protein complexes, or for that matter, to any chlorophyllous system above the level of chlorophyll in solution.

Influence of Linolenic Acid on the State of Chlorophyll in Membranous Systems

Effect of Linolenic Acid on Digitonin-Isolated Particles Containing System I. In Fig. 6 are seen C.D. spectra of (*a*) system I particles suspended in 0.01 M KCl, 0.05 M Tris-HCl buffer, pH 7.8 (solid line), and (*b*) system I particles incubated for a period of 30 min at 25°C in buffer containing 4×10^{-5} M linolenic acid (curve given by dashes). In the control there is seen a negative band at 679 nm, a positive band at 662 nm (presumably a chlorophyll *a* dimer doublet, in analogy with the in vitro situation), and a negative band at 643 nm (chlorophyll *b*).[3] Incubation in linolenic acid results in decrease in intensities of all three bands (these decreases are accompanied by shifts in maxima from 662 to 659 nm, and from 679 to 674 nm).

Effect of Linolenic Acid on Particles Containing Both Systems I and II. The spectrum of the control 10,000 *g* fraction of digitonin-isolated particles (which contain both systems I and II) is similar in the red region of the spectrum to the digitonin-isolated particles containing only system I, with the difference that in the case of the particles also containing system II, the C.D. band of chlorophyll *b* at 650 nm is stronger (about 1.4 times the ellipticity found at 678 nm). The chlorophyll *b* band of the control subchloroplast particles (obtained by sonication) in STN buffer, pH 7.8 (Fig. 7 A, solid line), is also intense, i.e., bears the same relationship to the 678 nm band as it does in the case of the digitonin-isolated particles containing both systems I and II. We note that an interesting situation exists in regard to the 678 nm band in control subchloroplast particles (obtained by sonication). In contrast with the 10,000 *g* digitonin-isolated particles, in which the intensity of the 678 nm band is 1.11 times the intensity of the 663 nm band, the sub-

[3] Chlorophyll determinations for intact chloroplasts, digitonin-isolated 10,000 *g* pellets (systems I and II), and digitonin-isolated 144,000 *g* pellets (system I) revealed chlorophyll *a*/chlorophyll *b* concentration ratios of 3.01, 2.32, and 5.19, respectively; in the system I particles the 643 nm chlorophyll *b* band is relatively weak compared with the chlorophyll *a* bands.

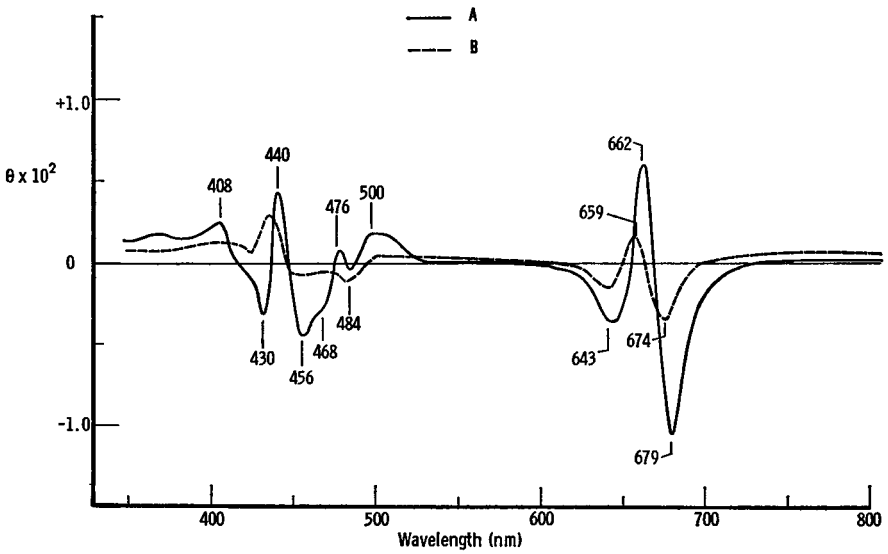

FIGURE 6 C.D. spectra (A) of digitonin-isolated system I particles suspended in 0.01 M KCl, 0.05 M Tris-HCl buffer, pH 7.8 (solid line), and (B) of the above particles after 10 min of incubation in buffer to which had been added 4.5×10^{-5} M linolenic acid (broken line). Chlorophyll concentration = 2×10^{-5} M; path length = 1 cm.

chloroplast particles (obtained by sonication) have only 0.75 times the intensity. This decreased negativity at 678 nm is accompanied by a second (new) minimum at 690 nm (see Fig. 7). These two minima form troughs around a more positive inflection at 683 nm; a possible interpretation of these findings will be considered in the discussion in relation to the data obtained with control chloroplasts (see below).

The blue region of the control 10,000 *g* digitonin-isolated fraction (systems I and II) is similar to that of the control spectrum of the subchloroplast particles (obtained by sonication) (solid line, Fig. 7). The band at 486 nm, which is negative in system I particles, has positive ellipticity in particles which contain both systems. Furthermore, the bands at 482, 486, and 500 nm, which exhibit negative ellipticity in C.D. spectra of barley "quantasomes" (see Dratz et al., reference 11), possess positive ellipticity in our subchloroplast and digitonin-isolated systems I and II particles. That these differences in sign are *not* due to differences in polarity can be inferred by observing that there exists no mirror-image relationship between the bands. One possible explanation for the difference in sign is the increase in light scattering which accompanies the greater structural organization found in those particles that exhibit positive ellipticity in the blue region of the spectrum. We have found (experiments to be reported elsewhere) that when the turbidity of suspensions of chlorophyll-containing systems (e.g., system I digitonin-isolated particles) is enhanced (either by increasing the concentration of the biological material, or by

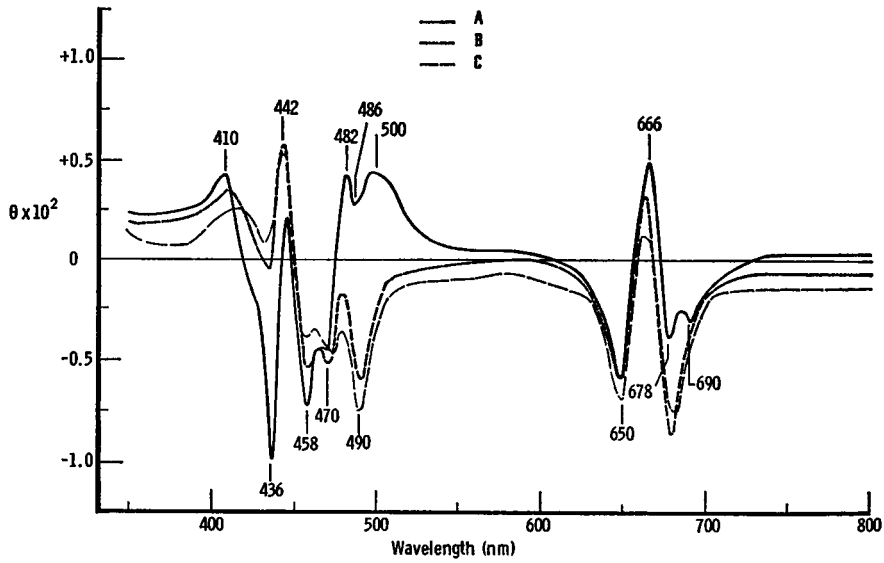

FIGURE 7 C.D. spectra of (A) subchloroplast particles (obtained by sonication) suspended in STN buffer, pH 7.8 (solid line); (B) subchloroplast particles after 5 min of incubation in STN buffer to which had been added 2.1×10^{-4} M linolenic acid (line given by short dashes); (C) subchloroplast particles after 1½ hr of incubation in STN buffer to which had been added 2.1×10^{-4} M linolenic acid (line given by long dashes). Chlorophyll concentration = 2.1×10^{-5} M; path length = 1 cm.

introducing inert material, such as talc) there occurs "interconversion" of negative to positive ellipticities, as well as strong shifts in band peaks.

Upon 5 min incubation of subchloroplast particles in STN buffer containing 2.1×10^{-4} M linolenic acid (see Fig. 7 B, short dashes), one observes an increase and narrowing of the C.D. band at 678 nm, and disappearance of the 690 nm band; these modifications are accompanied by changes in the C.D. bands at shorter wavelengths (the latter changes also occur in the case of fatty acid–treated, digitonin-isolated particles, containing both systems I and II). With longer incubation (see Fig. 7 C, long dashes) all bands decrease in intensity, but no further modification in sign occurs. (That the observed change in C.D. sign results from a reduction in light scattering is in good agreement with the findings of Molotkovsky and Zheskova [26] who, in 1966, reported that treatment of [nonintact] chloroplasts with fatty acids yields a decrease in the scattering of visible light by these chloroplasts.)

Effect of Linolenic Acid on Spinach Chloroplasts. In Fig. 8 A (solid line) may be seen a C.D. spectrum of largely intact spinach chloroplasts (chlorophyll concentration = 1.3×10^{-5} M) in STN buffer, pH 7.8. We note, with this control, the presence of the 664 nm band, but not its corresponding (doublet) negative 679 nm band. The latter is most likely obscured by the negative 672 nm band. This

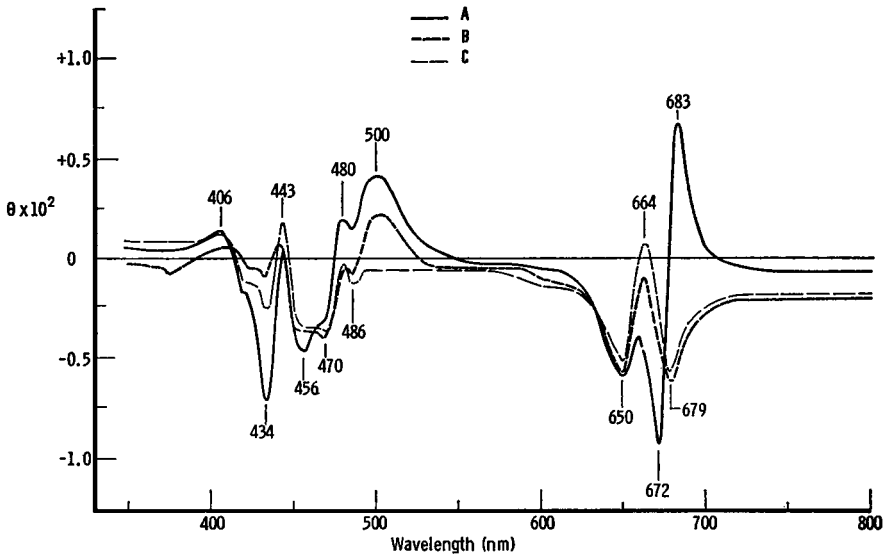

FIGURE 8 C.D. spectra of (A) chloroplasts suspended in STN buffer, pH 7.8 (solid line); (B) chloroplasts after 10 min of incubation in STN buffer to which had been added 1.1 × 10^{-4} M linolenic acid (line given by short dashes); (C) chloroplasts after 1 hr of incubation in STN buffer to which had been added 1.1 × 10^{-4} M linolenic acid (line given by long dashes). Chlorophyll concentration = 1.3 × 10^{-5} M; path length = 1 cm.

672 nm band, and the very intense positive band at 683 nm,[4] probably constitute yet another doublet (the zero crossover point between them corresponding to the absorption maximum seen at 678 nm in control chloroplasts; additionally, they behave similarly to treatment by fatty acids). The C.D. band with maximum at 664 nm, strongly positive in all other membranous systems, is now only a positive inflection between the 650 and 672 nm negative peaks. The blue region of control spinach chloroplasts is not dissimilar to that of control subchloroplast particles (obtained by sonication).

When chloroplasts are incubated for 10 min at 22.5°C, in the presence of linolenic acid at a fatty acid/chlorophyll ratio of 10 (Fig. 8 B), the 672 and 683 nm bands disappear, the 664 nm band becomes more positive, and the 679 nm band becomes evident. These latter changes continue with further incubation (Fig. 8 C).

DISCUSSION AND CONCLUSIONS

The deaggregating effects of ethanol on chlorophyll dimers in CCl_4 were shown earlier (13) in C.D. and visible absorption studies, as well as in I.R. studies (23).

[4] With chloroplasts, wide variations in C.D. spectra are possible. For example: a sample containing 13 μg of chlorophyll/ml has bands with maxima at 683 and 672 nm; these bands, in suspensions containing 30 μg of chlorophyll/ml, are shifted to 688–690 and 676–678, respectively; maxima of the other bands do not shift in wavelength, but their intensities, relative to each other, do change.

In the present work we show, in C.D. and visible absorption spectra, the existence of dimers and higher aggregates of chlorophyll in CCl_4-hexane. (The presence of the latter is in agreement with the I.R. and NMR data of Katz et al. [23, 24].) Additionally, we show that ethanol has the ability to convert the larger aggregates into dimers.

Since we have observed that introduction of linolenic acid to chlorophyll in CCl_4 brings about changes in C.D. similar to those brought about by the addition of ethanol, we conclude that there is also a direct action by this fatty acid on the chlorophyll chromophores. Although decrease in the 1650 cm^{-1} I.R. band (which accompanies deaggregation) *is* noted upon addition of fatty acid, increase at 1700 cm^{-1} is difficult to discern directly because of strong absorption by linolenic acid at this wavelength. From absorption studies in the visible region of the spectrum we detected the production of pheophytin when linolenic acid is added to chlorophyll in CCl_4; no pheophytin was produced during deaggregation by ethanol. (Trosper and Sauer [27] have demonstrated that glycolipids are capable of breaking up chlorophyll dimers either in mixed monolayers or in CCl_4.) In contrast to the single solvent, in which it is difficult to determine whether deaggregation occurs, experiments with chlorophyll in the solvent pair CCl_4-hexane (in which aggregates larger than dimers occur) allow one to observe directly that addition of fatty acids results not only in conversion to pheophytin but also in formation of dimers from higher chlorophyll aggregates. (Since a band with maximum at 695 nm is present [Norris, et al., reference 28] neither in visible absorption nor in C.D. spectra, it is unlikely that aggregates of pheophytin are formed.)

Regarding the situation of chlorophyll in solution, we see that deaggregation is brought about by direct action on the chromophore. In the case of chlorophyll attached to protein, we have shown that another mechanism for deaggregation by fatty acids occurs, namely, a conformational change of the protein, secondarily bringing about this conversion.

We turn attention now to our C.D. data on control chlorophyll-protein complexes, for these are also of interest. It may be recalled that the system I complexes, prepared according to Thornber, have, in addition to the bands at 662 and 676 nm, another band at 690 nm. We wish to tentatively assign the first two bands to a dimer doublet, in analogy with the doublet observed when chlorophyll is dissolved in CCl_4. The 690 nm band may arise from another aggregate, possibly a different type of dimer (29). In contrast, the C.D. spectrum of HP-700 chlorophyll-protein complexes (which have demonstrable P-700 activity [18]), has aggregate bands at 695–700 and 736 and 755 nm. The latter two bands may also be considered to be a doublet (see Results); their state of chlorophyll aggregation is probably very high. This doublet strongly resembles the one seen in our C.D. spectrum of chlorophyll in CCl_4-hexane, in which aggregates larger than dimers occur. The C.D. bands of the chlorophyll-protein complexes at 690 or 695–700 nm probably represent dimers. (In some of our HP-700 preparations, in which the concentration of aggre-

gate species was greatly increased [see footnote 5], there was no evidence of monomer absorption [i.e., no band at 660 nm] in the C.D. spectrum.) Quinlan (30), as well as Sherman and Fujimori (31), has shown that aggregates larger than dimers can occur in solvents such as dioxane and aqueous formamide; these two groups of workers have suggested that dimers (absorbing at 705 nm) may pass through intermediate stages of aggregation (e.g., with absorption maxima at 715 nm) in the formation of higher order aggregates (absorbing at 740 nm). Dratz et al. have given C.D. and absorption spectra for microcrystals of chlorophyll (11) in isooctane; these have an absorption maximum at 745 nm and C.D. maxima at 718 (positive band) and 751 nm (negative band). It is also interesting to note that, earlier, Brody and Brody (32) attributed the shifting (fluorescence and absorption) maxima in greening *Euglena* to transitional populations of chlorophyll aggregates of increasing size.

Now we turn our attention to the effect of linolenic acid on chlorophyll-protein complexes. In both types of complexes used in the present work, the long wavelength C.D. bands (aggregates) disappear. In HP-700 complexes, only the monomer band, with maximum at 660 nm, can be detected after addition of fatty acid. In the chlorophyll-protein complexes prepared according to Thornber, a band grew in at 671 nm. This band, which replaced the negative 660 and positive 676 nm (presumed dimer doublet) bands, as well as the negative 690 nm (separate dimer) band, may be (*a*) a resultant of incomplete deaggregation, consisting largely of monomer (absorbing at 660 nm) shifted to longer wavelengths by some residual absorption at 680 nm, or (*b*) in analogy with the situation observed when ethanol is added to chlorophyll in CCl_4, pure monomer, absorbing maximally at 671 nm.

In 1961 (33; see also 34), M. Brody postulated that chlorophyll dimers and monomers are attached periodically to protein helices, and that conformational changes in the protein could bring about changes in proportion of these chlorophyll species. That the linolenic acid–induced deaggregation of protein-containing chlorophyllous systems arises primarily from a conformational change is suggested by the similarity of results obtained by treatment with urea.

The simplest membranous chlorophyll-containing systems with which we worked were digitonin-isolated system I particles. In the control for these particles, the C.D. bands differ from both those of the (nonmembranous) chlorophyll-protein complexes and those of chlorophyll in solution, in one obvious regard. In the nonmembranous systems, the C.D. bands are opposite in sign to the corresponding C.D. bands of the system I digitonin particles. Indeed, all membranous systems resemble each other in this respect. The additional structural array brought about

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[5] In preparing HP-700 chlorophyll-protein complexes according to Yamamoto and Vernon (18), chloroplasts which contain 5 mg of chlorophyll are homogenized in 16 ml of Triton X-100. In addition to such complexes, we prepared others, especially enriched in aggregate, by using chloroplasts containing 10 mg of chlorophyll and homogenizing them in 16 ml of Triton X-100.

by organization into membranes seems to confer, on chlorophyll, the kind of configuration which results in this observed mirror-image relationship.

In subchloroplast particles (which additionally contain system II, and are more highly structured than system I digitonin-isolated particles), it may be recalled that in the blue region of the spectrum, where light scattering is greatest, C.D. bands were found to have positive ellipticity. A somewhat similar "distortion" of C.D. bands (in the ultraviolet) was ascribed by Menke (35) to light scattering by thylakoid suspensions. It is interesting to note that addition of linolenic acid to subchloroplast particles not only results in deaggregation, but confers negative ellipticity on the C.D. bands in the blue region of the spectrum, presumably by decreasing light scattering (26). (See also the work of Urry and Krivacic [36], in which is discussed the effect of light scattering on C.D. spectra of poly-L-glutamic acid suspensions.)

The C.D. spectra of our spinach chloroplasts, have, in addition to the bands reported earlier by Dratz (11, 12), a large positive band at 683 nm and a large negative band at 672 nm; these may represent an aggregate doublet. Chloroplasts used in the present work were prepared according to the method of Spencer (14), in which laminae are chopped with razor blades, resulting in production of chloroplasts which are ~85% whole (i.e., have intact chloroplast envelopes). We observed that when no special precautions are taken to preserve the integrity of the chloroplasts, e.g. when they are isolated by means of a Waring Blendor, these long wavelength bands of aggregated chlorophyll are lost. In subchloroplast particles, we observed two minima troughs (at 678 and 690 nm) around a positive inflection at 683 nm; one might expect such a condition to obtain where there remains only a residual quantity of the form of chlorophyll absorbing at 683 nm, and this absorption is superimposed upon a much stronger negative band at ~679 nm.

Incubation of chloroplasts in linolenic acid results in disappearance of the C.D. bands with maximum at 683 and 672 nm, appearance (disclosure?) of the 679 nm band, and increase in intensity of the 664 nm band. The work of Thomas and Van Hardeveld (37) is pertinent to our observations; they reported that treatment of spinach chloroplasts with lipase results in decrease of dichroism at 682 nm (and explained their results as being due to release of chlorophyll-containing lipoprotein particles from the lipid matrix).

Not only are the lamellae of chloroplasts particularly rich in galactolipids, but these galactolipids may contain as much as 96% of their fatty acids in the form of linolenic acid (38). As Benson (38) has suggested, it is likely that galactolipids and linolenic acid in chloroplasts exist in some dynamic equilibrium between the bound and free state. In their free state, they may well act to bring about conformational changes (such as those seen here) in the proteins to which chlorophyll is attached, causing shifts in populations of system I and system II pigments.

It was proposed earlier (5, 6) that a molecule similar in action to the protein fraction of RLE has the role, in vivo, of regulating the transfer of electrons and

distribution of energy between the two photosystems. Although several substances (3, 4) other than long-chain fatty acids can serve as models for the action of the protein fraction of RLE, the latter are present in high concentrations in chloroplasts and may well serve to demonstrate the mechanism of action of the effector molecule in vivo.

This work was submitted by B. Nathanson in partial fulfillment of the requirements for the Ph.D. degree at the City University of New York

Dr. Brody was supported in part by a City University of New York Faculty Research Grant.

Received for publication 21 May 1971.

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