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IN VITRO PREPARATION AND CHARACTERIZATION OF A 700 nm ABSORBING CHLOROPHYLL-WATER ADDUCT ACCORDING TO THE PROPOSED PRIMARY MOLECULAR UNIT IN PHOTOSYNTHESIS*

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SUMMARY

1. This study characterizes chlorophyll *a*-H_2O adducts in vitro in order to establish their generic relationship to the recently proposed [15, 18–20, 31] primary molecular adduct in photosynthesis. The effects of water titration and temperature on the absorption, fluorescence, excitation, and redox properties of the various in vitro chlorophyll *a* aggregate species are investigated.

2. From fluorescence measurements, we conclude that the driest chlorophyll *a* sample contains an equimolar amount of water. This conclusion is consistent with earlier experimental work [2, 3, 14, 17, 31], and clarifies the origin of the controversial [15] Katz model [14] of chlorophyll *a*-H_2O interactions.

3. With increasing water concentration or as the temperature is lowered below room temperature, the *A*-663 monohydrate chlorophyll $a \cdot H_2O$ (species absorbing at 663 nm) is favored at the expense of the *A*-678 anhydrous aggregate according to the equilibrium $2H_2O$ + chlorophyll $a_2 \rightleftarrows 2$ chlorophyll $a \cdot H_2O$. Under excess water conditions, *A*-663 is converted to *A*-743 (chlorophyll $a \cdot 2H_2O)_n$.

4. On slow sample cooling to $T \lesssim 200$ °K, we observe the growth of *A*-700 at the expense of *A*-663. There is a direct correspondence between the increasing (decreasing) absorption by *A*-700 (*A*-663) and increasing (decreasing) fluorescence at 720 nm (664 nm).

5. It is concluded that *A*-700 is most probably the dimer participating in the equilibrium 2 chlorophyll $a \cdot H_2O \rightleftarrows$ (chlorophyll $a \cdot H_2O)_2$. The *A*-700 band consists of two exciton components (separated by ≈ 280 cm^{-1}) that are interpretable in terms of the dimeric origin of *A*-700.

6. The deconvoluted *A*-700 absorption spectrum and the excitation spectrum of the 720 nm fluorescence are compared with the light-minus-dark spectra of *P*-700.

7. It is found that *A*-700 is reversibly bleached by I_2 ($E_0 = 0.54$ V). The significance of this observation is discussed in terms of the redox properties of monomeric chlorophyll *a* and *P*-700.

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INTRODUCTION

In 1948, Livingston reported that hydrated chlorophyll *a* fluoresces upon light excitation whereas anhydrous chlorophyll *a* in a hydrocarbon solvent is practically nonfluorescent [1]. In a detailed study, Livingston et al. [2] showed that the activation of fluorescence in a nonpolar chlorophyll solution by the controlled addition of polar solvents provides a convenient means of estimating trace quantities of adventitious water [2]. It has long been recognized [1–3] that it is difficult to purify in vitro solutions of chlorophyll *a* sufficiently to reduce the water content to a level lower than that of the order of the chlorophyll concentration.

At room temperature, in vitro chlorophyll *a* solutions exhibit a fluorescence maximum at 665 nm. At chlorophyll *a* concentrations $C_0 \gtrapprox 10^{-5}$ M, a long-wavelength absorption band in the vicinity of 700 nm (*A*-700) with a corresponding fluorescence band in the 720–740 nm region becomes evident upon sample cooling [4–10]. This long-wavelength fluorescence has generally been attributed to some uncharacterized aggregate states of chlorophyll *a* [4–10]. At $C_0 \approx 10^{-6}$ M, a low-temperature aggregate fluorescence band has not been observed [11].

A number of investigators [12–14, 32] have reported the preparation of a red-absorbing (in the 740–745 nm region) crystalline form of hydrated chlorophyll *A*-743 when films or concentrated (10^{-3}–10^{-2} M) Nujol mulls of chlorophyll are allowed to become saturated with water. The most probable structure for the *A*-743 is a polymeric aggregate of the chlorophyll dihydrate chlorophyll $a \cdot 2H_2O$ [15–17]. During the present decade, the question of chlorophyll-water interactions has become one of increasing interest. In 1974, it was proposed [15, 18–20, 31] that a probable structure for the photoactive aggregate *P*-700 in photosystem I [21, 22] in plant photosynthesis is a symmetrical dimeric chlorophyll *a*-water adduct in which the two parallel chlorin rings of the chlorophyll *a* molecules are held in position by two H_2O molecules through the formation of two reciprocal ring V ester C=O · · · HO · Mg(H) linkages.

The basic premise of the present study is that if the proposed [15, 18–20] (chlorophyll $a \cdot H_2O)_2$ adduct does in fact correspond to the Photosystem I reaction center pigment *P*-700 [21], it should be possible to synthesize in vitro a hydrated chlorophyll dimer whose long-wavelength absorption band occurs in the vicinity of 700 nm. We report the results of a series of experiments dealing with the aggregation behaviour of chlorophyll *a*-H_2O adducts as a function of water concentration C_w and temperature. We show that the frequently observed [9, 10] appearance of *A*-700 is a result of chlorophyll $a \cdot H_2O$ aggregation. We describe the experimental conditions under which *A*-700 becomes the predominant component and compare the optical (absorption, fluorescence and excitation) and redox properties of *A*-700 with those of *P*-700 [21]. From a consideration of multiple chlorophyll *a*-H_2O equilibria, we show that the observed *A*-700 effects are consistent with the equilibrium 2 chlorophyll $a \cdot H_2O \rightleftarrows$ (chlorophyll $a \cdot H_2O)_2$.

Experimental procedure, materials and methods

Chlorophyll *a* was extracted from spinach and purified using standard procedures [23]. The final purification was performed by removing the chromatographed chlorophyll *a* zone from the last sugar column, eluting it with 5 % ethylene hydroxide

in *n*-pentane, washing the ethylene hydroxide from the *n*-pentane solution with water, and recrystallization at dry ice temperature. The precipitate was filtered with a pressurized Millipore filtration apparatus and stored under vacuum. Absorption spectra of the purified chlorophyll *a* in anhydrous ether (AR grade) solutions had absorption maxima at 4286±1 Å and 6604±1 Å with blue/red absorbancy ratios of 1.29±0.01. These data have been consistently reproduced using the above recrystallization technique, and they agree with established criteria for chlorophyll *a* purity [24].

Samples for the optical spectroscopic study were prepared as follows. From a fresh CCl_4 stock solution, whose chlorophyll *a* concentration was determined spectrophotometrically by diluting an aliquot ($\approx$ 0.25 ml) with anhydrous ether (10 ml), a predetermined amount of sample was transferred quantitatively under dry nitrogen atmosphere into a cylindrical pyrex sample tube (internal diameter $\approx$ 0.8 cm) that had previously been evacuated and torched to eliminate traces of absorbed water. (The nitrogen gas was dried by passage through $CaSO_4$, KOH, and a liquid nitrogen trap.) This solution was evaporated under reduced pressure and the solid chlorophyll *a* which deposited on the walls of the vessel, was heated under vacuum ($\approx$ 1 μm) at 75 °C for time periods up to 24 h. From room-temperature fluorescence intensity measurements (see below), using the procedure after Livingston et al. [2], it was found that samples heated for periods longer than 30 min at this temperature do not show any appreciable changes in the amounts of adventitious water. The two component solvents, methyl cyclohexane and *n*-pentane, were rigorously dried by distillation over $LiAlH_4$ in a dry nitrogen atmosphere. Dry nitrogen was admitted to the vacuum line and the solvent, a 1 : 1 mixture of rigorously dried methyl cyclohexane and *n*-pentane, was transferred through vacuum-tight septa using syringes. A stock solution (suspension) of high purity water in methyl cyclohexane was prepared by sonication. Aliquots of this solution were added along with the solvents to obtain solutions of known added water and chlorophyll *a* concentrations. The samples were then frozen at liquid nitrogen temperature, sealed under vacuum, and finally sonicated for 1 h at 0 °C. Samples containing $2 \cdot 10^{-6}$–10^{-4} M chlorophyll *a* and 10^{-4}–$2 \cdot 10^{-2}$ M added water were prepared and employed in the study. During the entire duration of this study, the samples were stored in the dark at 77 °K. On completing the experiments, the sample tubes were opened, the solvent was evaporated, and anhydrous ether solutions of the solid residue were prepared. Absorption spectra of these samples established, by agreement with the purity criteria for the starting material, that there was no detectable photodegradation during the course of the experiments.

All absorption spectra were recorded on a Cary 14R spectrophotometer with no reference beam compensation. Fluorescence and excitation spectra were obtained with a high intensity tungsten-halogen lamp, a 0.25 meter Jarrell Ash monochromator, a Spex 0.75 meter double monochromator and appropriate infrared and visible filters. The analyzing system has been described elsewhere [25]. Owing to the spectral band widths in absorption and fluorescence, peak positions are accurate only to within ±0.5 nm. In the temperature dependent experiments, the sample temperature was adjusted by regulating the flow rate of cold nitrogen gas around the sample tube situated in an optical Dewar. The 1 : 1 methylcyclohexane and *n*-pentane glass transition temperature was measured to be approximately 90 °K, thus assuring that the samples were fluid at all temperatures reported in the temperature dependent experiments. The temperature was monitored by a thermocouple placed in thermal

contact with the sample tube. Spectra at 77 °K were obtained by immerision in a liquid nitrogen bath which was cooled below 77 °K to prevent boiling. In chlorophyll *a* redox experiments, I_2 was employed because of its solubility in the hydrocarbon solvent system.

Experimental results

The observed spectroscopic properties of the $2 \cdot 10^{-6}$ M sample generally agree with those reported by earlier authors [2, 11]. No long-wavelength aggregate absorption or fluorescence bands were found upon sample cooling. At $C_0 = 10^{-4}$ M a 700 nm absorption band and a long-wavelength fluorescence band at $\approx$ 730 nm were observed on cooling. However, problems with low light transmittance and self-absorption were encountered in the absorption and fluorescence measurements at this concentration due to the large molar extinction coefficient (86 000 at 660.5 nm) of chlorophyll *a*. We therefore concentrate in the following on the results we obtained for the $C_0 \approx 2 \cdot 10^{-5}$ M sample.

In agreement with Livingston et al. [2], we find that the addition of water to hydrocarbon chlorophyll *a* solutions activates the fluorescence intensity I_f of the chlorophyll. This intensity increases monotonically with increasing water concentration C_w. At $C_w = 3 \cdot 10^{-3}$ M, the value I_f for the $2 \cdot 10^{-5}$ M chlorophyll *a* solution is approximately half that $I_{f,max}$ for the fully activated ($C_w = 1.5 \cdot 10^{-2}$ M) sample. The ratio $\eta = I_f/I_{f,max}$ (signifying the fraction of chlorophyll *a* in the form of the monohydrate [2]) is found to be 0.18 for the "dry" preparation. This result is in excellent agreement with the corresponding values obtained by Livingston et al. [2] who found that $\eta (\simeq 0.18)$ remains practically invariant in benzene solutions of chlorophyll *a* ranging in concentrations from $4.8 \cdot 10^{-5}$ to $2.3 \cdot 10^{-4}$ M.

The absorption spectra of six representative samples with varying amounts of added water are shown in Fig. 1. Two salient features of this spectrum are the main peak at $\approx$ 663 nm and the shoulder at 678 nm. The 678 nm shoulder is seen to diminish with increasing water concentration and apparently vanishes at $C_w \gtrapprox 10^{-2}$ M (Fig. 1e). The diminution of the 678 nm shoulder is accompanied by a corresponding increase in the 663 nm peak (Fig. 1, a–e). The interconversion of the 678 nm shoulder and the 663 nm peak thus manifests the dynamical equilibrium that exists between the anhydrous and hydrated species of chlorophyll *a*, respectively.

Of particular interest is the observation of *A*-743 at high water concentrations. At $C_w = 2 \cdot 10^{-2}$ M, a significant portion of the chlorophyll exists as *A*-743 in the freshly made solution. At $C_w \gtrapprox 5 \cdot 10^{-3}$ M, *A*-743 appears as a major component upon aging of the sample (see Fig. 1, e and e'). In all cases, we observe that the appearance of *A*-743 is accompanied by the precipitation of chlorophyll *a*, the latter presumably in the form of crystalline chlorophyll *a* dihydrate (chlorophyll $a \cdot 2H_2O)_n$ [15–17].

As the sample is cooled, the absorption spectrum undergoes a dramatic change resulting in the emergence of a major long-wavelength band in the vicinity of 700 nm. This behavior is observed in all the samples with or without the addition of water, except that there is a minor shift in the peak position of the "700 nm" band. In the "dry" sample, this peak occurs at 698 nm near room temperature and at 702.5 nm at 121 °K. In "wet" samples, this peak generally occurs at $\approx$ 712 nm. For the sake of simplicity, we apply the generic terms *A*-663, *A*-678, *A*-700 and *A*-743 bearing in

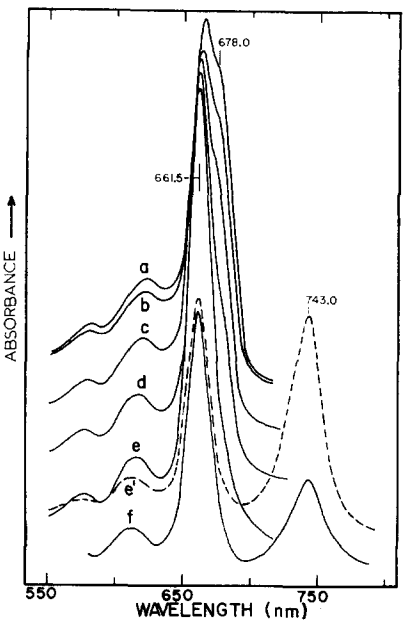

Fig. 1. The effects of water titration on the room-temperature absorption spectrum of chlorophyll *a* solution ($2.0 \cdot 10^{-5}$ M) in 1 : 1 methylcyclohexane and *n*-pentane: (a) "dry"; (b) $4.0 \cdot 10^{-4}$ M added H_2O; (c) $2.0 \cdot 10^{-3}$ M added H_2O; (d) $7.0 \cdot 10^{-3}$ M added H_2O; (e) $1.0 \cdot 10^{-2}$ M added H_2O; (e') $1.0 \cdot 10^{-2}$ M added H_2O; (f) $2.0 \cdot 10^{-2}$ M added H_2O. The solid curves are for freshly prepared samples. The dashed curve (e') illustrates the spectral changes observed in the sample in (e) upon aging for two weeks in the dark at room-temperature.

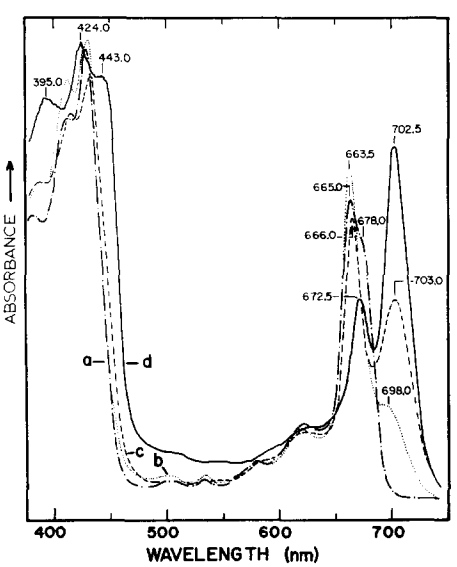

Fig. 2. Temperature dependence of the absorption spectrum of "dry" chlorophyll *a* ($2.0 \cdot 10^{-5}$ M) in 1 : 1 methylcyclohexane and *n*-pentane: (a) room temperature; (b) 245 °K; (c) 167 °K; (d) 121 °K.

mind the minor wavelength shifts that do occur due to changes in temperature and water concentration.

The temperature dependence of the absorption spectrum of the "dry" sample is illustrated in Fig. 2. As the temperature is lowered from 298 °K, we observe a diminution of the *A*-678 along with an increase in *A*-663 and an emergence of *A*-700 (see Fig. 2b). As the temperature is lowered further, we observe that *A*-700 begins to grow at the expense of *A*-663. At 121 °K (Fig. 2d), *A*-700 is the predominant species, contributing to over 85 % of the total absorption spectrum.

The spectral changes in the red wavelength region are accompanied by significant changes in the 380–450 nm wavelength region. As the temperature is lowered, there is a general broadening of the Soret absorption. The changes in the spectral distribution are complex, reflecting the interconversion of a multiplicity of chlorophyll *a* aggregates.

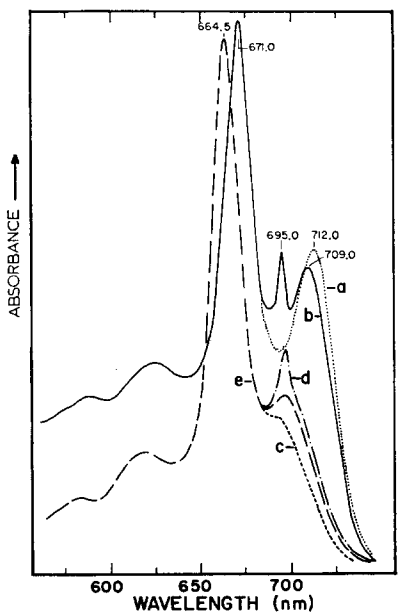

Fig. 3. The effect of the rate of cooling on the low temperature absorption spectrum of chlorophyll *a* solution ($2.0 \cdot 10^{-5}$ M) and H_2O ($1.0 \cdot 10^{-2}$ M) in 1 : 1 methylcyclohexane and *n*-pentane: (a) spectrum measured at 121 °K after slow cool-down from room temperature at 1.0 °C/min; (b) spectrum measured at 77 °K after rapid cool-down by plunging into liquid nitrogen; (c) spectrum measured at 251 °K after slow cool-down from room temperature at 1.0 °C/min; (d) spectrum measured at 245 °K after rapid cool-down at ≈ 25 °C/minute from 251 °K (c); (e) spectrum measured at 245 °K after the system had been allowed to equilibrate for 15 min after (d).

The rate of sample cooling has a dramatic effect upon the spectral distribution in the low temperature spectra. The predominance of *A*-700, normally obtained in slowly cooled samples (see Fig. 3a), is not observed in samples that are plunged into liquid nitrogen. Instead, in all samples to which water has been added, a new well defined band maximum at 695 nm is observed (see Fig. 3b). That *A*-695 is a metastable

intermediate formed during the conversion of *A*-663 to *A*-700 is suggested by the kinetic effects shown in Fig. 3, c–e. The *A*-695 becomes evident (Fig. 3d) immediately after the sample is cooled from 251 to 245 °K. (The 6° increment drop took place over a period of approx. 15 s.) The *A*-695 subsequently decays over a 15-min period at the end of which we observe the equilibrium spectrum shown in Fig. 3e. It is interesting and possibly significant that this *A*-695 kinetic effect is not observed in the dry sample or at low temperatures (< 200 °K).

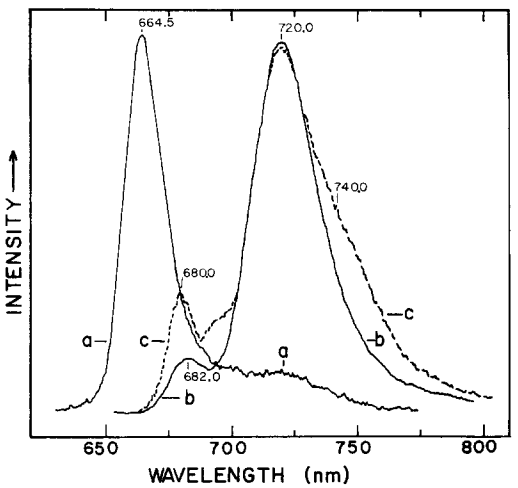

Fig. 4. The temperature dependence of the fluorescence spectrum of "dry" chlorophyll *a* solution ($2.0 \cdot 10^{-5}$ M) in 1 : 1 methylcyclohexane and *n*-pentane: (a) room temperature; (b) 121 °K. The solid curve (b) is the fluorescence spectrum obtained with a cooling rate of 1.0 °C/min. The dashed curve (c) was obtained when the same sample was cooled by plunging into liquid nitrogen. Curves a and b were obtained with 430 nm excitation and the 77 °K spectrum (c) was excited at 450 nm. The shoulders at 695 nm and 740 nm are indicative of the presence of metastable intermediate species. In rapidly cooled samples containing added water, the main fluorescence band peaks at 740 nm.

The observed fluorescence behavior of the $2 \cdot 10^{-5}$ M chlorophyll *a* solutions agrees in broad outline with that described by previous authors [4–10]. The room-temperature fluorescence spectrum of the dry sample is shown in Fig. 4a. This spectrum has a characteristic peak at 664.5 nm (*L*-664) that can be attributed to the *A*-663 chlorophyll *a* monohydrate [2, 11]. When the sample is cooled, the long-wavelength fluorescence band at 720 nm (*L*-720) becomes prominent. The *L*-720/*L*-664 intensity ratio increases as the temperature decreases. At 121 °K (see Fig. 4b), the fluorescence spectrum is dominated by *L*-720. In addition to *L*-720, only a minor component at 682 nm is observed. In "wet" samples, the 720 nm band red shifts to 740 nm.

Quick plunging of the dry sample into liquid nitrogen produces the 77 °K fluorescence spectrum shown in Fig. 4c. In addition to the 680 nm and 720 nm bands, we observe the appearance of a new band at 695 nm. A 740 nm shoulder to the red of *L*-720 is also apparent in Fig. 4c.

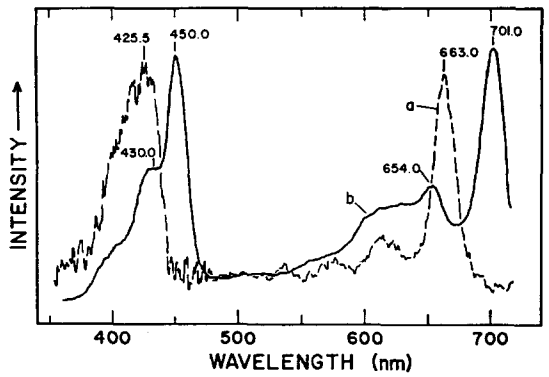

Fig. 5. Excitation spectra of the fluorescence at 720 nm of a $2.0 \cdot 10^{-5}$ M chlorophyll *a* solution in 1 : 1 methylcyclohexane and *n*-pentane: (a) room temperature; (b) 121 °K. The Soret bands of the uncorrected spectra were measured at higher detector gain.

The respective correspondences of *L*-720 and *L*-664 to *A*-700 and *A*-663 are demonstrated by the excitation spectra shown in Fig. 5. The low-temperature spectrum in Fig. 5b is associated with the 720 nm band in Fig. 4b while the excitation spectrum in Fig. 5a corresponds to the room-temperature fluorescence spectrum in Fig. 4a.

The addition of an equimolar amount of I_2, an oxidizing agent with a standard reduction potential 0.54 V, to a $1.5 \cdot 10^{-5}$ M chlorophyll *a* solution in 1 : 1 methylcyclohexane and *n*-pentane dramatically changes the normal cooling behavior illus-

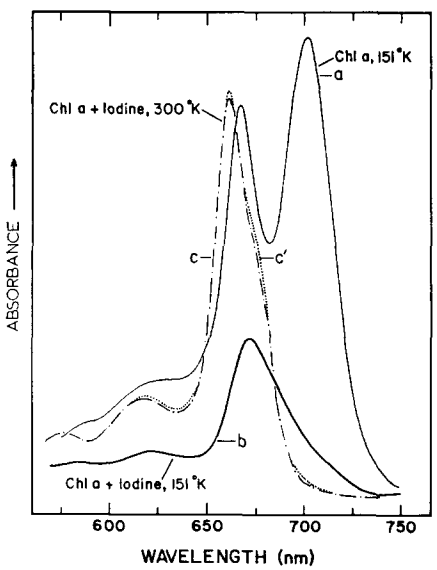

Fig. 6. The 151 °K absorption spectrum of a $1.5 \cdot 10^{-5}$ M 1 : 1 methylcyclohexane and *n*-pentane is shown in (a). When an equimolar amount of I_2 is added to the solution in (a), substantial bleaching of the *A*-700 component is observed at 151 °K in (b). The room-temperature absorption spectra of the 1 : 1 chlorophyll : iodine sample before and after the cooling cycle are shown in (c) and (c') respectively.

trated in Fig. 2. Instead of the emergence and eventual predominance of the *A*-700 component (Fig. 6a) in the absence of I_2, sample cooling (at 151 °K) has resulted in the bleaching of chlorophyll *a* when I_2 is present (Fig. 6b). This bleaching process is reversible. The room temperature absorption spectra of the 1 : 1 chlorophyll *a* : I_2 sample before and after cooling are shown in Figs 6c and 6c′ respectively.

DISCUSSION

According to Livingston et al. [2], *A*-663 corresponds to chlorophyll *a* · H_2O. The growth of *A*-663 at the expense of the *A*-678 shoulder (arising from anhydrous dimer chlorophyll a_2 [15, 16]) as water is added (Fig. 1, a–e) or as the temperature is lowered (Figs 2a, b) is thus a manifestation of the solvation process:

$$2H_2O + \text{chlorophyll } a_2 \overset{K_1}{\rightleftarrows} 2 \text{ chlorophyll } a \cdot H_2O. \tag{1}$$

The emergence and eventual predominance of *A*-700 along with the disappearance of *A*-663 as the temperature is further lowered provides proof that *A*-700 has originated from the aggregation of chlorophyll *a* · H_2O. The virtual absence of the anhydrous chlorophyll in Figs 2b–d, manifested by the absence of the *A*-678 shoulder, thus indicates that we have $C_w \gtrapprox C_0 = 2 \cdot 10^{-5}$ M, i.e., at least an equimolar amount of water in the driest sample. Earlier, it was established from fluorescence experiments [2] that $0.5\,C_0 \lessapprox C_w \lessapprox C_0$ for the driest chlorophyll *a* sample. Since our fluorescence ratio $\eta = I_f/I_{f,max} = 0.18$ (see Experimental Results) for our dry sample is in agreement with the corresponding ratio in Livingston's work [2], it is reasonable to suppose that the above two conditions for C_w should be consistent, and we arrived at the estimate $C_w \approx C_0 = 2 \cdot 10^{-5}$ M for the "dry" sample. This estimate appears to account for the observation [14] that *A*-743 releases one water of hydration upon drying. (See Acknowledgments.) The appearance of *A*-743 under excess-water conditions (Fig. 1, e′ and f) is accompanied by a diminution of the *A*-663, consistent with the equilibrium:

$$n \text{ chlorophyll } a \cdot H_2O + nH_2O \overset{K_2}{\rightleftarrows} (\text{chlorophyll } a \cdot 2H_2O)_n \tag{2}$$

in which *A*-743 is given by (chlorophyll *a* · $2H_2O)_n$ [15–17].

Upon slow cooling, the broad features of the respective growth and disappearance of *A*-700 and *A*-663 appear to be insensitive to C_w. The temperature dependence of the *A*-700 emergence is examined by the analog computer spectral deconvolutions for the $C_w/C_0 = 100$ sample shown in Fig. 7. In Fig. 7 the Gaussian component bands at 620 nm (A) and 660 nm (B) are independent of temperature changes, and are presumably unimportant in our consideration of the chlorophyll *a*-H_2O interactions. Intermediate in wavelength between the 263 °K 667 nm (a) component (which corresponds to *A*-663), and the main component α of *A*-700 appears a "680" nm (b) component. At 263 °K, this component occurs at 678 nm. At lower temperatures, this component undergoes a red shift to about 682 nm (*A*-682). To the red end of the component α, we find the component β. The α and β components peak at 699 nm and 713 nm at 263 °K, respectively. At lower temperatures, these components shift to constant peak positions at 703 nm and 718 nm, respectively. The area ratio ρ of α and β appears to be a constant, averaging 3.03 ± 0.5 in 36 different determinations at varying temperatures and water concentrations. We therefore conclude that the α

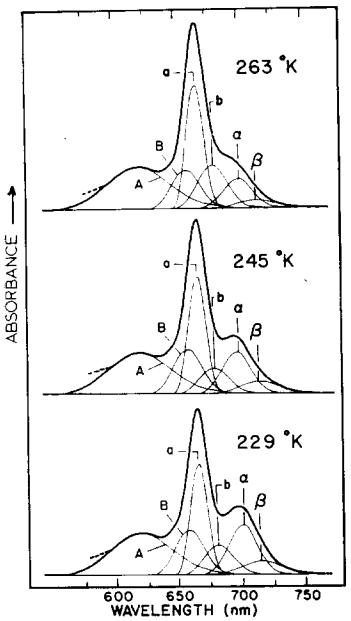

Fig. 7. Computer deconvolutions of the absorption spectrum of a $C_w/C_0 = 100$ sample at three temperatures, illustrating the spectral details accompanying the emergence of the *A*-700 species. The α and β exciton components peak at 699 nm and 713 nm, respectively.

and β components belong to the same absorbing species, the *A*-700. (A detailed exciton theoretical analysis of the α, β bands has been given elsewhere [26].) At a given temperature, the ratio ϕ of the area under the sum of the α and β components to that of the square of a [$\phi = (\alpha+\beta)/a^2$] appears to be an invariant of C_w. At 245 °K, for example, we obtain $\phi = 1.7 \pm 0.1$ (this value has been obtained by normalizing the component areas with respect to the sum of the areas of a, α and β) at $C_w = 2 \cdot 10^{-5}$, 10^{-4}, $4 \cdot 10^{-4}$, and $2 \cdot 10^{-3}$ M.

The invariance of ϕ can only be accounted for by the hypothesis that *A*-700 corresponds to the empirical structure (chlorophyll $a \cdot H_2O)_2$ and results from the dimerization of chlorophyll $a \cdot H_2O$ according to the equilibrium

$$2 \text{ chlorophyll } a \cdot H_2O \overset{K_3}{\rightleftarrows} (\text{chlorophyll } a \cdot H_2O)_2 \tag{3}$$

Since, according to the present development, the reactant and product concentrations in [3] are directly proportional to the areas under a and $(\alpha+\beta)$ respectively, it is easy to show that the equilibrium constant K_3 can be written

$$K_3 = \phi C_0^{-1} \tag{4}$$

which is equal to $8.5 \cdot 10^4$ at 245 °K.

At low temperatures, the *A*-700 absorption has a maximum at 703 nm (Fig. 2, c and d). At temperatures close to room temperature, the corresponding maximum occurs at 698 nm (Figs 2b and 7). Likewise, the *P*-700 has a room-temperature red absorption maximum at 697.5 nm [27]. The spectral distribution of the *A*-700 red absorption band is skewed on the long wavelength side. This non-Gaussian behavior

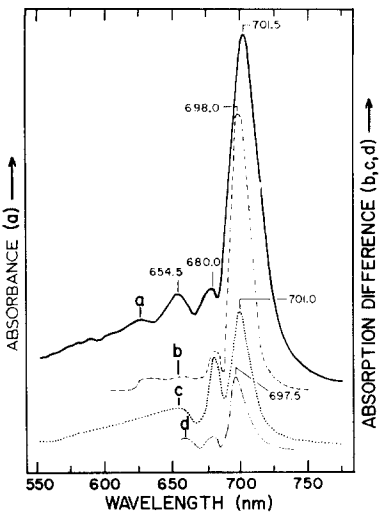

Fig. 8. Comparison of the 136 °K absorption spectrum (a) of the "pure" *A*-700 (chlorophyll *a* · $H_2O)_2$ adduct with three independent determinations of the light-minus-dark spectrum of *P*-700 in the red-wavelength region according to (b) Kok [21]; (c) Ke [28]; and (d) Philipson et al. [27]. See text for a discussion for the observed variations in the 680 nm band intensity and the manner in which spectrum (a) has been obtained. The *P*-700 spectra were measured at room temperature.

has been accounted for in the resolution of the α and β components (Fig. 7). A similar asymmetry in the *P*-700 red band [27, 28] has been observed [27].

A comparison of the red absorption of the "pure" *A*-700 and that of the *P*-700 is made in Fig. 8. The "pure" *A*-700 spectrum displayed in Fig. 8 has been obtained by subtracting from the 136 °K absorption spectrum of the "dry" sample the computer deconvoluted components a and b. Three different versions of the *P*-700 red absorption, derived from light-minus-dark spectra of Photosystem I reaction centers, are given in Fig. 8, b–d. The Soret region of the *A*-700 absorption is complicated (see Fig. 2) by the presence of three major components (*A*-663, *A*-682 and *A*-700). It is not possible to obtain a meaningful deconvolution in this region*.

The comparison in Fig. 7 shows good qualitative agreement between the absorption characteristics of *A*-700 and *P*-700. The deconvoluted *A*-700 absorption spectrum also agrees in broad outline with the corresponding spectral region in the *L*-720 excitation spectrum (Fig. 5b) except that the 680 nm band is absent in Fig. 5b. The 680 nm band, which is present in all the four difference determinations shown in Fig. 8, may have resulted from some adventitious source in view of the variability of the 680 nm/700 nm intensity ratio of the *A*-700 and *P*-700 absorption (Fig. 8). The maximum of the *P*-700 Soret band is reported to occur at $\approx$ 430 nm [21, 27, 28]. There is a 430 nm band in the Soret region of the *L*-720 excitation spectrum (Fig. 5b),

* Monomeric chlorophyll *a* in polar solvents displays three main resolvable bands in the Sore t region. The corresponding Soret region for each dimeric chlorophyll *a* adduct would consist of twic e as many components due to exciton splitting. The existence of at least three different chlorophyll *a* aggregation species precluded the possibility of a meaningful deconvolution analysis.

although the maximum excitation intensity in this spectral region occurs at ≈ 450 nm*.

The important implication of the redox experiment summarized in Fig. 6 is that solvated chlorophyll *a* monomer ($E_0 = 0.64$ V [30]) is not oxidized by I_2 ($E_0 = 0.54$ V). A control experiment, conducted on a $1.5 \cdot 10^{-5}$ M chlorophyll *a* solution in EPA (where no *A*-700 formation has been detected upon cooling in the absence of I_2), reveals that the chlorophyll in the monomeric form is not bleached by I_2 at temperatures less than or equal to 300 °K**. The I_2 bleaching under conditions conducive to *A*-700 (chlorophyll $a \cdot H_2O)_2$ formation therefore suggests that this species has an oxidation potential $\lesssim 0.5$ V. It is noteworthy that the in vitro *A*-700 species appears to have redox properties similar to the in vivo *P*-700 pigment ($E_0 = 0.43$ V) [21]. Experiments dealing with the photoactivity of *A*-700 and the electron spin resonance activity of the bleached *A*-700 species are underway.

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* It may be appropriate to note that the difference spectral determination of *P*-700 in the Soret region is complicated by carotenoid absorption [21].

** Monomeric chlorophyll *a* bleaching by $FeCl_3$ ($E_0 = 0.76$ V) has been the subject of earlier investigations [29, 30].

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