LOW TEMPERATURE ABSORPTION SPECTRA OF CHLOROPHYLL *a* IN POLAR AND NONPOLAR SOLVENTS

S. S. BRODY *and* S. B. BROYDE

From the Photobiology Laboratory, Biology Department, New York University, New York, New York 10003

ABSTRACT Absorption spectra of chlorophyll *a* were measured in polar and nonpolar solvents, as a function of temperature from 298° to 77°K. Both dilute and concentrated solutions were examined. In both types of solvents at room temperature, the absorption spectra of concentrated solutions differ from dilute ones in that the half width of the main red absorption band is greater, and all bands are shifted to longer wavelengths. These differences are largely due to the presence of dimers when the pigment concentration is high. In dilute ethanol solutions, where the chlorophyll is unassociated, cooling causes a red shift in all bands which is due to the increased polarity of the solvent at low temperature. On cooling at high concentrations in ethanol and EPA, a new band appears near 700 nm. This band is attributed to dimers present prior to cooling, but absorbing at shorter wavelengths at room temperature. In nonpolar solvents, a band near 700 nm appears at the solvent freezing point. In these solvents, the "700" nm absorption is attributed to dimers, and/or small polymers, partly formed by cooling. A change in aggregate geometry when the solvent becomes viscous or frozen can account for the appearance of this "700" nm absorption band at low temperature, in polar and nonpolar media.

INTRODUCTION

Over the years, a number of workers have measured the absorption spectra of chlorophyll and its derivatives at low temperature. Freed and Sancier (1–4) in a series of studies begun in 1951 reported the appearance of some new bands of chlorophyll *a* and *b* at low temperature, and studied the solvent dependence of these spectral changes. Linschitz (5) measured the absorption spectra of ethyl chlorophyllide *a* and *b* in EPA, as a function of temperature, down to 80°K. Stensby and Rosenberg (6) measured the absorption spectrum of dilute and concentrated (8×10^{-3} M) solutions of chlorophyll *a* in ethanol as a function of temperature from 298° to 77°K. Amster and Porter (7)[1] obtained low temperature absorption spectra of dilute solutions of chlorophyll *a* and *b* dissolved in purified 3-methyl pentane.

[1] Amster, R., and G. Porter. Private communication. To be published.

An interesting feature of the spectra, obtained with all concentrated solutions (6) and dilute nonpolar solutions (7),[1] is the appearance of a new absorption band in the vicinity of 700 nm on cooling. Other evidence for the existence of a low temperature absorption band near 700 nm has been obtained from fluorescence excitation spectra of concentrated ethanolic solutions of chlorophyll *a* (8). These solutions exhibit an intense long-wavelength emission with maximum near 725 nm, and the fluorescence excitation spectrum for this emission has a maximum near 700 nm. Both the 725 nm emission and the 700 nm band in the excitation spectrum have been ascribed to chlorophyll aggregates. Stensby and Rosenberg (6), and Amster and Porter (7)[1] have assigned the 700 nm absorption band to an aggregated state of the pigment. Forms of chlorophyll *a* absorbing near 700 nm have been of interest because the sensitizer for one of the photochemical reactions in vivo is of this type (9).

We have now delineated the conditions which give rise to the "700" nm band in solution. A systematic study has been made of the effects of temperature, pigment concentration, and solvent type on the absorption spectra of chlorophyll *a*, and the results have been related to the state of aggregation of the pigment. The terms "polar" and "nonpolar" are used to denote solvents which are respectively basic and neutral in the Lewis sense.

EXPERIMENTAL

Absorption spectra were measured with a Cary Model 14R recording spectrophotometer equipped with a scattered transmission accessory. Optical densities exceeding 2.0 were measured by placing a wire mesh neutral density screen, of known optical density, in the reference beam.

To conveniently measure absorption spectra at any temperature between 298° and 77°K, the following procedure was used.

A small, stoppered, partially silvered Dewar flask constituted the thermally insulated sample chamber (Fig. 1). A cuvette containing the sample was held rigidly within the Dewar by a brass block which also helped to avoid temperature gradients along the cuvette. The Dewar was closed with a tightly fitting rubber stopper provided with connectors for a thermocouple and to allow circulation of nitrogen. Before cooling the Dewar was flushed with dry nitrogen to minimize condensation. To cool the sample, nitrogen gas, refrigerated by passing it through liquid nitrogen, was flushed through the Dewar until a desired temperature was reached. To warm the sample, dry nitrogen gas at room temperature was similarly employed. The temperature was measured with a calibrated copper-constantan thermocouple which was attached to the sample cuvette with a small piece of clay. The other end of the thermocouple was maintained at 273°K in an ice-water mixture. For dilute solutions, a 1 mm path-length rectangular silica cuvette was used, except for EPA, in which case a 1 cm path-length cell was employed. For concentrated solutions, about 20 μl of solution was pressed between two $\frac{1}{4}$ inch thick optical glass plates, and the edges were sealed with wax. Very little distortion of the spectra due to scattering occurred with these plates because of the short optical path length (about 10^{-4} cm). While scattering was also small in the case of the 1 mm cells, a correction was made in the calculation of optical density ratios using a run in which solvent alone was cooled.

Samples were allowed to equilibrate at each temperature for a few minutes, till large changes in temperature ceased. The temperature was measured at the beginning and at the end of each spectrum and an average value determined; it took about 3 min to measure a complete spectrum and the temperature changed about 5°. At the melting point of the solvent, the temperature measured in this way differed by less than 5° from the true melting point. Melting was evidenced by large changes in optical density and by constant temperature. A typical cooling or warming cycle took about 45 min between 298° and 77°K. Unless otherwise indicated, samples were cooled and then warmed, spectra being measured at intervals in both cycles.

Chlorophyll *a* was prepared by a combination of methods (10, 11) as described previously (12). Concentrated solutions were made by dissolving a weighed sample of crystalline chlorophyll in a measured volume of solvent. To completely dissolve the chlorophyll at high con-

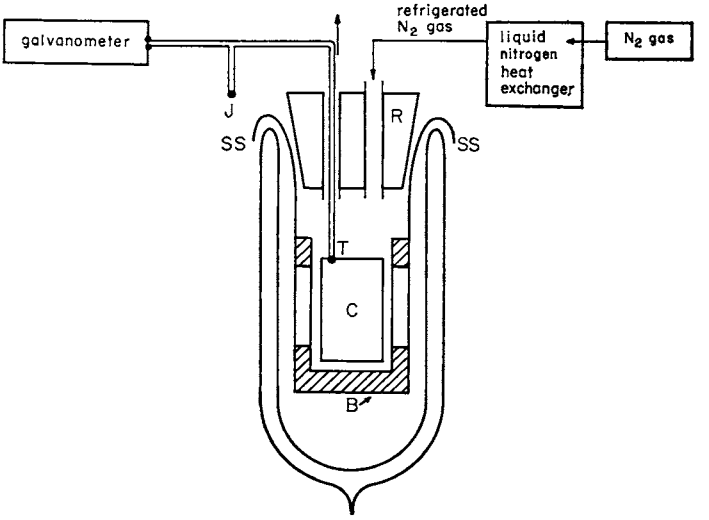

FIGURE 1 Schematic diagram of the apparatus. The following symbols are used: C = cuvette, $S.S$ = stainless steel supporting wires, B = brass cuvette holder, R = rubber stopper, T = thermocouple junction, J = reference thermocouple junction in ice bath.

centration, it was necessary to "pump" the solution (300 μl) about 50 times with a syringe. In the case of ethanol this was done in a glove bag filled with nitrogen saturated with ethanol in order to avoid allomerization of the pigment. Concentrations of dilute solutions were determined using the extinction coefficients summarized by Seeley and Jensen (13) or by Sauer, Smith, and Schultz (14).

Spectranalyzed benzene and 1,2 dichloroethane were obtained from Fisher Scientific Company, Millford, N. J., as was anhydrous ethyl ether. Carbon tetrachloride was the Spectroquality Reagent, from Matheson Coleman and Bell, East Rutherford, N. J. Isopentane was practical grade from City Chemical Corp., New York. Ethanol was absolute, reagent quality solvent from U.S. Industrial Chemical Co., New York. EPA consists of ether, isopentrane, and ethyl alcohol in the volume ration of 5:5:2, and it forms a glass on cooling. Solvents were used as received. The limits of solubility of water, the most prevalent polar impurity in the nonpolar solvents (15), are 6.5×10^{-4} M in CCl_4, 9.2×10^{-3} M in benzene, and

8.6×10^{-3} M in 1,2 dichloroethane. These are small compared to the 5.0×10^{-2} M of chlorophyll employed for the high concentration samples; as a result, the effect of water in these systems should not be large.

RESULTS

Polar Solvents: EPA and Ethanol

Dilute Solutions: The absorption spectra of a 5.6×10^{-6} M solution of chlorophyll *a* at 298° and 120°K in EPA are shown in Fig. 2. The absorption maxima are at 663 and 431 nm at 298°K, and at 667 and 442 nm at 120°K. Thus, cooling causes a red shift in the absorption bands. The temperature dependence of the peak positions is given in Fig. 3 for cooling and warming. Also, on cooling from 298° to 120°K, the half width of the red band decreases from 19 to 17 nm, and there is an

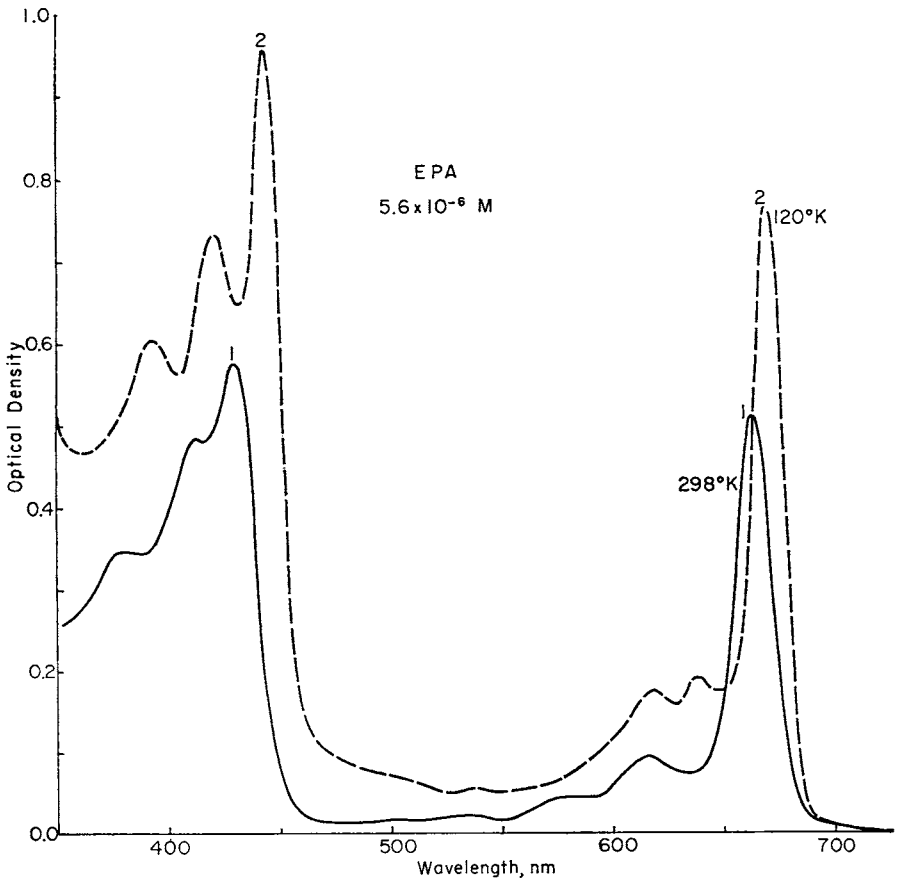

FIGURE 2 Absorption spectra of chlorophyll *a* in EPA, 5.6×10^{-6} M. Curve 1, 298°K; curve 2, 120°K.

increase in optical density by a factor of 1.45, partly due to solvent contraction. The satellite on the short-wavelength side of the blue peak is better resolved at low temperature.

Similar changes occur in ethanol. Here, on cooling at 77°K the optical density of the red maximum increases by a factor of 1.40. This increase arises in part from the contraction of ethanol on cooling. The density of ethanol increases from 0.79 at

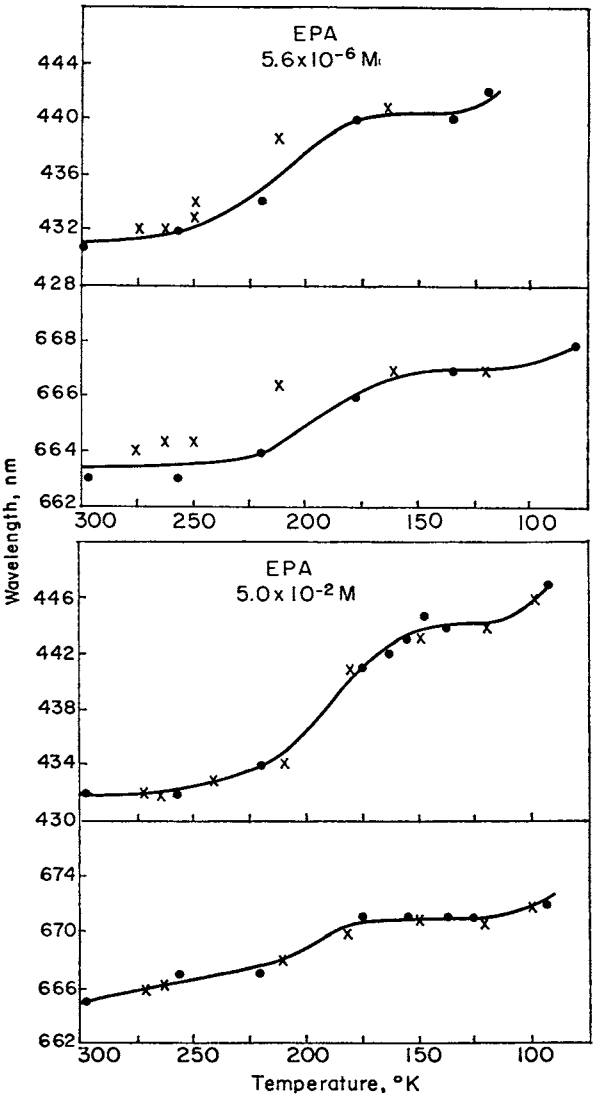

FIGURE 3 Absorption maxima of chlorophyll *a* in EPA as a function of temperature for 5.6×10^{-6} M (left) and 5.0×10^{-2} M (right). Cooling cycle is given by dots. Warming cycle is given by crosses.

TABLE I

ABSORPTION PROPERTIES OF CHLOROPHYLL *a*

Solvent	Chlorophyll Concentration	Temperature	Absorption maxima	Half band width*	$\frac{\epsilon \text{ blue}‡}{\epsilon \text{ red}}$
CCl_4	2.7×10^{-5} M	298°K	664 ± 1 nm 617 432 413	20 ± 1 nm	1.37
		77	707 671 628 437 421		
CCl_4 plus 0.5% ethanol	2.7×10^{-5}	298	665 618 432 416	19	1.20
		77	673 655 625 445 423		
CCl_4	5.0×10^{-3}	298	668 625 433 417	35	1.42
		121	709 671 626 438 420		
CCl_4	5.0×10^{-2}	298	670 626 434 418	33	1.21
		92	712 678 630 441 423		
CCl_4 plus 10% ethanol	4.5×10^{-2} M	298	667 617 433 417	27	1.02
		77	707 676 625 448 426		

TABLE I—*Continued*

Solvent	Chlorophyll Concentration	Temperature	Absorption maxima	Half band width*	$\frac{\epsilon \text{ blue}‡}{\epsilon \text{ red}}$
Ethanol	4.5×10^{-5} M	298°K	664 ± 1 nm	21 ± 1 nm	1.10
			616		
			432		
			417		
		77	672	15	
			655		
			618		
			444		
			423		
Ethanol	6.0×10^{-2}	298	666	30	1.10
			618		
			437		
		77	705		
			673		
			655		
			622		
			443		
			433		
EPA	5.6×10^{-6}	298	663	19	1.03
			615		
			431		
			418		
		120	667	17	1.26
			637		
			617		
			442		
			420		
EPA	5.0×10^{-2}	298	665	23	1.13
			617		
			430		
			417		
		98	721		
			672		
			640		
			624		
			446		
			423		

TABLE I—*Continued*

Solvent	Chlorophyll Concentration	Temperature	Absorption maxima	Half band width*	ϵ blue‡ / ϵ red
Benzene	4.8×10^{-5}	298°k	665 ± 1 nm 618 432 419	20 ± 1 nm	1.35
		116	706 671 624 440 420		
Benzene	5.0×10^{-2}	298	666 620 432 416	30	1.32
		89	715 671 625 439 426		
1,2 dichloroethane	4.3×10^{-5}	298	663 616 431 412	21	1.21
		83	710 669 647 437 426	30	

* Main red absorption maximum.
‡ Optical density ratio of main blue band to main red band.

298°K to 1.02 at 77°K, a factor of 1.29, so that a comparable increase in chlorophyll concentration is expected. However, this is still less than the observed increase in optical density. Correcting for the density change of the solvent and the associated shorter optical path the extinction coefficient of chlorophyll *a* in ethanol at its red absorption maximum is higher by a factor of 1.19 at 77°K than at 298°K.

Freezing usually caused irregular variations in optical density; in concentrated solutions where the volume was very small, holes often formed. Therefore, changes in the ratios of absorption maxima are reported, except where optical densities varied regularly, i.e. in dilute solutions of EPA and absolute ethanol, both of which form glasses on cooling.

At low temperature a new band occurs in both solvents near 640 nm in EPA and near 655 nm in ethanol as previously noted by Freed and Sancier (1) and Stensby and Rosenberg (6). The band first appears at 210°K in EPA and at 190°K in ethanol. On the other hand, there is no increase in absorbance near 700 nm upon cooling. On warming back to room temperature, the original spectral characteristics reappear.

A summary of all results is given in Table I.

Concentrated Solutions. In Fig. 4 are given the absorption spectra in the red region of a 6.0 × 10^{-2} M solution of chlorophyll *a* in ethanol at various temperatures. The solution was frozen rapidly by plunging it into liquid nitrogen and spectra were obtained while warming. Again, all bands are red-shifted at low temperature, maxima occurring at 666 and 437 nm at 298°K and at 673 and 443 nm at 77°K. The most striking change is the appearance of a new band at 700–705 nm which will be referred to as the "700" nm band.

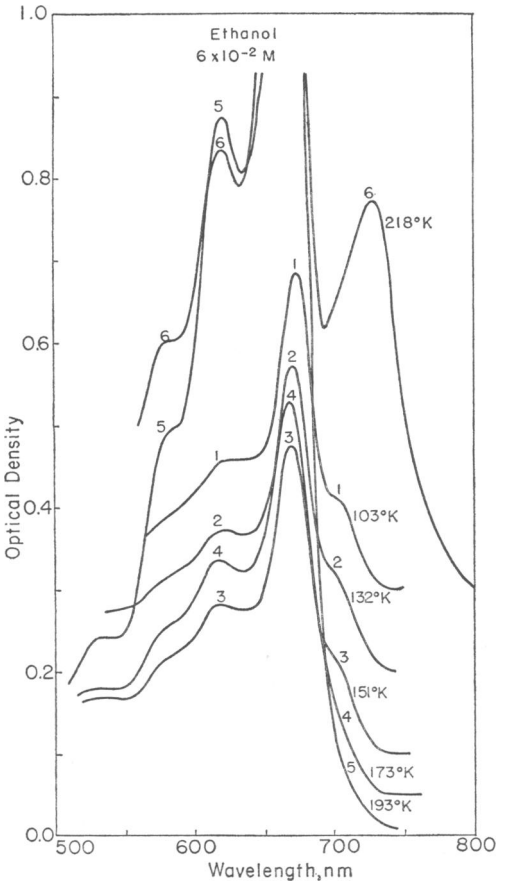

FIGURE 4 Absorption in the red region of the spectrum of a 6.0 × 10^{-2} M solution of chlorophyll *a* in ethanol at various temperatures during warming from 77°K. Optical densities of main red band for curves 5 and 6 are 1.53 and about 1.5 respectively. Curves 1–4 and 6 are displaced upward to facilitate comparison; the optical density at 800 nm is actually zero in each case.

As the solution warms the main red band broadens, all bands are shifted to the blue, and the "700" nm band becomes less resolved. At 193°K, the "700" nm shoulder has completely disappeared and there is a large increase in optical density (presumably holes in the light path through the solution were filled in on melting).

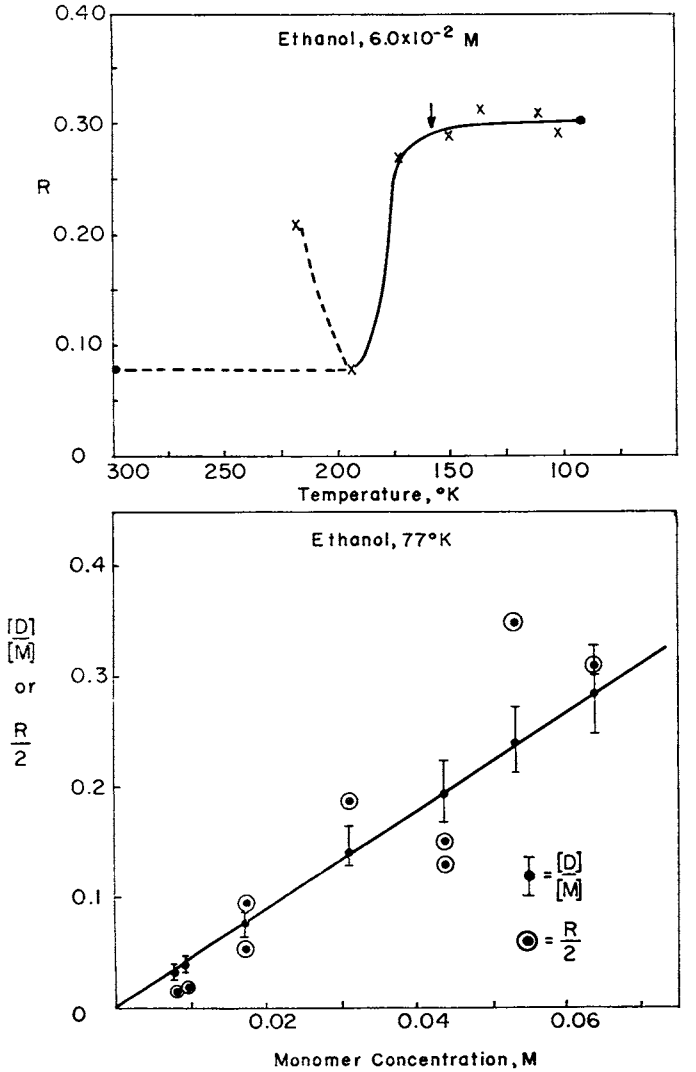

FIGURE 5 Upper curve; ratio, R, of absorbancies of "700" nm band to main red absorption band as a function of temperature, for a 6.0×10^{-2} M solution of chlorophyll a in ethanol. Cooling cycle is given by dots and warming cycle is given by crosses. Lower curve: ratio of dimer to monomer concentration $[D]/[M]$ and $R/2$, as a function of monomer concentration of chlorophyll a in ethanol, at 77°K. Former obtained from equilibrium constant for dimerization in ethanol.

On further warming to 218°K chlorophyll microcrystals absorbing maximally at 728 nm appear. Absorption by this species increases as the temperature is raised. If the system is again cooled, absorption by microcrystalline chlorophyll remains.

Fig. 5, upper curve, gives the ratio R, of optical densities of the "700" nm band to the main red absorption near 670 nm, as a function of temperature. The "freezing point" of ethanol at 158°K is indicated by the arrow. The value of R at 77°K is

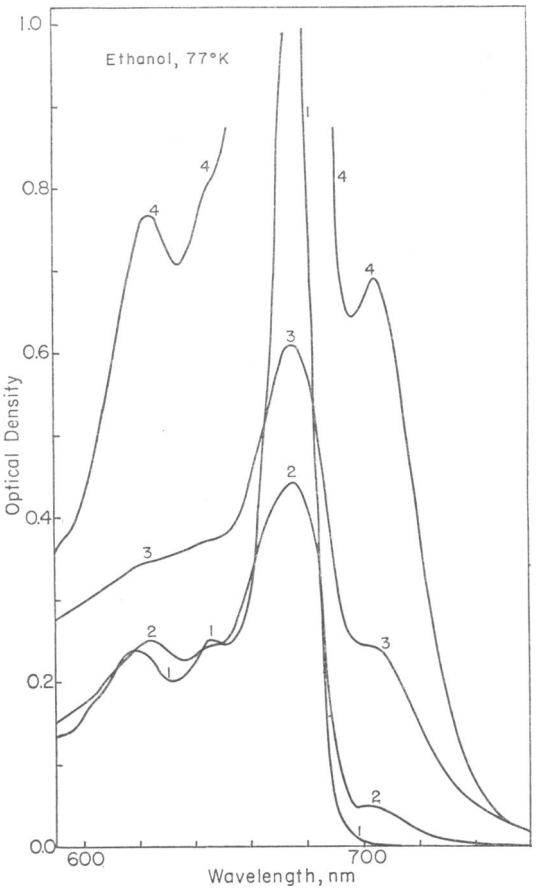

FIGURE 6 Absorption at 77°K of chlorophyll *a* in ethanol in the red region of the spectrum, at various concentrations. Curve 1; 9.0×10^{-4} M, optical density 672 nm, 1.29; curve 2, 2.0×10^{-2} M; curve 3, 4.0×10^{-2} M; curve 4, 6.0×10^{-2} M, optical density 673 nm, 2.66.

rather constant for a given concentration when the solution is frozen rapidly. When the solution is cooled slowly, R is higher by a variable factor, sometimes as great as two.

A study was made of the absorption spectra of chlorophyll *a* in ethanol as a function of concentration, in the range from 1.9×10^{-4} to 10^{-1} μl. For these measurements samples were immersed into liquid nitrogen immediately after preparation. Fig. 6 shows the red region of some of the spectra obtained at 77°K. Table II gives

chlorophyll concentrations and R for these measurements; R, at 77°K increases with increasing chlorophyll concentration. The position of the "700" nm band increases from 700 nm in the less concentrated solutions to 706 nm at the highest concentration. A 655 nm band is present irrespective of concentration.

When cooling a 5.0×10^{-2} M EPA solution of chlorophyll to 154°K, the "700" nm band begins to appear at 712 nm (Fig. 7, curve 2). On further cooling an additional absorption band near 721 nm (Fig. 7 curve 3) appears so the "700" nm band is no longer clearly resolved. On warming both bands disappear at about 220°K. Hysteresis in the temperature dependence of the absorption bands is probably related to hysteresis in the temperature dependence of the properties (e.g.

TABLE II

VARIATION OF R WITH CHLOROPHYLL CONCENTRATION IN ETHANOL AT 77°K

Chlorophyll concentration	R
1.9×10^{-4} M	0.004
8.0×10^{-3}	0.029
	0.022
1.0×10^{-2}	0.035
2.0×10^{-2}	0.18
	0.11
4.0×10^{-2}	0.37
6.0×10^{-2}	0.26
	0.30
8.0×10^{-2}	0.71
1.0×10^{-1}	0.63

viscosity [16] of EPA itself. The wavelengths of the main red and blue absorption maxima, as a function of temperature, are given in Fig. 3.

Carbon Tetrachloride. Fig. 8 shows the absorption spectra of a 2.7×10^{-5} M solution of chlorophyll *a* in CCl_4 at room temperature (curve 1) and at 77°K (curve 2). As in the polar solvents, all bands are red shifted at low temperature, maxima occurring at 664 and 432 nm at room temperature and at 671 and 437 nm at 77°K. The peak positions of all bands are given as a function of temperature in Fig. 9. In contrast to the polar solvents, the "700" nm band occurs also in dilute solution, being at 707 nm at 77°K (Fig. 10, curve 1, and Fig. 8, curve 2). On warming from low temperatures, R is constant until about 15°K below the melting point (Fig. 11) when it begins to decrease. When the solution melts the "700" nm band disappears completely. Further warming to room temperatures produces no change in R.

Fig. 12 shows absorption spectra of a 5.0×10^{-2} M solution of chlorophyll *a* in CCl_4, at various temperatures. These spectra were taken from the warming cycle.

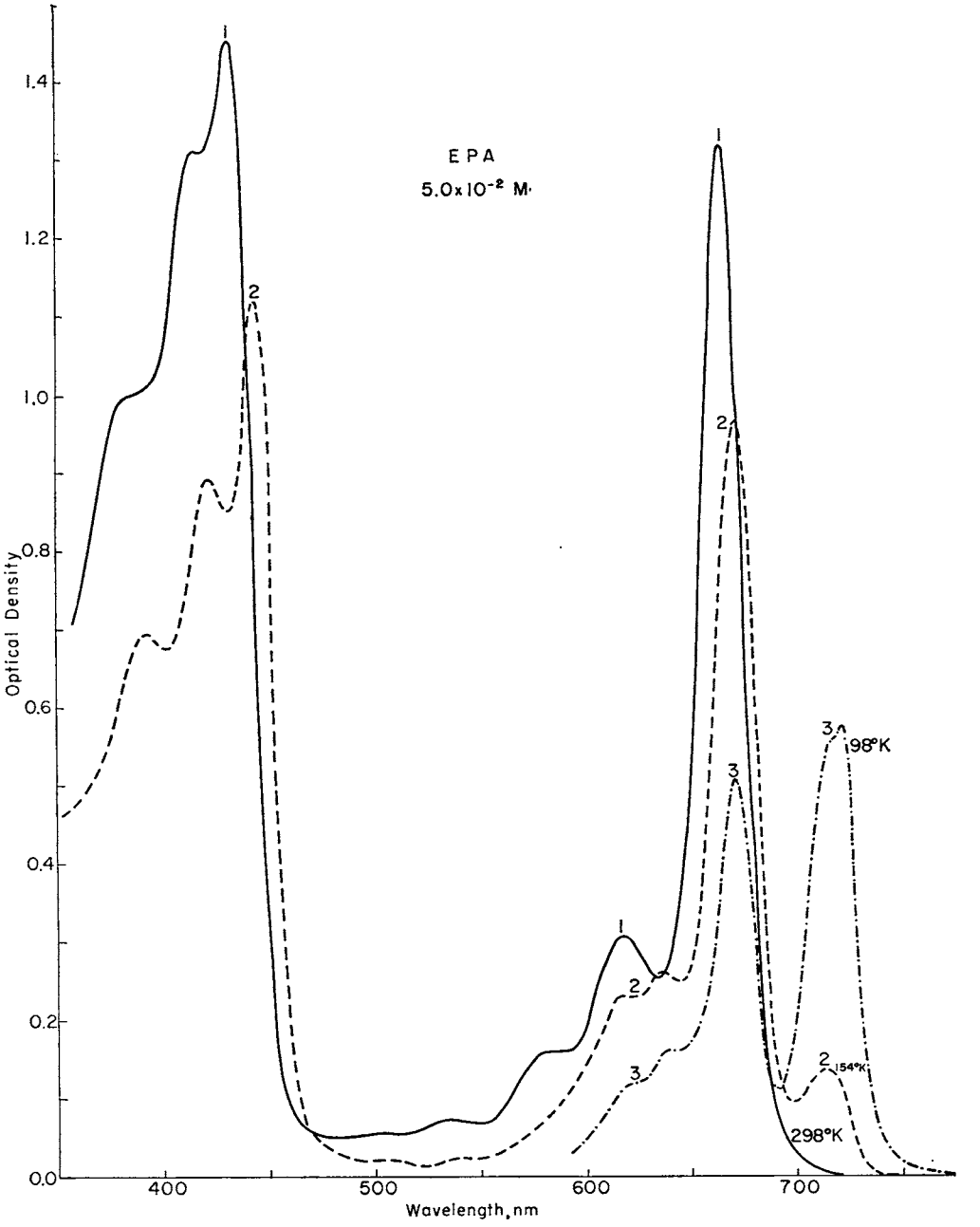

FIGURE 7 Absorption spectra of chlorophyll *a* in EPA, 5.0×10^{-2} M. Curve 1, 298°K; curve 2, 154°K; curve 3, 98°K.

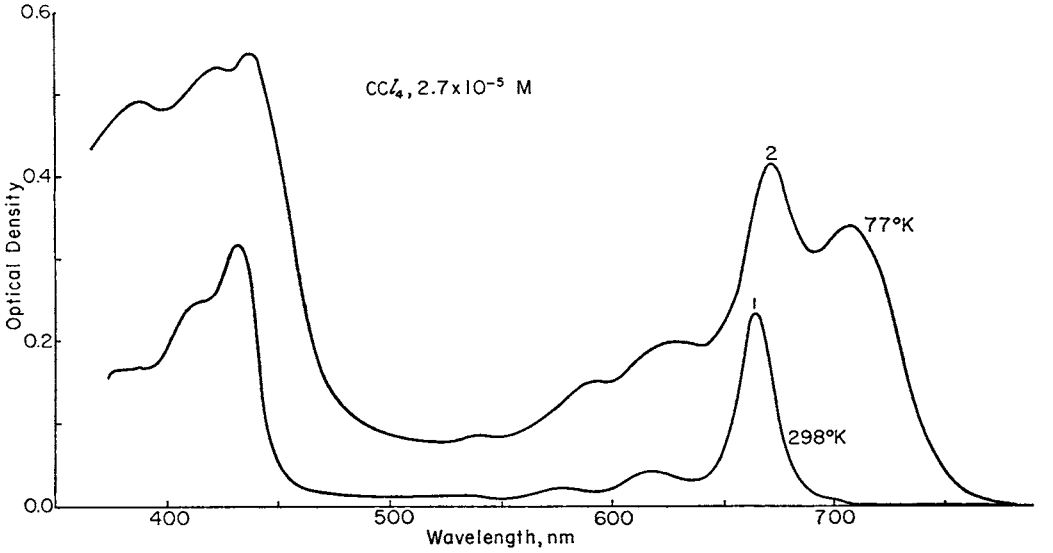

FIGURE 8 Absorption spectra of chlorophyll *a* in CCl_4, 2.7 × 10^{-5} M. Curve 1, 298°K; curve 2, 77°K.

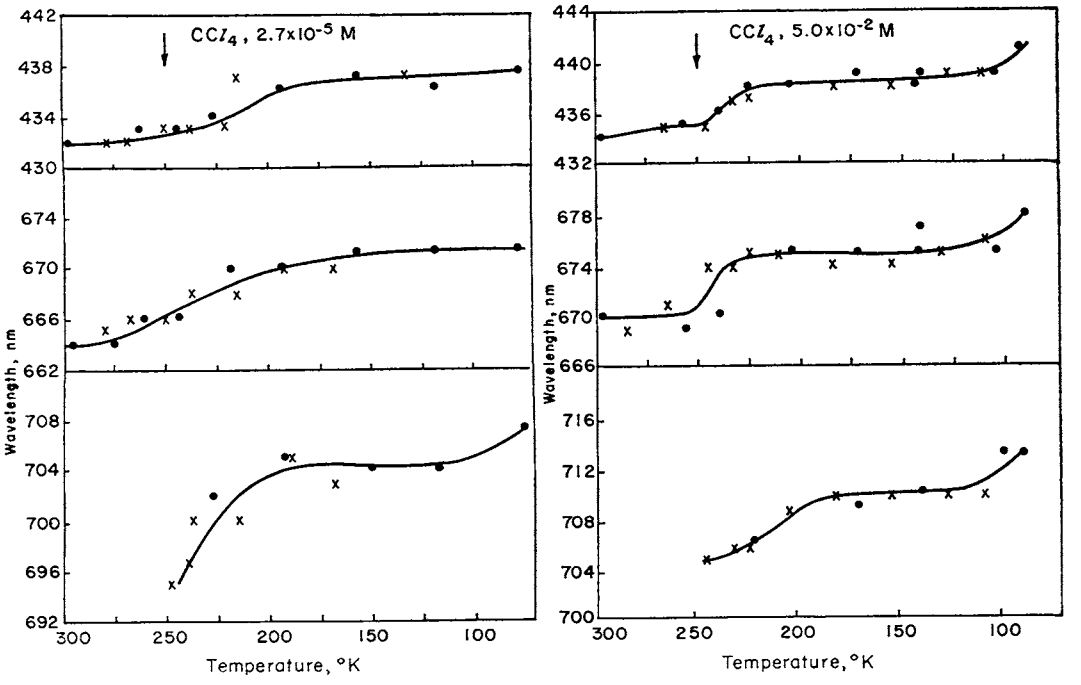

FIGURE 9 Absorption maxima of chlorophyll *a* in CCl_4, as a function of temperature for 2.7 × 10^{-5} M (left) and 5.0 × 10^{-2} M (right). Cooling cycle is given by dots, warming cycle is given by crosses.

At the lowest temperature, 96°K (curve 4) there is a band at 712 nm, and the main red and blue maxima which occur at 670 and 434 nm at room temperature are red shifted to 678 and 441 nm. The main red absorption band is broader and more assymetric at low temperature, and the satellite on the short-wavelength side of the Soret band is more prominent. The temperature dependence of the peak positions is given in Fig. 9. Similar changes occur in a 5.0×10^{-3} M solution; the tem-

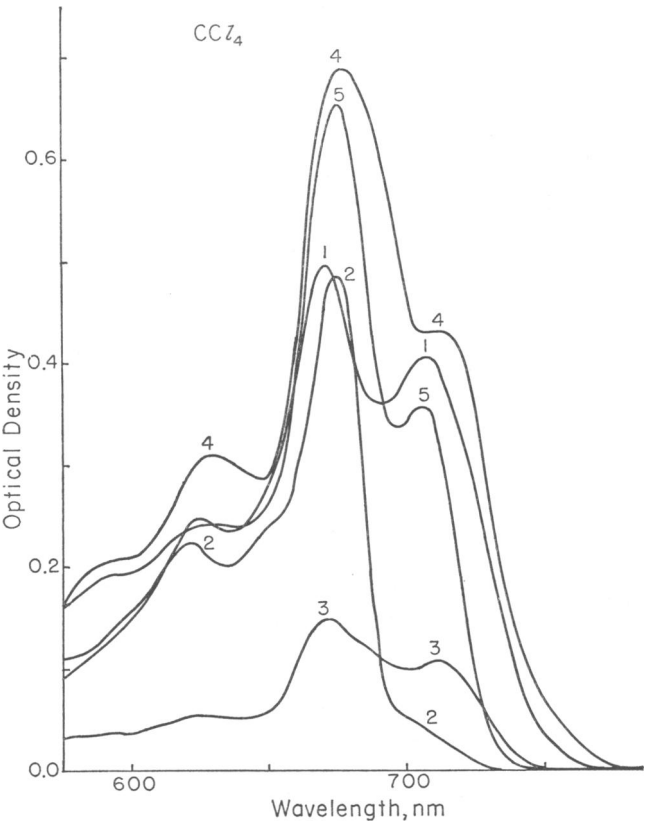

FIGURE 10 Absorption in the red region of the spectrum of chlorophyll *a* in CCl_4. Curve 1, 2.7×10^{-5} M, 77°K; curve 2, 2.7×10^{-5} M, plus 0.5% ethanol, 77°K; curve 3, 5.0×10^{-3} M, 121°K; curve 4, 5.0×10^{-2} M, 92°K; curve 5, 4.5×10^{-2} M, plus 10% ethanol, 77°K.

perature dependence of *R* for this concentration is given in Fig. 11. *R* is constant on warming up to the melting point of CCl_4, 250°K. Above this temperature the "700" nm band disappears completely; nevertheless *R* decreases slightly on further warming to room temperature. No differences were noted in *R* between the warming and the cooling cycle except for the few points near the melting point, which probably result from supercooling of the solvent. The 5.0×10^{-3} M solution differs from the 5.0×10^{-2} M in two respects. In the former, the "700" nm band at the lowest tem-

perature (121°K) is at shorter wavelengths, namely 709 nm (Table I). Also the room temperature absorption spectra are somewhat different, the assymetry of the red band being more pronounced in the 5.0×10^{-3} M solution (compare Fig. 12, curve 1 and curve *A*).

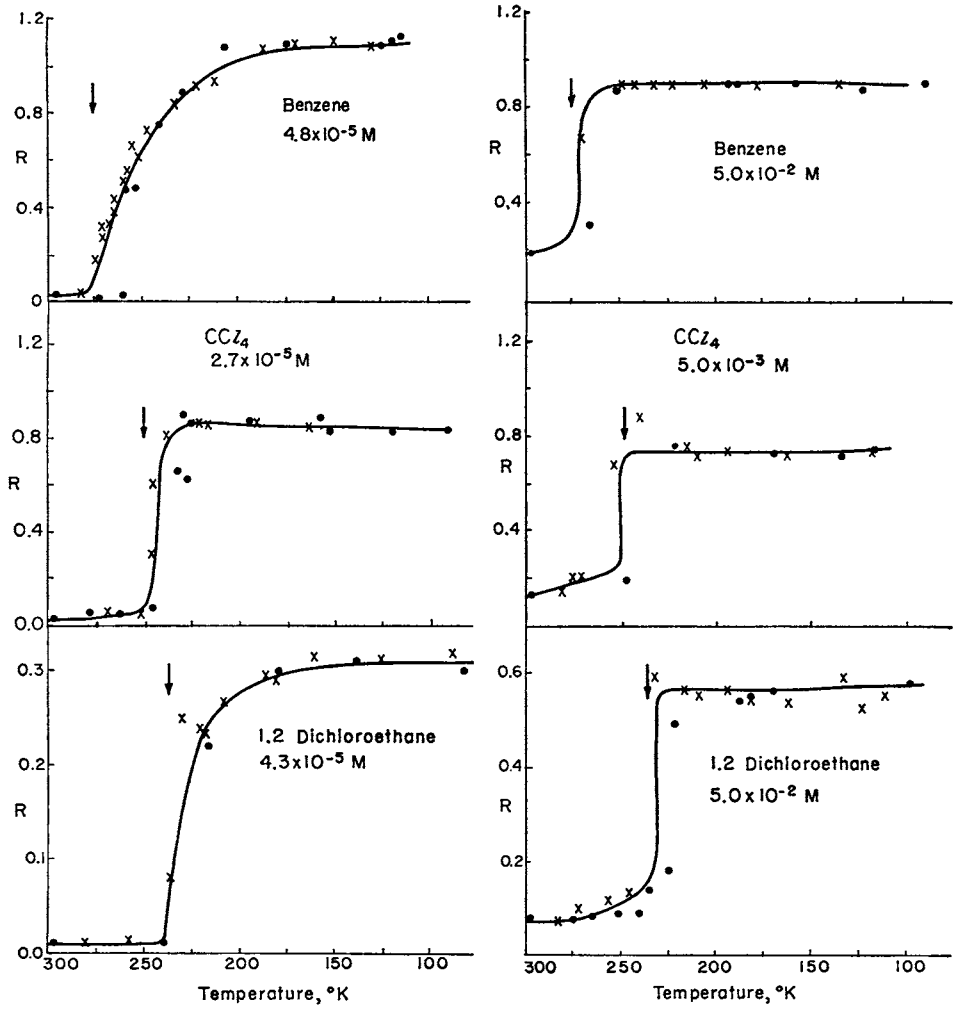

FIGURE 11 Ratio, *R*, of "700" nm band to main red absorption band as a function of temperature in various solvents. Cooling cycle is given by dots, warming cycle is given by crosses. Solvent melting points are indicated by arrows.

When 0.5% ethanol is present in a dilute (2.7×10^{-5} M) CCl_4 solution of chlorophyll *a*, cooling still causes a red shift in all bands. However, the "700" nm band is not resolved even at 77°K although there is a slight increase in absorbance near "700" nm (Fig. 10, curve 2). However, a band near 655 nm, similar to that found in pure ethanol solutions, does occur.

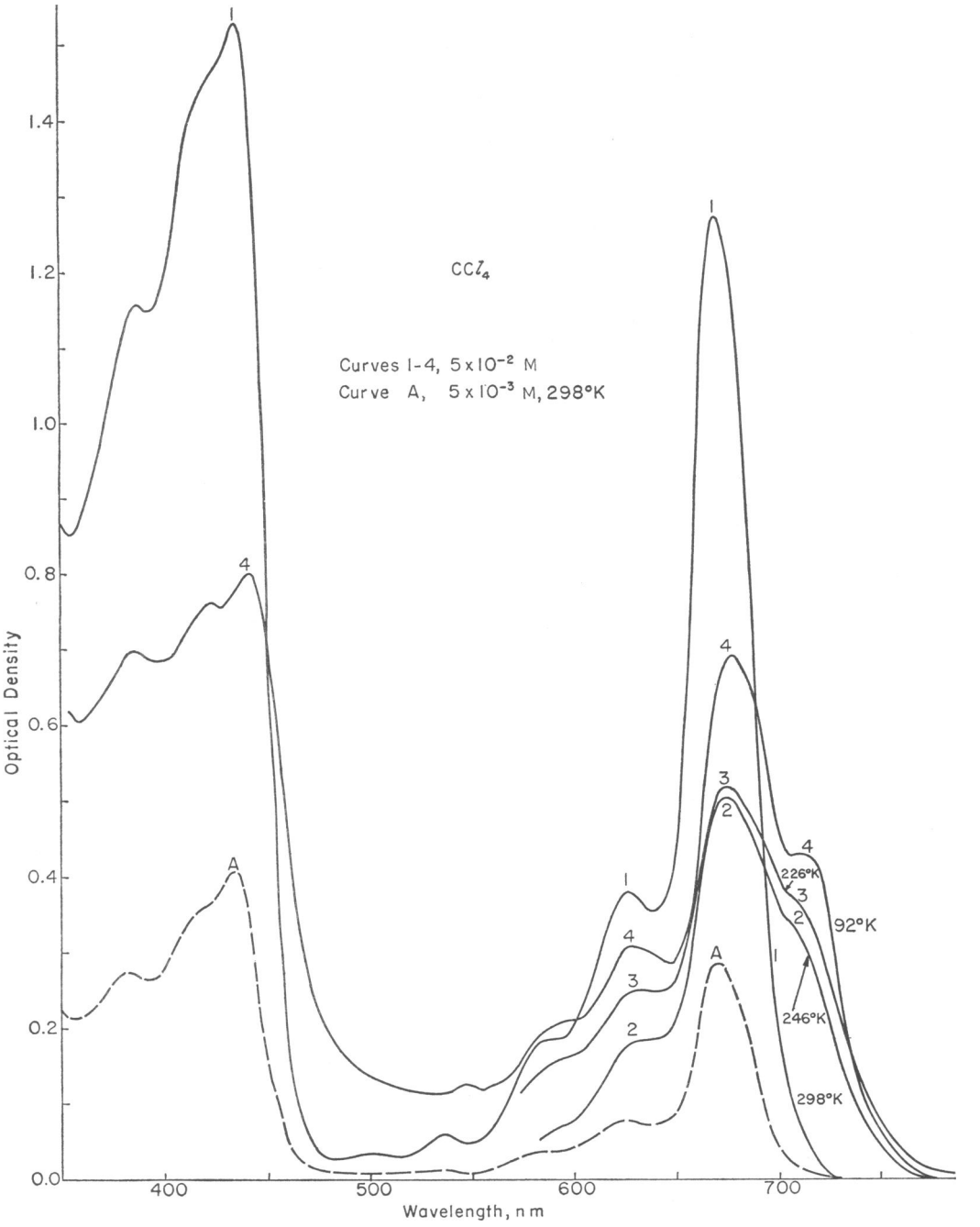

FIGURE 12 Absorption spectra of chlorophyll *a* in CCl_4. Curves 1–4, 5.0×10^{-2} M at temperatures given in the figure. Curve *A*, 5.0×10^{-3} M, 298°K.

When as much as 10% ethanol is added to the 5.0×10^{-2} M CCl_4 solution, cooling still causes the "700" nm band to appear (Fig. 10, curve 2) although it now appears over a larger temperature range beginning at about 220°K and reaching full intensity at about 170°K. The temperature dependence of R is qualitatively like that obtained for the 6.0×10^{-2} M ethanol solution (See Fig. 5, upper curve). At 77°K the maximum of the "700" nm band is at 707 nm.

Benzene. Results for this solvent are similar to those described for CCl_4. See Fig. 11 for the variation of R with temperature for concentrated and dilute solutions. In the concentrated solutions, R begins to decrease on warming over a narrow temperature range (about 15°) near the melting point (278.5°K) while in the dilute system R varies over a larger temperature range, from 200° to 278°K. In both cases the "700" nm band disappears at the melting point. The maximum of the "700" nm band, at low temperature, is again at shorter wavelengths in the dilute (4.8×10^{-5} M) than in the concentrated (5.0×10^{-2} M) solution, being at 706 nm in the former case and at 715 nm in the latter.

To determine if the changes in R were time dependent, warming was allowed to proceed gradually in the dilute solution taking about 2 hr for the entire temperature range, while cooling was rapid. As shown in Fig. 11, the variation of R with temperature is reversible, at least once the solvent has frozen. In the cooling cycle, benzene was supercooled. To further examine the possibility of time-dependent changes in R dilute solutions were rapidly immersed in liquid nitrogen or in an ice-salt mixture (252°K) and maintained at these temperatures for ½ hr. In both cases R was constant for this period of time. However, the value of R on rapid freezing to 77°K was 0.84 which is 20% lower than when cooling is slow.

1,2 dichloroethane. In dilute solutions of this solvent, the "700" nm band is not resolved on cooling but the optical density near 700 nm rises noticeably with respect to the main red absorption band. On the other hand, in concentrated solution the "700" nm band does appear. The variation of R with temperature for dilute and concentrated solutions is given in Fig. 11. R changes near the melting point in a manner similar to that observed for CCl_4 and benzene, i.e. over a temperature range of 40°K in the dilute solution, while in the concentrated system almost the entire change is at the melting point. At temperatures above the melting point, R continues to decrease slightly in concentrated but not in dilute solution, as occurs in the other solvents, and the "700" nm band is not resolved in this temperature range.

DISCUSSION

Effect of Cooling on Absorption Spectra of Monomers

Dilute solutions of chlorophyll *a* in polar solvents are not aggregated at room temperature or at 77°K. Therefore, changes in their absorption spectra on cooling are due to changes in the monomer bands.

The red shifts suffered by the monomer bands in polar media may arise because of an increase in the polarity of the solvent at low temperature. It is well-known that the absorption bands of chlorophyll are red-shifted in more polar solvents; these shifts have been correlated with the refractive index and to a lesser extent with the dielectric constant of the medium (see, e.g., reference 13). On cooling, the dielectric constant of ethanol increases markedly, from 25.8 at 292°K to 56.6 at 163°K, so that this increased polarity probably accounts for the red shift at low temperature. Regions of highly polar environment may also contribute to the red shifts in the absorption bands of chlorophyll in the plant as compared to their positions in solution. In nonpolar solvents the dielectric constant is practically constant with temperature and the red shifts at low temperature are due to further aggregation (see below).

The low temperature absorption band, near 640 nm in EPA and 655 nm in ethanol, and in CCl_4 containing a small amount of ethanol is reminiscent of a similar band occurring at room temperature in pyridine at 638 nm (17). This band seems to be characteristic of chlorophyll *a* in polar solvents: the red shift and the narrowing of the main red band permit it to be resolved at low temperature in EPA and ethanol. In pyridine the main red absorption band is already at 671 nm at room temperature. The occurrence of this band is no doubt related to a coordination of chlorophyll with the polar solvents (13, 18–20). In fact, its presence is a sensitive test for trace amounts of polar solvent (Fig. 10, curve 2).

Differences in the Absorption Spectra of Dilute and Concentrated Solutions at Room Temperature

On going from dilute to highly concentrated chlorophyll solutions there is a red shift of the main red absorption band and an increase in its half bandwidth, as well as a slight red shift in the blue band (see Table I). These changes are due to the presence of dimers which absorb at longer wavelengths than the monomers (14, 21, 22). In fact, in nonpolar solvents at high concentration the chlorophyll is largely dimeric (14, 19). In CCl_4, for example, where the equilibrium constant for dimerization is 10^4 M (14), solutions containing 5.0×10^{-3} and 5.0×10^{-2} M are 90 and 96% dimeric, respectively. Thus, the spectra in Fig. 12, curves 1 and *A*, are essentially those of the dimer. While the spectrum of the 5.0×10^{-3} M solution is very similar to the one calculated for chlorophyll dimers in CCl_4 (14), the red band of the 5.0×10^{-2} M solution is less assymetric and narrower. The dimer red band is assymetric because it is comprised of two components whose relative intensities are determined by the geometry of the dimer (see also below). Thus, it appears that the dimer structure in 5.0×10^{-2} M solutions is different from that in 5.0×10^{-3} M solutions. Higher aggregates have not been found in CCl_4 solutions, even at concentrations approaching 10^{-1} M for chlorophyll *a* (19) so these differences in their spectra at the two concentrations are not due to different degrees of aggregation.

The "700" nm Low Temperature Band

Relation to the State of Aggregation of Chlorophyll a. Chlorophyll is solvated at the magnesium when polar materials are present and the state of solvation is a factor in the state of aggregation at room temperature (14, 19, 20, 23). When the molecules are solvated aggregation is prevented in dilute solutions. In concentrated solutions, however, solvation reduces but does not eliminate aggregation (22).

The "700" nm band arises on cooling in those systems where chlorophyll can dimerize, i.e., in nonpolar solvents and in polar solvents at high concentration. In dilute nonpolar solutions as little as 0.5% ethanol prevents the "700" nm band from appearing, while at high concentration it appears even when 10% ethanol is present. Thus, the earlier evidence (6–9, footnote 1) that it originates from an aggregated form of the pigment is further substantiated.

Origin of the Band in Polar Solvents. Since the "700" nm band appears at low temperature, one must consider the possibility that it is due to chlorophyll that comes out of solution on freezing. However, the band occurs in EPA which has no definite phase transition and, in ethanol, it reaches full intensity before the solution solidifies. Furthermore, absorption by chlorophyll microcrystals is not near 700 nm, but at 725 nm and beyond (24). When such microcrystals form in our ethanol solutions (Fig. 4, curve 6) the spectra are no longer reversible with respect to temperature, i.e., crystallization is not a reversible process. Similarly, the reversibility argues against a colloidal suspension causing the "700" nm low temperature absorption. Love and Bannister (25) have found that the formation of chlorophyll colloids is an irreversible process.

Next we consider the possibility that the "700" nm band is characteristic of chlorophyll dimers present prior to cooling but absorbing at shorter wavelengths at room temperature as shown earlier (22). Assuming this band is the long wavelength–red absorption maximum of chlorophyll dimers at low temperature in ethanol, then, as a first approximation, $R = 2\,[D]/[M]$, where $[D]$ and $[M]$ are dimer and monomer concentrations, respectively. The factor of two enters because the dimer extinction coefficient is approximately twice that of the monomer. It is also assumed that absorption at about 670 nm at low temperatures is primarily due to monomers. This is valid only for systems where dimers are a minor constituent, such as in ethanol. At room temperature $[D]/[M]$ can be calculated from the equilibrium constant for dimerization in ethanol, 4.5 ± 0.8 M (22). In Fig. 5, lower curve, $[D]/[M]$ and $R/2$ are plotted as a function of monomer concentration. It can be seen that the concentration of the "700" nm absorbing species (at 77° K) and the concentration of dimer (at 298° K) are governed by a similar equilibrium constant for solutions brought rapidly to 77°K. An analysis of fluorescence excitation spectra (8) has also led to the conclusion that the "700" nm band is due to dimers in polar solvents.

Measurements of fluorescence quantum yields (26) have shown that dimers do

not form on cooling in ethanol and the present absorption studies confirm this for solutions that are frozen rapidly. On the other hand, when concentrated ethanol solutions are cooled *slowly*, higher values of R are obtained, indicating that aggregation does occur while the solvent is in the liquid state.

Origin of the Band in Nonpolar Solvents. In nonpolar solvents the intensity of the "700" nm band cannot be correlated with the degree of dimerization at room temperature. In fact, the band is very prominent in dilute solutions where, for example, in CCl_4 only 15% of the total chlorophyll (2.7×10^{-5} M) is dimerized at room temperature.

Although the "700" nm band beings to appear at the solvent freezing point it does not seem that solute has frozen out because the absorption properties of this band ere not those of chlorophyll microcrystals. Furthermore, the reversibility in the temperature dependence of R speaks against its being due to a phase change by chlorophyll. This band has also been noted by Amster and Porter (7, 8) in 3-methyl pentane which forms a glass on cooling.

Next we consider the possibility that dimers or small polymers are giving rise to the 700 nm band. Fig. 11 shows that R increases somewhat on cooling even before the freezing point in concentrated solutions although the "700" nm band is not resolved. This increase in R reflects an increase in the half bandwidth of the 670 nm band which occurs in the concentrated solutions. The half bandwidth is a sensitive indicator of aggregation (21) so it appears that further aggregation (to form more dimers and/or polymers) is taking place in the concentrated solutions while the solvent is liquid. In dilute solutions on the other hand, (see Fig. 11) aggregation does not occur at temperatures above the freezing point. However, the "700" nm band is very intense at 77°K although the degree of dimerization at room temperature is small. Consequently, aggregates are also forming in these dilute solutions presumably at the freezing point. Fluorescence spectra have previously shown that aggregation takes place on cooling in nonpolar solvents (26). The rate of cooling influences the extent of this aggregation as shown by the lower value of R which is obtained when freezing is rapid as compared to cooling slowly. (The variation of R is much greater for ethanol than for nonpolar solvents probably because ethanol is liquid over a much greater temperature range.) It should be possible to determine whether aggregates higher than dimers are present in nonpolar solvents below the freezing point from low temperature infrared spectra since dimers and higher polymers have different infrared absorption peaks. At toom temperature, dimers absorb at about 1655 cm^{-1} while higher aggregates have a band near 1640 cm^{-1} (19).

Differences in Aggregate Structure at Room and Low Temperatures. The presence or formation of aggregates does not explain the increase in intensity of the "700" nm band after the solvent has frozen. These further changes must be due to another process since continued aggregation is not like when the medium is rigid.

Theoretical analyses of the spectral properties of aggregates have shown that aggregation causes the absorption bands of the monomer to split (see e.g., references 27 and 28). The position of the aggregate bands with respect to that of the monomer and the relative intensities of the components depend on the orientation of the monomer units with respect to each other, and on the degree of coupling between them. When coupling is strong, large shifts in the center of the transition are predicted.

As determined by Sauer, Smith, and Schultz (14) the red absorption band of the chlorophyll *a* dimer has two components in CCl_4 at 665 and 682 nm at room temperature and similar dimer spectral properties in ethanol were found in our laboratory (22). Fluorescence excitation spectra at 77°K have shown that the "700" nm band is accompanied by another new band, at 676 nm in ethanol and at 682 nm in pyridine (8). In CCl_4 such a band is also indicated by the increased assymetry on the long-wavelength side of the 670 nm band (Fig. 12) at low temperature. This absorption near 676 nm at 77°K may well be the short wavelength component of the "700" nm band. The absorption spectrum of the "700" nm form alone could not be determined due to difficulty in measuring quantitatively accurate absorption difference spectra at low temperature.

Changes in the orientation of the monomers in the aggregate could cause the changes in absorption spectra on cooling. A change in geometry, whereby coupling between the monomer units is increased, would explain the large red shift in the center of the transition when the "700" nm band appears. Such a reorientation may well occur at the freezing point in the nonpolar solvents; further changes can take place even when the medium is rigid, with attendant increases in the long wavelength ("700" nm) component at the expense of the short (676 nm) wavelength component. A similar geometric rearrangement could occur in ethanol which becomes viscous in the temperature range where the "700" nm band is resolved. (However, for polar solvents the red shift in the dimer band may be at least partly due to the high dielectric constant at low temperature). Differences in orientation could also be responsible for the slightly different peak positions of the "700" nm band at different chlorophyll concentrations. Of course, a difference in the size of the aggregate would also produce different peak positions as well as the shift in the transition center. At room temperature, concentration dependent differences in spectra, which could be related to geometry were noted above for dimers in CCl_4. Stensby and Rosenberg (6) have pointed out that the quenched fluorescence of chlorophyll dimers at room temperature in ethanol would be accounted for if the structure of this species at 298°K were different from that at 77°K.

We gratefully acknowledge support for this research by the National Science Foundation under Grant GB-7244. S. S. Brody was supported (in part) by a Public Health Service Research Career Program Award K3-GM-17, 918.

Received for publication 23 June 1968.

REFERENCES

1. FREED, S., and K. SANCIER. 1951. *Science.* **114**:275.
2. FREED, S., and K. SANCIER. 1952. *Science.* **116**:175.
3. FREED, S., and K. SANCIER. 1954. *J. Am. Chem. Soc.* **76**:198.
4. FREED, S., and K. SANCIER. 1957. *Science. 125*:1248.
5. LINSCHITZ, H. 1956. *In* Photosynthesis. Vol. II, 2. E. Rabinowitch. Interscience Publishers, Inc., New York. 1802.
6. STENSBY, P., and J. ROSENBERG. 1961. *J. Phys. Chem.* **65**:906.
7. AMSTER, R., and G. PORTER. 1966. *Proc. Roy. Soc. A.* **296**:38.
8. BRODY, S. S. 1968. *Biophys. J.* **8**:210.
9. VERNON, L. P., B. KE, and R. CLAYTON. 1966. *In* The Chlorophylls. L. P. Vernon and G. R. Seely, editors. Academic Press, Inc., New York.
10. JACOBS, E., A. VATTER, and A. S. HOLT. 1954. *Arch. Biochem. Biophys.* **53**:228.
11. ANDERSON, A., and M. CALVIN. 1963. *Nature.* **194**:1097.
12. BROYDE, S., and S. BRODY. 1966. *Biophys. J.* **6**:303.
13. SEELY, G., and R. JENSEN. 1965. *Spectrochim. Acta.* **21**:1835.
14. SAUER, K., J. SMITH, and A. SCHULTZ. 1966. *J. Am. Chem. Soc.* **88**:2681.
15. STEPHEN, H., and T. STEPHEN, editors. 1963. Solubilities of Inorganic and Organic Compounds. The Macmillan Co., New York. Vol. I.
16. GREENSPAN, H., and E. FISCHER. 1965. *J. Phys. Chem.* **69**:2466.
17. MCCARTIN, P. J. 1963. *J. Phys. Chem.* **67**:513.
18. LIVINGSTON, R. 1960. *Radiation Res. Suppl.* **2**:196.
19. KATZ, J., G. CLOSS, F. PENNINGTON, M. THOMAS, and H. STRAIN. 1963. *J. Am. Chem. Soc.* **85**: 3801.
20. CLOSS, G., J. KATZ, F. PENNINGTON, M. THOMAS, and H. STRAIN. 1963. *J. Am. Chem. Soc.* **85**: 3809.
21. SEELY, G. 1967. *J. Phys. Chem.* **71**:2091.
22. BROYDE, S., S. BRODY, and M. BRODY. 1968. *Biochim. Biophys. Acta.* **153**:183.
23. ANDERSON, A., and M. CALVIN. 1964. *Arch. Biochem. Biophys.* **107**:251.
24. JACOBS, E., A. HOLT, R. KROMHOUT, and E. RABINOWITCH. 1957. *Arch. Biochem. Biophys.* **72**: 495.
25. LOVE, B., and T. BANNISTER. 1963. *Biophys. J.* **3**:99.
26. BROYDE, S., and S. BRODY. 1967. *J. Chem. Phys.* **46**:3334.
27. KASHA, M. 1963. *Radiation Res.* **20**:55.
28. DEVOE, H. 1964. *J. Chem. Phys.* **41**:393.