Plant Physiol. (1972) 49, 421-429

Four Universal Forms of Chlorophyll *a*[1]

Received for publication June 14, 1971

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ABSTRACT

We have matched the red absorption band measured at −196 C in a variety of chloroplast preparations with four major component curves representing forms of chlorophyll *a* having peaks at 661.6, 669.6, 677.1, and 683.7 nanometers. Chloroplast fractions enriched in one or the other of the two photochemical systems both contain these four major components, but system 1 preparations contain relatively more chlorophyll *a* 684. Chlorophyll *a* 677 and chlorophyll *a* 684 have greater bandwidths in system 1. Bands at longer wavelengths near 693 and 704 nanometers also often occur, but with far smaller heights than the above major bands. The longer wavelength bands are more common in system 1 than in system 2. In system 1 the half-widths of the four major bands in typical spectra average 11.3, 10.0, 10.3, and 10.8 nanometers while in system 2 they are 11.6, 9.8, 9.4, and 9.6 nanometers. Some spectra with sharper and some with wider bands were found, but the wavelengths were identical.

The wide variations in the shape of the red absorption band of chlorophyll *a* in plants are due to the presence, in various proportions, of a number of forms of chlorophyll *a* with different absorption maxima in the 660 to 720 nm region. We wanted to determine the individual absorption spectra of these different forms of chlorophyll and to see if these forms are identical in various species. The question is whether there are only a few specific chlorophyll complexes always having constant absorption peak wavelengths and constant half-widths or if there are a large number of complexes with variable spectroscopic properties. With complex spectra it is possible, by a curve analysis, to determine the precise peak position, the half-widths, and the relative proportions of the components themselves.

A long standing objective has been to see how far it is profitable to go in the interpretation of absorption spectra by fitting the data with the sums of simpler curves such as Gaussian components. We try here to define explicitly the potentialities and limits of this approach to the study of the native forms of chlorophyll.

We have been particularly interested in the spectra of chloroplast fractions 1 and 2 that are enriched either in photosystem 1, which produces reducing power and has more long wavelength pigments, or in photosystem 2, which is directly responsible for O_2 evolution and contains more chlorophyll *b* than does fraction 1.

Various articles illustrate the diversity of opinions about the different forms of chlorophyll (1, 2, 6, 14, 15, 20). Two forms of chlorophyll *a*, "C*a*[2] 670" and "C*a* 680," have generally been considered to constitute most of the green color of plants. Previously we have made many curve analyses of the absorption spectra of various algae, chloroplasts, and chloroplast fractions to determine the peak position and the half-widths of C*a* 670 and C*a* 680. This was done by fitting the observed absorbance spectra with the sums of Gaussian curves. The results always showed that C*a* 670 had a greater half-width than C*a* 680 and that in system 1 fractions of chloroplasts C*a* 680 always had a greater half-width than in system 2 fractions (8, 9, 12, 13). Furthermore, the peak positions and the half-widths of both these hypothetical C*a* 670 and C*a* 680 components varied over a wide range of wavelengths when spectra of different preparations were compared. Such a variation in peak wavelengths and in half-width would result if each of the two assumed components were, in fact, composed of two separate chlorophyll forms with fixed peak positions and widths occurring in different proportions in the various samples.

Recent curve analyses have, however, made it seem necessary to interpret chlorophyll absorption spectra *in vivo* as being composed of four rather than two major forms of chlorophyll *a* (22). These four components now seem to be widely distributed and to have constant peak wavelengths, but their half-widths differ between different preparations. Variations in the proportions of these components account for the wide diversity of spectra that are found.

We report here close fits to absorption spectra, measured at −196 C, in the 640 to 690 nm region using four major bands for chlorophyll *a* with average peak positions of 661.6, 669.6, 677.1, and 683.7 nm and two bands for chlorophyll *b* with average peak positions of 640.1 and 649.5 nm. Other longer wavelength bands in the 690 to 720 nm region are present in some, but not in all, preparations. The results support the constant component hypothesis in a variety of algae and in several representative higher plants. Furthermore, good fits have been obtained with bands at those wavelengths for many less distinct spectra. We will present several typical spectra from which the wavelength peaks and half-widths of these bands were derived and will describe the curve analysis methods used for doing so. Part of this work has been discussed in a preliminary report (10), but at that time the significance of the differences in width of the components was not appreciated. The wavelengths reported here are corrected for a spectrophotometer calibration error. Previously published wavelength values were 0.8 nm too high.

CONSIDERATIONS GOVERNING THE CURVE-FITTING PROCEDURE

Since the component bands of chlorophyll *a* absorption are appreciably sharper at liquid nitrogen temperature (−196 C) than at room temperature, the low temperature spectra

[1] C.I.W.-D.P.B. publication No. 479.

[2] Abbreviation: C*a*: chlorophyll *a*.

are far better for identifying the components. Only low temperature spectra are discussed in this article. The following comments on curve fitting and on the individual bands themselves refer to data and components similar to those shown in Figure 1.

The absorption of light by plants in the orange and red part of the spectrum is due almost entirely to chlorophyll. In the blue, where chlorophyll also has characteristic bands (14), there is strong overlapping absorption by carotenoids and other colored substances. For this reason, we are concerned only with the region from about 570 to 740 nm where, except for the prominent 650 and the very small 640 nm bands of chlorophyll *b*, the spectrum is composed of overlapping spectra of different forms of chlorophyll *a*. Figures 1 to 3 show some absorption spectra illustrating the wide variety of shapes that may be found.

The Shape of Individual Bands. The fits of Gaussian curves to chlorophyll absorption spectra are far better than those of Lorentzian curves. The tails of Gaussian curves are slightly too low, while those of Lorentzian curves are far too high. Addition of Gaussian and Lorentzian curves of the same width and peak wavelength in an adjustable proportion may give a better fit in the long wavelength tail region. In the present work, however, only pure Gaussian curves have been used in order to reduce the number of parameters needed to describe the components. However, it is highly likely that unsymmetrical bands, broader on the short wavelength side, would be more realistic than are symmetrical curves. Such a skew may be introduced by adding a small Gaussian component on one side of a main Gaussian band.

The Red Tail. The main red absorption band in spectra of chloroplast fractions can be reasonably well matched from 655 to 690 nm by the sums of four Gaussian curves. The red tail beyond about 690 nm may often require one or more extra components. As far as we now know these long wavelength forms may have their peaks anywhere from 690 to beyond 710 nm. It is often difficult to decide whether a small difference between the measured absorbance spectrum and the sum of the long wavelength tails of the 677.1 and 683.7 nm components is due to the presence of a real component or to the inadequacy of Gaussian curves for representing the tail of a single absorption band with sufficient precision.

The 625 nm Region. A more serious difficulty than with the long wavelength tails occurs in the 625 nm region. Here we presume there must be one or two vibrational bands for each of the four main forms of chlorophyll. But since these side bands of each chlorophyll form are much wider than those of the main peaks, we cannot yet resolve them. All these bands in the 625 nm region fuse together, and their sum can be roughly approximated by a single broad band. The peak position and shape of this composite 625 nm band vary with the proportions of the various chlorophyll forms. In analyzing the shape of the main peak some sort of a secondary band near 625 nm must be included because its long wavelength side overlaps the 662 nm band. Therefore, an estimate of the composite 625 nm band is used in the present series of curve analyses. G. R. Seely (personal communication) has found that the vibrational band in spectra of pure chlorophyll dissolved in certain organic solvents may have two components. A band at 628 nm about 35 nm in half-width and a quarter of the main peak's height, as shown without a label in Figure 1, will fit most of the spectra.

The 655 nm Region. Between the main peak and this first obvious vibrational band, near 625 nm *in vivo* and at 615 nm in ether or acetone, there is a hidden band showing clearly only at low temperature (20). However, its inclusion is necessary for a Gaussian curve analysis even of room temperature spectra for pure chlorophyll *a* (9). In spectra of pure chlorophyll *a* at room temperature measured in 80% acetone this hidden band comes at 652 nm. Therefore, to fit spectra of natural chlorophyll complexes a corresponding composite 655 nm band was included in Reference 22 and is discussed in Reference 10. Like the 625 nm composite vibrational band, this one also represents the sum of all the chlorophyll forms present. Adequate fits can, however, sometimes be realized without this 655 nm band, and it has been omitted in the work reported here. When the 655 nm band is omitted a corresponding increase in height and a minor increase in width of the curves representing all the major components shorter than 684 nm take place. The error curves in Figure 17 of Reference 10 show that a better fit results from using a 655 nm band in the analyses of fraction 1 for *Scenedesmus*. Without it the 650 nm chlorophyll *b* band and the 662 nm chlorophyll *a* band may, in part, be broadened and raised in height, since they both may be forced to substitute partially for the omitted broad 655 nm components. That effect on the 662 band appears strongly in curve analyses of spectra lacking C*b* 650. For this reason, some of the 662 components of Table IV are not considered to represent the true width of actual forms of chlorophyll *a*. Furthermore, the uncertainty in the area of the chlorophyll *b* bands due to omission of the 655 nm component makes it impossible to estimate reliably the relative proportions of chlorophylls *a* and *b* from curve analyses alone.

Criteria for Selection of Significant Spectra for Curve Analysis. Spectra of normally green whole chloroplasts or of whole algae are inevitably flattened by the sieve effect, so that they cannot be considered to represent the sums of the spectra of the individual pigments in the preparation (18). Therefore, we discuss here only spectra of very small particles prepared from finely disintegrated chloroplasts.

To get a rough estimate as to whether or not there is a serious distortion from the flattening effect of optically dense particles in suspension we compare the peak heights of the 625 and of the 670 to 680 maxima. The height, H, of the 625 nm peak expressed as a fraction of the major peak height is 0.2 for chlorophyll *a* in 80% acetone. A common range for H in chloroplast fractions is 0.25 to 0.30 (18). Values much above 0.30 suggest possible complications from the flattening effect.

For measurements of absorption spectra of scattering samples some arrangement for collecting a representative fraction of the light scattered in all directions is desirable. The opal glass system of Shibata has been used for measurements made in this laboratory with the spectrophotometer described in Reference 11. The slit width was 1 nm. Spectra useful for determining the the characteristics of their component bands must have peaks distinct enough to establish the peak wavelengths and widths of several, although not necessarily all, the components in one spectrum. The more common spectra that have the components present in such proportions as to smear out the measured spectrum into a nearly smooth curve can be analyzed by the use of known components, but such spectra are not suitable for determining the peak wavelengths and widths of the components.

MATERIALS AND METHODS

Preparations were made by forcing various algae or leaf chloroplasts through a needle valve and centrifuging the resulting homogenate at 3000*g* for 10 min to yield a relatively clear, green supernatant. This supernatant was either used directly or was fractionated by centrifuging in a sucrose density gradient (10–50%, w/v) at 60,000*g* for 30 to

60 min. This procedure (5) produced an upper, light fraction 1 composed of stroma lamellae fragments, enriched in photosystem 1, and a heavier fraction 2 below containing grana stack fragments, enriched in photosystem 2 (19). The algae, originally obtained from the Indiana Culture Collection, Indiana University, Bloomington, Indiana, were *Anacystis nidulans, Chlorella pyrenoidosa, Euglena gracilis, Plectonema boryanum, Scenedesmus obliquus* D_3, and *Stichococcus bacillaris*. They were grown autotrophically in 1-liter batches, and the cells were harvested near the end of the logarithmic phase. Of these algae, only the spectrum of *Euglena* changes appreciably with different growth conditions (4). *Scenedesmus* mutant 8 was supplied by Prof. Norman Bishop, Oregon State University, and was grown heterotrophically. The *Oenothera* mutants were obtained from Prof. W. Stubbe and prepared according to the method of Fork *et al.* (7). The sorghum bundle sheath or mesophyll chloroplast preparations were made by Dr. J. Berry according to the method of Woo *et al.* (23) with differential grinding. The chlorophyll protein from *Brassica oleracea* (mustard) was prepared by Mrs. Murata (16).

CURVE-FITTING PROCEDURE

Digitization of Recorded Curves. A curve digitizer was used to tabulate the curve heights at intervals of 1 nm. The least count of this device is 0.00842 inch per unit, thus giving a resolution of about 0.1% of full scale for a curve 8 inches high. Visual setting of the cursor facilitates averaging the minor irregularities in the plotted spectra while digitizing. There is both an automatic readout system depending on a shaft angle encoder connected to the ACME computer and a counter that can be read by the operator.

The RESOLV Program. Some useful capabilities of the curve analysis program obtained through the kindness of Dr. D. D. Tunnicliff of the Shell Development Laboratory, Emeryville, California, have been described (9, 10). For the work summarized here that program is used to transform the wavelength data to wave number, to modify estimated Gaussian input bands as necessary to achieve an optimal fit of their sum to the absorbance data, to calculate the error of fit at each point, and to tabulate and plot the results. We have modified the program to permit specifying the allowed variation of the individual input bands. Thus the adjustment of the peak wavelength and the width of each band can be limited either for each iteration or for the final result. For the curve analyses reported here, the estimated input bands' wavelength and half-widths usually were: 590.0, 45.0; 628.0, 35.0; 640.0, 10.0; 650.0, 10.0; 663.0, 10.0; 670.0, 10.0; 678.0, 10.0; 684.0, 10.0; (and when necessary) 695.0, 12.0; 705.0, 15.0. For repeated analyses, widths of 8.0 or 9.0 sometimes were used for the 670.0 and 678.0 input bands. For Figure 3 the bands derived in Tables I and II were used as input bands. We have allowed 12 iterations for each analysis and permit the major peaks to change in wavelength position or in width by 0.3 nm per iteration. The broader bands at 590, 628, and 655 nm and the long wavelength bands, usually at 695 and 705 nm, are allowed from

Table I. *Peak Wavelengths of Components*

Curve No. and Material	SE, % of Peak	Chloropyll *b*		Chlorophyll *a*					
		Cb 640	*Cb* 650	*Ca* 662	*Ca* 670	*Ca* 677	*Ca* 683	*Ca* 691	*Ca* 704
					nm				
Fraction 1 preparations									
Very Sharp spectra									
C34G, *Stichococcus*	0.34	639.9	649.5	661.7	669.6	676.9	683.4	691.6	...
C71C, *Scenedesmus*	0.45	(640.9)[1]	649.2	661.5	669.4	677.2	683.6	693.3	(702.5)
Typical green algal spectra									
C27D, *C. pyrenoidosa*	0.32	641.2	649.7	661.4	669.3	677.2	683.6	692.7	(704.3)
Typical green leaf spectra									
C5D, spinach	0.37	640.9	650.6	662.1	669.6	677.0	683.7	691.4	705.4
C106A, sorghum bundle sheath	0.40	640.5	649.2	661.7	669.6	676.9	683.6	691.5	704.6
C36B, *Oenothera* system 1	0.42	639.0	649.8	661.4	669.7	677.0	683.7	691.7	706.4
Fraction 2 preparations									
Very sharp spectra									
C35H, *Stichococcus*	0.35	640.3	649.6	661.7	669.8	676.9	683.1	690.2	...
C72A, *Scenedesmus*	0.45	(639.8)	648.9	661.3	669.7	677.7	684.3	692.4	...
Typical green algal spectra									
C28E, *C. pyrenoidosa*	0.30	640.0	648.4	660.9	669.2	677.4	683.2	693.7	...
Typical green leaf spectra									
C6J, spinach	0.41	640.0	649.3	661.3	669.6	676.7	683.3	687.6	...
C37D, *Oenothera* system 2	0.37	640.1	649.5	661.3	669.4	677.0	683.0	691.9	...
Unfractionated preparations									
Typical green algal spectra									
C76J, *Chlorella vulgaris*	0.34	(639.3)	649.1	662.3	669.7	677.8	685.1	692.0	...
C62G, *Scenedesmus* mutant 8	0.47	(642.2)	649.5	662.0	669.8	677.6	683.9	692.2	...
Typical green leaf spectra									
C47A, spinach	0.49	640.0	649.7	661.3	669.6	677.2	684.6	690.5	705.5
C107A, sorghum mesophyll	0.27	640.0	649.9	661.5	669.6	676.7	683.5	690.7	701.0
Average		*640.1*	*649.5*	*661.6*	*669.6*	*677.1*	*683.7*	*691.5*	*704.6*
Standard Deviation		*0.20*	*0.13*	*0.09*	*0.05*	*0.09*	*0.16*	*0.39*	*0.90*

[1] Values in parentheses were omitted from averages; curves too small to be significant.

1- to 5-nm adjustments in position and in width per iteration. These limitations make it possible to keep the computer from modifying the estimated input bands so much that they might lose their physical significance.

RESULTS

Derivation of the Peak Positions and Widths of the Main Peaks. Sixty-five spectra of chloroplast homogenates or fractions from leaves and algae that were measured at liquid nitrogen temperature have been studied. Many of them have been repeatedly subjected to curve analyses with various input bands and with different program specifications. Fifteen spectra have been selected that fall into groups with characteristic shapes. The curve analyses giving the bands of Tables I, II, and III are illustrated in Figures 1 and 2 and in Figure 18 of Reference 10. The most useful curves for explicitly defining the shape and position of each component are the spectra of fractions from *Stichococcus* and from *Scenedesmus*. The next sharpest group are the *Chlorella* type spectra. Another typical shape is that of the green leaf type illustrated by fractions 1 and 2 of *Marchantia, Oenothera,* and spinach. The contrasts between these groups of spectra have been illustrated (3, 5).

Because the relative heights of each band differ in the various spectra, some spectra are more favorable than others for showing any one band. Table I shows the wavelength position of the major chlorophyll bands in spectra that were selected as being particularly suitable for band characterization. Tables II and III give the half-widths and the relative proportions, respectively, of component curves fitting these spectra. In Table III the proportions of the components are reported as the percentage that each one contributes to the sum of the bands listed for chlorophyll *a*. The largest deviations of the sum curve from the experimental points are usually about 1% of the peak heights. Because the agreement between different curve analyses is often closer than 1 nm we have reported the results to 0.1 nm even though the spectrophotometer calibration is not that accurate. Its scale was found to read 0.8 nm too high after this work was completed and that correction is included in the wavelengths here reported, but not in the scales of the figures.

The Components Found.

Cb 640. In spectra with a large C*b* 650 band it is often necessary to add a small 640 nm component with about a 10-nm half-band width. This may represent free chlorophyll *b*, perhaps dissolved in lipids. Because of its small size, the exact peak position and width of the band are less accurately determined than those of some other bands.

Cb 650. The peak position of chlorophyll *b* averages 649.5 nm with a half-width of about 11.7 nm in both fractions 1 and 2. Fraction 2 spectra contain more chlorophyll *b* than do those of fraction 1.

Ca 662. The peak wavelength of this form suggests that it may be free chlorophyll *a* dissolved in lipid. If, however, it is a protein complex, the chlorophyll must be in monomeric

Table II. *Half widths of Components*

Curve No. and Material	Chlorophyll *b*		Chlorophyll *a*					
	C*b* 640	C*b* 650	C*a* 662	C*a* 670	C*a* 677	C*a* 683	C*a* 691	C*a* 704
Fraction 1 preparations								
Very sharp spectra								
C34G, *Stichococcus*	8.3	11.0	10.8	8.9	8.4	11.3	20.0	...
C71C, *Scenedesmus*	12.6	11.7	11.1	9.8	8.8	9.9	13.0	(14.2)[1]
Average sharp, fraction 1	*10.4*	*11.4*	*11.0*	*9.3*	*8.6*	*10.6*	*17.0*	*(14.2)*
Typical green algal spectra								
C27D, *C. pyrenoidosa*	12.1	12.4	11.6	10.1	9.8	10.6	13.1	(15.1)
Typical green leaf spectra								
C5D, spinach	10.3	11.8	11.3	9.7	10.6	10.8	14.3	17.4
C106A, sorghum bundle sheath	10.9	11.9	11.1	10.0	10.3	10.3	10.5	23.4
C36B, *Oenothera* system 1	11.1	11.4	11.3	10.2	10.4	11.5	14.1	15.4
Average typical, fraction 1	*11.1*	*11.9*	*11.3*	*10.0*	*10.3*	*10.8*	*13.0*	*18.7*
Fraction 2 preparations								
Very sharp spectra								
C35H, *Stichococcus*	(7.3)	10.5	10.7	8.9	7.6	8.8	19.3	...
C72A, *Scenedesmus*	(11.3)	11.2	11.6	10.4	8.0	8.2	14.9	...
Average sharp, fraction 2	*(9.0)*	*10.9*	*11.1*	*9.7*	*7.8*	*8.5*	*17.1*	...
Typical green algal spectra								
C28E, *C. pyrenoidosa*	11.3	11.2	12.0	10.2	9.3	11.0	17.1	...
Typical green leaf spectra								
C6J, spinach	8.5	11.5	11.2	8.9	9.0	8.5	17.7	...
C37D, *Oenothera* system 2	8.4	11.6	11.6	10.2	9.9	9.4	13.4	...
Average typical, fraction 2	*9.4*	*11.4*	*11.6*	*9.8*	*9.4*	*9.6*	*16.1*	...
Unfractionated preparations								
Typical green algal spectra								
C76J, *C. vulgaris*	(7.0)	11.8	10.9	9.6	8.4	10.6	18.8	...
C61B, *Scenedesmus* mutant 8	(13.1)	12.1	11.3	9.8	8.6	10.1	18.8	...
Typical green leaf spectra								
C47A, spinach	9.6	11.3	11.0	9.9	10.2	10.1	15.9	16.4
C107A, sorghum mesophyll	10.5	11.6	11.4	9.8	9.7	8.6	9.4	25.1
Average typical, unfractionated	*10.0*	*11.7*	*11.2*	*9.8*	*9.2*	*9.9*	*15.7*	*20.8*

[1] Parentheses indicate curves too small to be significant.

Table III. *Proportions of Chlorophyll a Components*

Curve No. and Material	% of Total Chlorophyll *a* as:					
	Ca 663	Ca 670	Ca 678	Ca 684	Ca 693	Ca 705
	%					
Fraction 1 preparations						
Very sharp spectra						
C34G, *Stichococcus*	24.7	21.4	30.7	15.8	7.4	0
C71C, *Scenedesmus*	21.9	26.1	27.4	14.9	8.0	1.7
Typical green algal spectra						
C27D, *C. pyrenoidosa*	23.3	24.4	27.2	16.2	6.9	2.0
Typical green leaf spectra						
C5D, spinach	19.0	21.1	30.3	19.7	6.9	3.0
C106A, sorghum bundle sheath	18.5	21.8	28.5	19.1	6.1	6.5
C36B, *Oenothera* system 1	19.9	21.7	27.3	19.5	7.8	3.8
Average fraction 1	*22*	*23*	*29*	*17*	*7*	*3*
Fraction 2 preparations						
Very sharp spectra						
C35H, *Stichococcus*	27.3	24.2	34.6	8.3	5.6	0
C72A, *Scenedesmus*	24.6	32.0	30.6	7.5	5.3	0
Typical green algal spectra						
C28E, *C. pyrenoidosa*	26.3	27.0	28.5	12.4	5.8	0
Typical green leaf spectra						
C6J, spinach	25.1	25.5	37.0	7.8	4.6	0
C37D, *Oenothera*	22.8	27.3	36.9	8.7	4.3	0
Average fraction 2	*25*	*27*	*33*	*8*	*5*	*0*
Unfractionated preparations						
Typical green algal spectra						
C76J, *C. vulgaris*	25.4	29.6	31.1	7.3	6.7	0
C62B, *Scenedesmus* mutant 8	24.5	27.3	29.2	11.8	7.2	0
Typical green leaf spectra						
C47A, spinach	20.5	24.6	37.4	9.8	5.3	2.4
C107A, sorghum mesophyll	20.7	24.0	32.0	13.5	5.0	4.8
Average unfractionated	*23*	*26*	*32*	*11*	*6*	*2*

form. This component shows clearly in both fractions 1 and 2 of spinach and of *Stichococcus* (C5, C6, C34, and C35) of Figure 18 in Reference 10. The inclusion of C*a* 662 in the analyses of other spectra makes possible a good fit with a single 670 nm form. Briantais (1), as well as Thomas and Bretschneider (21), also gives evidence for the reality of this 662 nm form of chlorophyll *a* although Thomas and Bretschneider give the wavelength as 665 nm. It remains to be seen if this form of chlorophyll *a* usually contributes to action spectra. The average peak position is 661.6 nm with an apparent 11.3-nm half-width, in both fractions. The apparently greater half-width of this form of chlorophyll *a* may be an artifact, as mentioned in the "Discussion."

Ca 670. This form, so obvious in many spectra, is now considered to be about 10 nm wide. Previously, a much greater width of C*a* 670 was required for curve fitting before the universality of a 662 form was realized. C*a* 670 seems to be identical in the two fractions. The average is 669.6 nm with a half-width of about 9.9 nm, significantly narrower than C*b* 650 or C*a* 662.

Ca 677. The 677 nm form was previously called "C*a* 680." As used now in conjunction with a 684 nm form it fits the spectra of both fractions 1 and 2 without significant change of peak wavelength. Apparently the bandwidth is 10% narrower in fraction 2 than in fraction 1, as seen in Table II. There may be an increased refractive index on the long wavelength side of the 670 nm band from the anomalous dispersion effect that might give an increased apparent absorbance through refractive scattering near the 677 nm band. This artificial peak would overlap with and modify the apparent band shape of C*a* 677. This effect rather than the existence of different C*a* 677 forms could conceivably account for the small differences in half-widths reported in Table II. The average peak position is 677.1 nm. The half-width averaged 10.3 nm in typical fraction 1 spectra and 9.4 nm in typical fraction 2 preparations.

Ca 684. A shoulder near 684 nm is most clearly seen in the low temperature spectra of Ogawa and Vernon (17) for fractions enriched in P700. This confirms without doubt the reality of the 684 component that we have found to be a necessary though often "hidden" component of all spectra. In fraction 1 preparations the proportion of this form is far greater than in fraction 2 preparations. Its presence in fraction 2 preparations may, perhaps, be due to incomplete separation of the functional pigment systems. This form of chlorophyll is less specifically defined because it rarely shows as a clear shoulder. Its average peak wavelength is 683.7 nm with a half-width of 10.8 nm in typical fraction 1 preparations and 9.6 nm in fraction 2.

Ca 692. The present work estimates the peak wavelength of the previously called C*a* 695 forms at about 691.5 nm with about a 13- to 18-nm width.

Ca 705. The characteristics of this small band have not been well established. Its apparent wavelength peak varied from 700 to 706 and its half-width from 14 to 25 nm.

Analysis of Less Distinct Spectra with Bands Derived from Sharper Spectra. While only the spectra with well defined

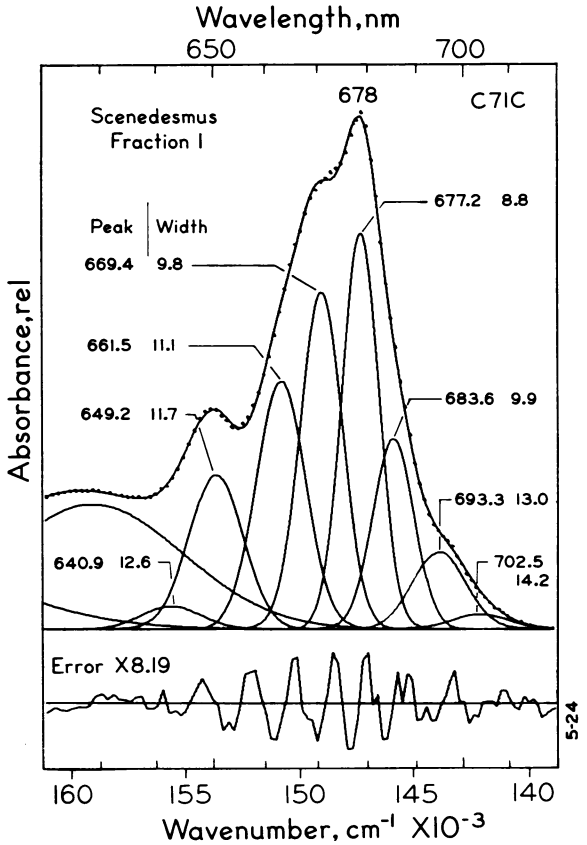

FIG. 1. The absorption spectrum at −196 C of fraction 1 from *Scenedesmus* chloroplasts fitted by the sums of Gaussian components. The observed data are plotted as points while the line through them is the sum of the component curves the characteristics of which are given in Table I to III. The error of fit at each point is shown below on a scale with the designated magnification. This is redrawn from part of Figure 17 of Reference 10.

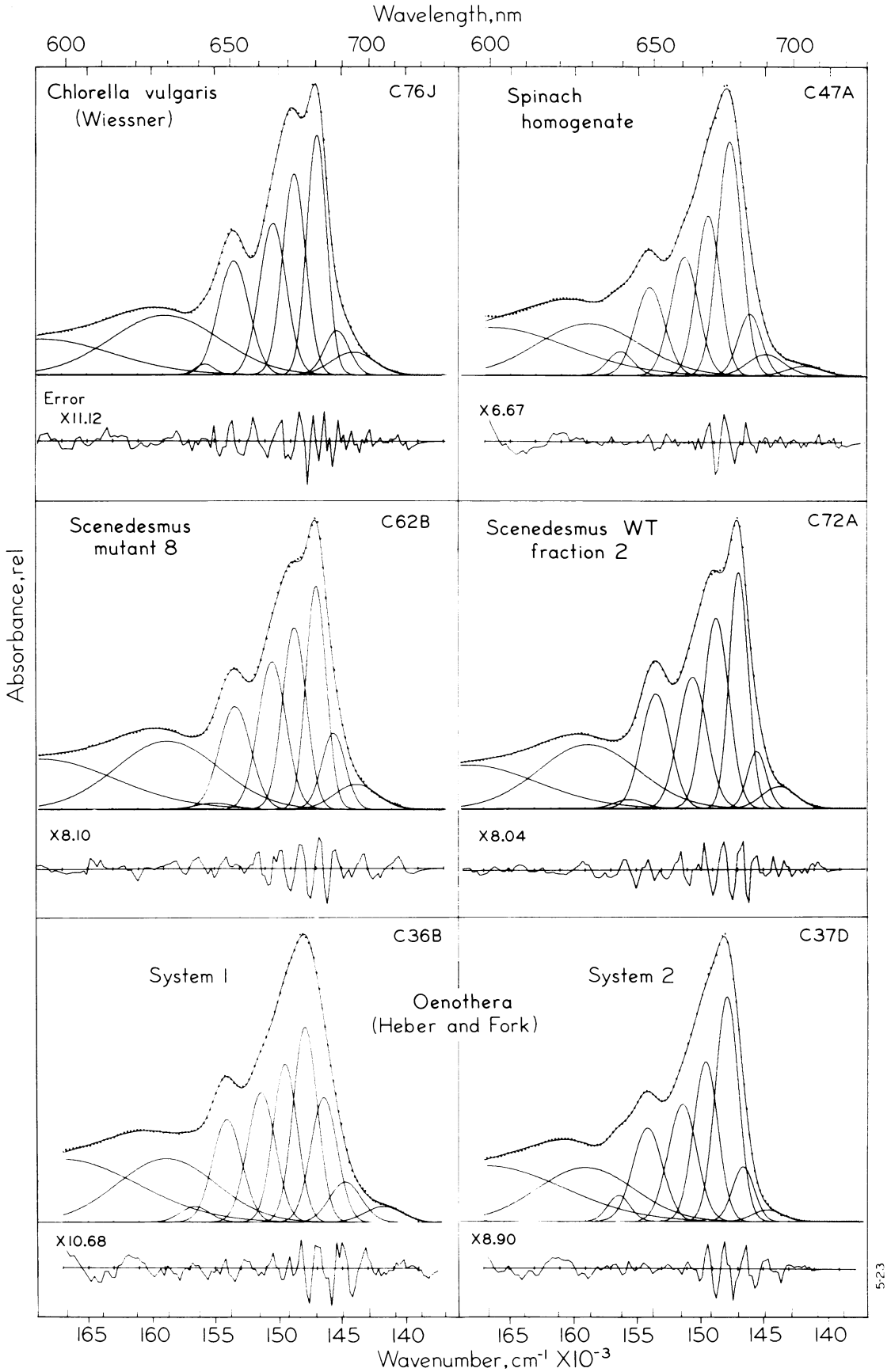

FIG. 2. The absorption spectra of various chloroplast preparations at −196 C. The component curves are specified in Tables I to III.

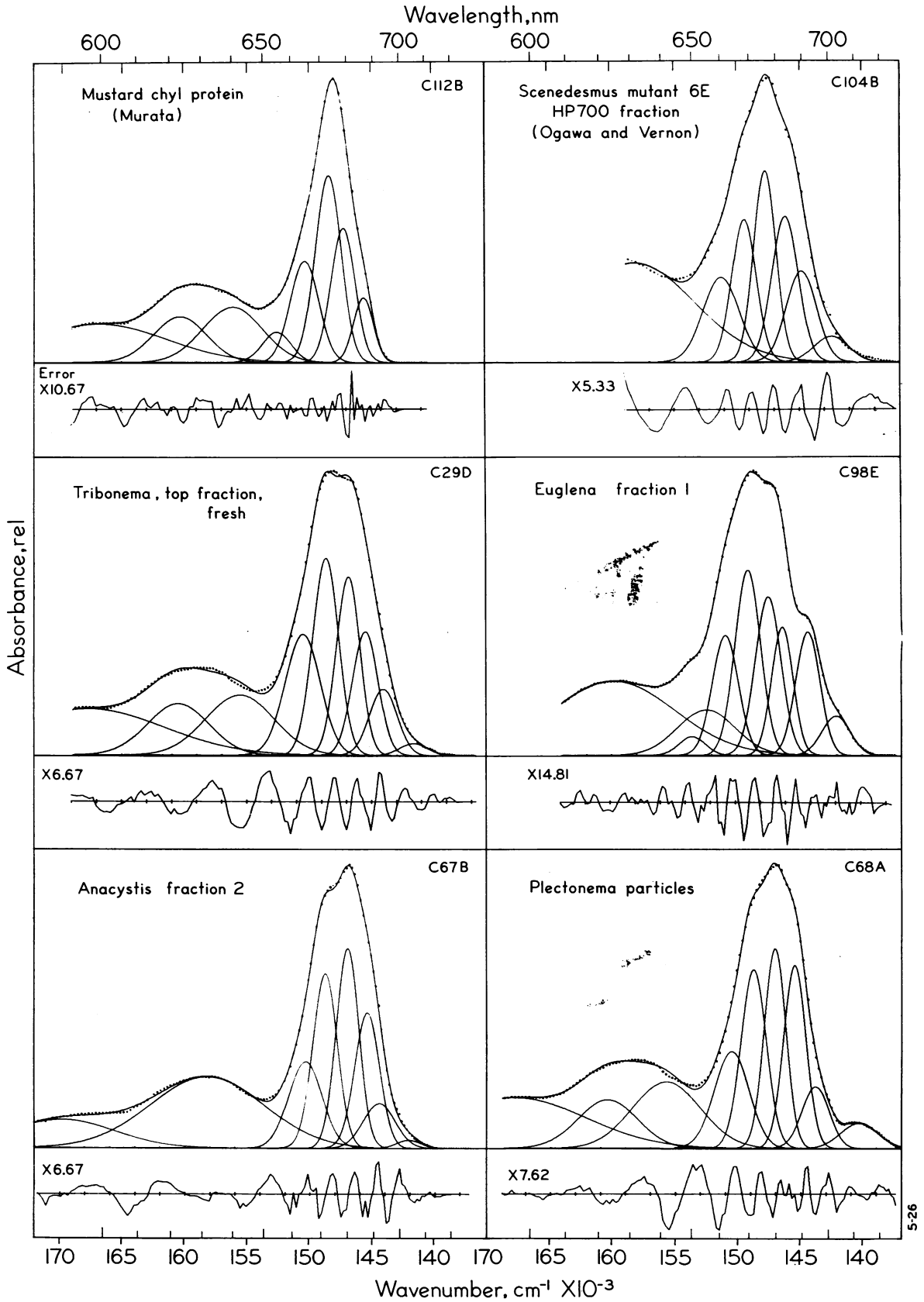

FIG. 3. Some low temperature spectra of preparations with little or no chlorophyll *b*. The Gaussian components used to match these curves are defined in Table IV.

Table IV. *Bands that Fit Indistinct Spectra Having Little or No Chl b*

Curve No. and Material	SE, % of peak	Chlorophyll *b*	Chlorophyll *a*					
		Cb 650	*Ca* 662	*Ca* 670	*Ca* 677	*Ca* 683	*Ca* 691	*Ca* 704
Peak wavelengths, nm								
Input bands from Table I		650.3[1]	661.6	669.6	677.1	683.7	691.5	704.6
C112B, Chl protein, mustard	0.33	(653.4)	662.1	670.4	675.8	684.5	...	...
C29D, *Tribonema* fr. fresh	0.80	0	661.2	669.3	677.5	683.9	690.7	702.4
C67B, *Anacystis* fr.	0.63	0	662.5	669.3	677.5	684.8	689.5	702.2
C104B, *Scenedesmus* mutant 6 HP700	1.14	0	661.5	669.7	677.2	684.7	690.8	702.4
C98E,[2] *Euglena* frl	0.37	651.6	662.1	670.1	677.5	682.8	692.3	703.1
C68A, *Plectonema* fr	0.70	0	661.3	669.2	676.9	684.2	692.0	(709.1)
Average		...	*661.8*	*669.7*	*677.1*	*684.0*	*691.1*	*702.2*
Half-widths of components, nm								
Input bands from Table II		11.7[1]	11.3	9.9	10.0	10.0	14.7	(15.0)
C112B, Chl protein, mustard		(11.6)	11.7	10.9	10.5	(8.8)	...	...
C29D, *Tribonema* fr. fresh		...	(14.5)	11.0	10.3	11.3	12.4	13.1
C67B, *Anacystis* fr.		...	(13.7)	10.3	9.8	10.9	14.3	11.1
C104B, *Scenedesmus* mutant P6 HP700		...	(14.9)	10.3	9.7	11.7	13.5	15.9
C98E,[2] *Euglena* frl		10.2	10.8	10.9	11.0	9.3	10.7	13.2
C68A, *Plectonema* fr		...	(14.2)	10.6	9.3	9.8	12.1	(17.5)
Average		...	*11.1*	*10.7*	*10.1*	*10.6*	*12.6*	*13.3*
Proportions of the components as % of the main bands' area								
C112B, Chl protein mustard		6.3	22.8	39.2	26.9	11.0	0	0
C29D, *Tribonema* fr. fresh		0	21.6	26.6	22.6	17.2	10.1	1.9
C67B, *Anacystis* fr.		0	16.6	25.1	27.3	20.7	8.9	1.3
C104B, *Scendesmus* mutant 6 HP700		0	15.9	18.5	23.3	21.5	15.5	5.2
C98E, *Euglena* frl		2.2	16.1	24.9	21.7	14.7	16.1	6.5
C68A, *Plectonema* fr		0	17.0	23.4	22.9	22.1	9.2	5.5

[1] For C112B and C98E only.

[2] Slightly different input bands were used.

peaks can be used to derive the shapes of components, an obvious question to ask is if the components that were derived as described will also fit the spectra that do not have clearly defined peaks and shoulders. Accordingly, six spectra of Figure 3 were analyzed using as the estimated input bands the average peak positions and half-widths from Tables I and II. These bands, after optimization for each curve by the RESOLV program, came out as shown in Table IV. There was no significant change of peak wavelengths (except for *Ca* 677 of C112 B, the "chlorophyll protein" preparation) while the half-widths did show some variation and averaged about 0.5 nm wider in these spectra. As previously mentioned, *Ca* 662 was artificially broadened where there was no chlorophyll *b* or "655" band so these widths were omitted from the averages.

DISCUSSION

From Table I it is evident that the four major forms of chlorophyll *a* show no significant variation of peak wavelength either from one alga to another or between the two fractions. The differences between the two fractions are due to the increased proportions of *Ca* 684 and longer wavelength pigments and to the greater half-widths in fraction 1 of *Ca* 677 and *Ca* 684.

Because of its wavelength position *Ca* 662 may be free chlorophyll *a*. The fact that its half-width, nevertheless, appears greater than that of the other major forms of chlorophyll *a* we attribute to the computer's attempt to use this band to play the part of the hidden 655 nm band.

The practical value and also the limits of usefulness of curve analysis for resolution into single band components have become evident in the course of this work. This approach has made it possible to identify the peak wavelength and half-width of the main red band of each form of chlorophyll. There is, however, a region of uncertainty on the short wavelength part of the main peaks. This results from the overlap of the secondary bands, not yet evaluated, of the 683.7 and 677.1 forms with the main bands of the short wavelength forms. To remove this uncertainty, one must use as components synthetic spectra made up of three or four Gaussian curves with a sum that represents the complete spectrum of each form of chlorophyll.

We do not yet have any well established means of deriving the shape of the components in room temperature spectra from those obtained by curve analysis of low temperature spectra. This is particularly important because we would like to compare the curve analyses of absorption spectra with those of action spectra for the photochemical steps of photosynthesis, both recorded at the same temperature. Having found the minimal number of components, their peak wavelengths, and half-widths that fit a low temperature spectrum, it should be possible to make a significant curve analysis of the corresponding room temperature spectra. Without the re-

sults of the low temperature analysis it would usually be impossible to decide on the relative merits of a number of equally close fits attainable with a variety of components.

Presumably a complete description of the red spectrum for a single form of chlorophyll *a* would include specifications for the main peak, the hidden vibrational band near 650 nm, and the two obvious shorter wavelength vibrational components, near 590 and 625 nm. Possibly also an extra Gaussian component on the long wavelength tail of the main peak should be added, not as a real absorption band, but merely to improve the fit of the long wavelength tail of the main component. Thus the eventual description should be given by listing the wavelength of the peak, the half-width, and the height in relation to the main peak for these five parameters that describe the spectrum from 570 to 730 nm for each form of chlorophyll. However, we have reported here the peak positions, widths, and relative areas only for the main bands of each form of chlorophyll. The present work can, we hope, be extended to give a complete description for each of these spectral forms. Our findings of *Ca* 677 in fraction 1 and of *Ca* 684 in fraction 2 may perhaps be due to incomplete separation. This disagrees with the conclusions of Briantais (1) that system 1 consists of *Cb* 650, *Ca* 665, *Ca* 670, *Ca* 685, and *Ca* 705, while system 2 contains *Cb* 650, *Ca* 670, and *Ca* 680 (his Fig. 34).

The results we report agree remarkably well with the conclusions of Gulyaev and Litvin (14) from their first derivative spectra of whole organisms at 20 C, with small wavelength differences between their assumed components and ours. Their second derivative analysis, however, lumps their *Ca* 685 component with *Ca* 678, leading to an apparent peak at 680 to 683 nm.

Acknowledgments—We are indebted to Dr. Leo Vernon for a copy of the spectrum from which part of Figure 3 was plotted; to Dr. Marie-Rose Michel-Wolwertz for curves C5, C27, and C28; and to Dr. Wolfgang Wiessner for C76. We wish to thank the National Science Foundation for Grant GB 8630 and Dr. D. D. Tunicliff of the Shell Development Laboratory, Emeryville, California, for the original computer program.

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